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Targeting the AKAP Function of PI3Kγ with a Mimetic Peptide for the Treatment of Obstructive Respiratory Diseases

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Tables of contents

Summary

Background: Obstructive respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), are a diversified group of pathological conditions that, with approximately 4 million deaths every year, represent the third cause of mortality and the leading cause of morbidity worldwide. Despite the diversity in terms of etiology, pathogenetic mechanisms and clinical manifestations, these conditions share common features such as airway hyper-reactivity, persistent respiratory tract inflammation, and mucus hypersecretion with consequent airway mucus plugging and airflow obstruction. The mainstay treatment for obstructive respiratory diseases includes β_2-adrenergic receptor (β_2-AR) agonists and phosphodiesterase (PDE) inhibitors, cAMP elevating agents known to regulate airway constriction and inflammation. Furthermore, these drugs are potent inducers of the cystic fibrosis conductance regulator (CFTR), the epithelial channel regulating mucus hydration and clearance whose dysfunction is causative for CF. More specifically, cAMP elevating agents increase CFTR activation and stabilization, as well as the efficacy of approved CFTR modulators targeting the most prevalent channel mutant in CF (F508del), by raising sub-cortical cAMP levels and mediating protein kinase A (PKA)-induced channel phosphorylation. However, their clinical use is limited by unwanted side effects due to global cAMP elevation in the airways and in distant organs. Thus, novel and safer approaches for the manipulation of the β_2-AR/cAMP signaling axis to treat chronic airway diseases are urgently needed.

A previous study from our group recognized phosphoinositide 3-kinase γ (PI3Kγ) as an A-kinase anchoring protein (AKAP), localized in a specific β_2-

AR microdomain, that negatively regulates cAMP levels in the heart via tethering PKA to the cAMP hydrolysing enzymes, phosphodiesterase 3 and 4 (PDE3; PDE4), thereby favouring their PKA-mediated phosphorylation and activation. Since PI3Kγ exerts the same function in pulmonary cells, we hypothesized that the pharmacological targeting of PI3Kγ scaffold activity could be exploited to achieve cAMP elevation in the airways, leading to concomitant bronchodilation, reduced inflammation and CFTR modulation.

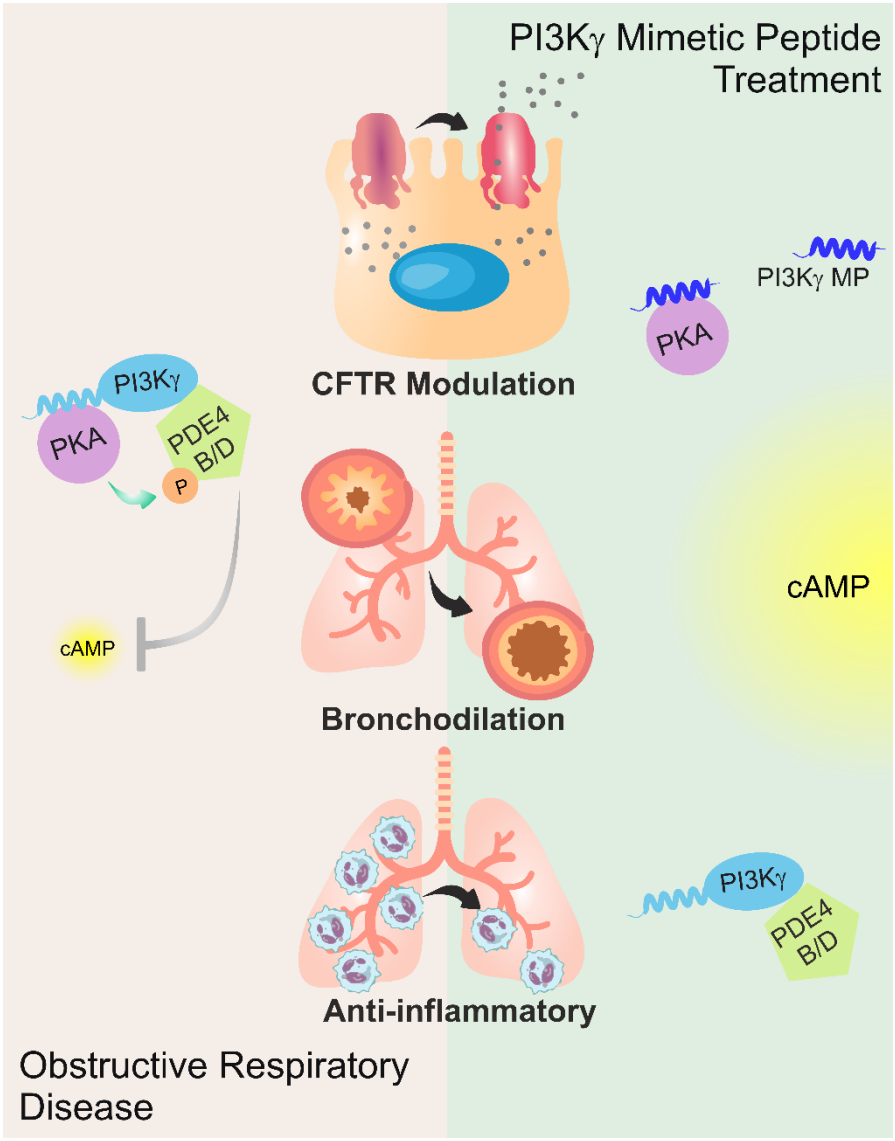
Materials and methods: We explored the ability of a cell-permeable mimetic peptide, namely PI3Kγ MP (Patent n° PCT/IB2015/059880 - WO/2016/103176), targeting the scaffold activity of PI3Kγ to function as a (i) bronchodilator, (ii) anti-inflammatory agent and (iii) CFTR modulator. A mouse model of chronic lung inflammation (OVA-sensitized mice) was used to assess the PI3Kγ MP bronchodilatory and anti-inflammatory effects. Biochemical and surface biotinylation assays were performed in immortalized cell lines (HEK293T, PANC-1, 16HBE14o- and CFBE41o-), while CFTR activity was measured in Ussing chamber and through organoid swelling assays performed in primary cells derived from healthy or CF-affected subjects.

Results and Discussion: We found that intratracheal instillation of the PI3Kγ MP in mice induced a significant and dose-dependent increase in cAMP in both tracheas and lungs, which persisted in the airways up to 24 hours without altering cAMP homeostasis in other organs, underlying the advantage of using this compound instead of β_2-adrenergic agonists or PDE inhibitors, known to cause tachyphylaxis and several other systemic side effects. Furthermore, in a mouse model of chronic lung inflammation, the PI3Kγ MP

limited methacholine-induced airway hyperresponsiveness and reduced infiltration of neutrophils, the major cell population accumulating in diseased lungs responsible for their remodeling and irreversible structural damage, indicating that the compound can concomitantly target two of the main pathological features of the disease. Notably, the peptide induced PKA-mediated phosphorylation of the CFTR channel exclusively at the S737 residue, differently from the adenylyl cyclase activator forskolin which promoted the phosphorylation of most of the CFTR phosphorylation sites, strengthening the thesis of a constrained cAMP increase upon peptide treatment. The peptide-mediated CFTR phosphorylation induced a dose dependent increase in Cl^- secretion in primary cells derived from healthy subjects that was not observed using a control peptide (CP). Moreover, no further CFTR activation was detected when a PDE4 inhibitor was applied on top of the PI3Kγ MP, suggesting that the peptide likely inhibits the PDE4 pool associated to CFTR regulation. In the same Ussing chamber experiments, the peptide was still able to induce a transient increase in short circuit current (I_{SC}) in the presence of the CFTR blocker CFTRinh-172 which is proper of the activation of Ca^{2+}-activated Cl^- channels (CaCCs). This result is consistent with the activation of alternative channels, via the capability of the cAMP/PKA signaling to increase cytoplasmic Ca^{2+} levels in airway epithelial cells, that can synergize with the CFTR, thus maximizing Cl^- secretion. In line with a well-known role of cAMP in promoting plasma membrane stabilization of CFTR, we also found that the peptide increases the amount of the channel at the cell surface. Notably, this process turned out to be independent from the activation of the cAMP/PKA axis and possibly mediated by protein kinase D1 (PKD1), target found to be phosphorylated

upon PI3Kγ MP treatment and known to play an important role in cellular trafficking and exocytosis. The ability of the peptide to increase the amount of CFTR at the plasma membrane as well as its gating prompted us to explore whether this compound can be exploited to increase the therapeutic efficacy of the gold standard CFTR modulators used in patients. As a matter of fact, the PI3Kγ MP was able to double Cl^- secretion induced by Trikafta® in primary bronchial epithelial cells derived from CF patients carrying the most prevalent mutation F508del.

Conclusions: These results unveil PI3Kγ as the orchestrator of a β_2-AR/cAMP microdomain central to smooth muscle contraction, immune cell activation and epithelial fluid secretion in the airways, eventually indicating the use of a PI3Kγ MP for compartment-restricted therapeutic cAMP elevation. In addition, these data strongly point to the use of the peptide as a promising strategy to not only ensure bronchorelaxation, anti-inflammatory effects and CFTR localization at the plasma membrane in obstructive respiratory diseases, but to concomitantly increase the therapeutic effects of CFTR modulators, especially needed for CF patients that do not fully benefit of current regimens.



Graphical abstract. Schematic representation of the mechanism of action and therapeutic effects of the PI3Kγ mimetic peptide (PI3Kγ MP).

Introduction

Obstructive Respiratory Diseases

Epidemiology

Obstructive respiratory diseases are a diversified group of pathological conditions manifesting with shortness of breath, wheezing, coughing, chest tightness and frequent respiratory infections that, with approximately 4 million deaths every year, represent the third cause of mortality and the leading cause of morbidity worldwide (Collaborators 2020). The World Health Organization estimates that currently hundreds of millions of people suffer from these diseases and a growing trend is expected for the upcoming years (Adeloye, Agarwal et al. 2021). In particular, the prevalent obstructive respiratory diseases are chronic obstructive pulmonary disease (COPD) and asthma that, accounting for more than 300 million patients each, correspond to the most common chronic illnesses in adults and in children, respectively. Cigarette smoke (CS), air pollution and occupational exposure to inhaled irritants, such as vapours, gases, dusts or fumes, have been found to be common causative factors for both insurgence and development of these pathological conditions, together with genetic polymorphisms affecting lung development and function, as well as the innate and adaptive immune response of patients (Rabe and Watz 2017, Papi, Brightling et al. 2018, Sakornsakolpat, Prokopenko et al. 2019). Regarding genetic alterations, mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) are responsible for causing cystic fibrosis (CF), another chronic obstructive respiratory disease with a devastating impact on patients' survival and quality of life. Albeit much less frequent compared to COPD and asthma,

CF is the most common rare genetic disease found in the Caucasian population which affects more than 100 thousand people worldwide (Shteinberg, Haq et al. 2021). In Italy, about 6 thousand subjects are currently living with this disease, with an incidence in 2018 of one newborn being diagnosed with CF for every 5.442 healthy births (Campagna, Amato et al. 2021). The most frequent CFTR mutation in this country, and also worldwide, is the deletion of phenylalanine in position 508 (F508del), with 68.7% of patients carrying the mutated allele at least in heterozygosity. However, the Italian frequency of F508del is lower than the European average (about 82%), as there is a higher percentage of patients affected by rarer mutations (Campagna, Amato et al. 2021).

Over the next decade, prevalence of asthma and COPD is predicted to significantly rise in developing countries (Celli and Wedzicha 2019), but so is the number of CF patients requiring long-term care, as survival is progressively improving as a result of intensive follow-up and better treatments targeting obstructive airway diseases main pathological features.

Main pathological features

Despite the diversity in terms of etiology, pathogenetic mechanisms and clinical manifestations, these conditions share common features such as airway hyperreactivity, persistent respiratory tract inflammation, and mucus hypersecretion with consequent airway mucus plugging and airflow obstruction (Rabe and Watz 2017, Papi, Brightling et al. 2018, Boucher 2019, Shteinberg, Haq et al. 2021).

Airway hyperresponsiveness

Airway hyperresponsiveness is the predisposition of the airways of patients affected by obstructive respiratory diseases to restrict excessively in response to stimuli that would not have any or very little effect in healthy subjects. This phenotype is associated with increased risk of exacerbation and level of treatment required to control symptoms, together with a decline in lung functionality. Airway smooth muscle (ASM) is one of the major player involved in the hyperresponsiveness process. In patients affected by respiratory diseases, the expression of contractile proteins such as α smooth muscle actin and desmin in smooth muscle cells correlates with airway hyperresponsiveness severity (Slats, Janssen et al. 2008). Moreover, ASM and airway epithelial cells are both affected by a substantial remodeling process caused by the chronic inflammatory response induced by persistent irritant stimuli causative of airway hyperresponsiveness. Particularly, the disruption of the epithelium not only dysregulates the ability of this tissue to release epithelial-derived relaxing factors, but as well strongly increases the amount of external stimuli able to interact with airway smooth muscle cells, leading to both their proliferation and hyperplasia. As a result, the airway muscle mass of patients affected by these respiratory diseases, in particular asthma, is much thicker and capable of generating an higher total force compared to the one of healthy subjects, thus potentiating bronchoconstriction (Chapman and Irvin 2015). Most of the stimuli inducing airway smooth muscle cells hyperplasia and hyperproliferation, including transforming growth factor (TGF-β_1), histamine, leukotriene D4, interleukin 1 beta (IL-

1β) and tumor necrosis factor alpha (TNF-α), are mediators of another feature of chronic respiratory disease, namely inflammation (Athari 2019).

Chronic Inflammation

Chronic inflammation is the hallmark causative for obstructive respiratory diseases progression. However, there is a distinctive signature of inflammatory cells among these conditions. The most prevalent form of asthma is the eosinophilic one, with 50% of asthmatic patients showing an increased number of eosinophils measured not only in their airways but also in their blood. Other subtypes are instead characterized by either the presence of both eosinophils and neutrophils or by neutrophilia alone. This latter type of asthma is rare, but it is often seen in patients with a severe disease condition and is also less responsive to standard treatments (Robinson, Humbert et al. 2017). Neutrophils and in particular neutrophil-derived reagents, which are indeed strongly implicated in airway remodelling and injury, are instead prevalent in both COPD and CF, together to a small extent with macrophages. The primary role of both these inflammatory cells is to phagocytose invading microorganisms and destroy them by the internal generation of reactive oxygen species and the action of proteases, such as neutrophil elastase (NE), matrix metalloproteinases and cathepsins, that are also though able to induce proteolytic degradation of the airway extracellular matrix (ECM) in a chronic pathological setting (Trivedi, Khan et al. 2021). These enzymes have also been found to affect mucus secretion.

Mucus obstruction

Oxidants and proteases have been shown to interfere with both the expression and function of the CFTR protein, the main regulator of mucus hydration and clearance in the airways. Disfunction of this chloride channel leads to defects in ion-fluid transport, in particular in water secretion across the airway epithelium, with the consequent production of a dehydrated, hyper-concentrated mucus that accumulates in the airways of patients causing airflow obstruction (Boucher 2019). Over 1900 mutations in the *CFTR* gene are probably causative of CF, with diverse molecular consequences ranging from defective synthesis, gating, conductance or reduced stability of the CFTR channel at the apical plasma membrane (Mall and Hartl 2014). However, CFTR dysfunction is also observed in asthmatic and COPD patients. A significantly high percentage of severe asthmatic patients affected by airway mucus hypersecretion have been found to express polymorphisms in the *CFTR* gene (Crespo-Lessmann, Bernal et al. 2021). Additionally CS, the leading cause of COPD, has been found to impair both CFTR expression and function in airway epithelia, spanning from reduced transcription to protein degradation, and resulting in "acquired" CFTR dysfunction. More precisely, CS can reduce CFTR activity up to 60% in the airways of healthy non-smokers and can decrease CFTR levels in lipid rafts in mice (Mall and Hartl 2014).

The extent of airway obstruction and chronic inflammation varies in asthma, COPD and CF, resulting in disease specific treatment guidelines.

Nevertheless, these regimens rely on several common pharmaceuticals including bronchodilators, anti-inflammatory drugs and mucolytics.

Current pharmacological treatments

Bronchodilators

The mainstay treatment for obstructive respiratory diseases include inhaled corticosteroids and β_2-adrenergic receptor (β_2-AR) agonists, which reduce airway inflammation and reverse airway constriction, respectively (Celli and Wedzicha 2019). β_2-AR agonists are the most effective bronchodilators in clinics that mediate airway smooth muscle relaxation via the production of cyclic adenosine monophosphate (cAMP). The main mechanism by which this second messenger acts as a bronchodilator is through the activation of protein kinase A (PKA), protein that, by phosphorylating several targets within the cell, leads to the activation of myosin light chain phosphatase and inhibition of myosin light chain kinase, and thus to ASM relaxation (Billington, Ojo et al. 2013).

Anti-inflammatory drugs

β_2-AR agonists can also engage their target in infiltrating leukocytes, eventually contributing to the resolution of inflammation (Barnes and Hansel 2004). Even more effective anti-inflammatory drugs are inhibitors of phosphodiesterases (PDEs), the enzymes responsible for cAMP hydrolysis into 5′ AMP, which are used in combination with β_2-AR agonists in severe cases of asthma and COPD to boost cAMP levels in inflammatory cells. This is because, on one hand, cAMP-mediated PKA activation leads to the phosphorylation of the cAMP-responsive element

binding protein (CREB), which translocates to the nucleus promoting the production of pro-resolving mediators, anti-inflammatory cytokines, and stimulation of immune cells polarization, efferocytosis and granulocyte apoptosis (Tavares, Negreiros-Lima et al. 2020). On the other hand, PKA can also reduce the expression of pro-inflammatory genes via inhibiting the transcriptional activity of NF-κB (Stellari, Sala et al. 2019).

CFTR modulating agents

Furthermore, both β_2-AR agonists and PDE inhibitors can also be exploited to improve mucus clearance in chronic obstructive diseases. cAMP elevating agents are potent modulators of the CFTR protein, since both channel opening and stability at the plasma membrane rely on the cAMP signaling pathway (Lobo, Amaral et al. 2016, Vijftigschild, Berkers et al. 2016, Chin, Hung et al. 2017). The CFTR protein is phosphorylated by PKA at multiple sites, and in particular PKA-dependent phosphorylation events occurring on sites located at the regulatory domain of the protein are necessary for nucleotide-dependent gating of the channel. ATP binding to both nucleotide-binding sites enhances dimerization of the nucleotide binding domain 1 (NBD1) and 2 (NBD2) which leads to CFTR opening and Cl^- secretion. Channel closure is instead mediated by the ability of one of the ATP binding sites to hydrolyze ATP leading to NBD dimer destabilization (Chin, Hung et al. 2017). The cAMP pathway has also been found to increase the amount of CFTR channel at the plasma membrane. On one hand, cAMP/PKA-mediated CFTR phosphorylation stabilizes the plasma membrane channel by enhancing its interaction with cytoskeletal scaffolding proteins such as the Na^+/H^+ exchanger regulatory

factor (NHERF1) and the ezrin/radixin/moesin (ERM) complex. On the other hand, cAMP increases the amount of CFTR at the membrane by attenuating channel endocytosis via the activation of the exchange protein directly activated by cAMP (EPAC1). cAMP-mediated activation of this protein leads to its translocation to the plasma membrane and binding with NHERF1, interaction that finally favors the anchoring of the CFTR channel to the actin cytoskeleton (Lobo, Amaral et al. 2016).

However, cAMP elevating agents can also be administered to CF patients since they strongly improve the efficacy of the newly approved CFTR modulators used in clinics. In 2019 the Food and Drug Administration (FDA) approved Trikafta®, a triple combination therapy targeting the most common mutant protein found in CF patients, namely the F508del-CFTR. Trikafta® comprises the correctors tezacaftor (VX-661) and elexacaftor (VX-445), able to restore the defective F508del-CFTR protein folding, and the potentiator ivacaftor (VX-770), which is instead capable of rescuing the gating defect of this mutated protein allowing Cl^- secretion (Bear 2020). Even if these breakthrough CFTR modulators have been found to be highly effective in clinics, with lung function improvements in F508del heterozygous patients up to 14%, they rescue CFTR activity only up to 60% of physiological values (Veit, Roldan et al. 2020), underlying the need to introduce in clinical practice new and/or combination treatments, in particular cAMP modulating agents, for this lethal disease.

Side effects of currently used cAMP modulating agents

Despite their proven capability to modulate the cAMP signaling pathway, the efficacy of β_2-AR agonists and PDE inhibitors is still limited. In clinics,

β_2-AR agonists are known to cause tachyphylaxis, which leads to a reduced therapeutic effect and adverse events, such as increased heart rate, palpitations, tachyarrhythmia, and tachycardia, stemming from systemic drug exposure (Cazzola, Page et al. 2013). Similarly, inhibition of cAMP breakdown by drugs targeting phosphodiesterase 4 (PDE4), the major cAMP-hydrolyzing enzyme in the airways, has been clinically tested, but exhibits unwanted side effects, such as emesis, nausea, diarrhea and weight loss, likely due to systemic PDE4 blockade (Oba and Lone 2013). Thus, novel and safer approaches for the manipulation of the β_2-AR/cAMP signaling axis to treat chronic airway diseases are urgently needed.

The cAMP regulating function of the A-kinase anchoring protein PI3Kγ

Previous work from our group identified phosphoinositide 3-kinase γ (PI3Kγ) as a key negative regulator of β_2-AR/cAMP signaling in the heart. In this tissue, PI3Kγ serves as an A-kinase anchoring protein (AKAP) that tethers PKA to PDE3 and 4, thereby favoring their PKA-mediated phosphorylation and activation. This mechanism of localized PDE stimulation eventually allows the restriction of β_2-AR/cAMP responses to discrete subcellular compartments (Perino, Ghigo et al. 2011, Ghigo, Perino et al. 2012). Accordingly, disruption of the scaffold but not the kinase activity of PI3Kγ results in β_2-AR/cAMP signaling amplification in cardiomyocytes (Ghigo, Perino et al. 2012). Intriguingly, PI3Kγ is also found in pulmonary cells (Fanelli, Puntorieri et al. 2010). Basal cAMP levels in tracheas of mice lacking PI3Kγ ($PI3K\gamma^{-/-}$) are 2-fold higher compared to wild-type animals and to mice expressing a kinase-inactive PI3Kγ ($PI3K\gamma^{KD/KD}$), indicating that the same kinase-independent

regulation of cAMP by PI3Kγ found in cardiomyocytes is also preserved in airway cells [Ghigo, Murabito et al, under revision]. Moreover, a significant reduction of the catalytic activity of PDE4B and PDE4D is detected in tracheas of PI3Kγ$^{-/-}$ mice, while their function is maintained in PI3Kγ$^{KD/KD}$ tissues, corroborating the notion that PI3Kγ restrains airway cAMP levels downstream of β_2-ARs through a kinase-independent activation of PDE4 (Ghigo, Murabito et al, under revision). In light of these data, we speculated that pharmacological targeting of PI3Kγ scaffold activity could be exploited to achieve therapeutic cAMP elevation in the airways.

Herein, I describe the activity of a cell-permeable PI3Kγ-derived mimetic peptide (PI3Kγ MP) (Patent n° PCT/IB2015/059880- WO/2016/103176 by Kither Biotech S.r.l.) that, by interfering with the interaction between PI3Kγ and PKA, inhibits PI3Kγ-bound PDE4B and D and, in turn, enhances β_2-AR/cAMP responses in human bronchial smooth muscle, immune and epithelial cells. Intratracheal instillation of the PI3Kγ MP limits bronchoconstriction and lung neutrophil infiltration in a mouse model of asthma. In human airway epithelial cells, the PI3Kγ MP not only increases both CFTR gating and channel levels at the plasma membrane, but also restores the function of the most prevalent CFTR mutant in CF (F508del) by potentiating the effects of approved CFTR modulators.

Results

A PI3Kγ mimetic peptide enhances airway β_2-AR/cAMP signaling

In order to disrupt the PKA-anchoring function of PI3Kγ and produce a compartmentalized β_2-AR/cAMP response, a peptide encompassing the PKA-binding motif of PI3Kγ (Perino, Ghigo et al. 2011) was fused to the cell-penetrating sequence Penetratin-1 (P1) (Fig. 1A and B) (Guidotti, Brambilla et al. 2017). A FITC-labeled version of the PI3Kγ mimetic peptide (PI3Kγ MP) was readily detectable *in vitro* in human bronchial smooth muscle cells (hBSMCs) within 30 min of administration (Fig. 1B). The internalization of the peptide was followed by the disruption of the interaction between the type II regulatory subunit of PKA (PKA-RII) and the catalytic subunit of PI3Kγ, p110γ, in a dose-dependent manner (Fig. 2A). Next, we assessed the ability of the PI3Kγ MP to inhibit cAMP-PDEs and boost β_2-AR/cAMP signaling in the airways using primary airway smooth muscle cells. PI3Kγ MP reduced the catalytic activity of PDE4B and PDE4D by 30% in murine tracheal smooth muscle cells (mTSMCs) isolated from wild-type animals, while no significant effect of the peptide was observed in cells lacking PI3Kγ ($PI3K\gamma^{-/-}$) (Fig. 2B). After evaluating the effects of the PI3Kγ MP *in vitro,* we assessed whether the PI3Kγ MP could be exploited to enhance airway β_2-AR/cAMP signaling *in vivo*. To do so, the peptide was instilled intratracheally in mice and cAMP levels were quantified in explanted tracheas and lungs 24 hours after treatment. The PI3Kγ MP induced a dose-dependent increase in cAMP, demonstrating 80 μg/kg as the lowest dose that could elicit a significant increase in cAMP levels in both tracheas and lungs (Fig. 3). Moreover, the PI3Kγ MP persisted in the airways up to 24 hours after a single-dose instillation (Fig. 4), when maximal cAMP accumulation was also detected (Fig. 5). Notably, the direct intrapulmonary application route

prevented the PI3Kγ MP from diffusing outside of the respiratory system as well as from altering cAMP homeostasis in other organs, such as the heart (Fig. 3-5). Furthermore, the PI3Kγ MP did not elicit a significant antibody response either after repeated systemic administration in the presence of adjuvants (Fig. 6A) or when applied locally (Fig. 6B).

Overall, these experiments demonstrate that inhalation of PI3Kγ MP can be safely employed to boost β_2-AR/cAMP signaling in the airways.

The PI3Kγ mimetic peptide induces airway relaxation in a mouse model of asthma

Since activation of the β_2-AR/cAMP signaling has well-established relaxant effects in airway smooth muscle, the impact of PI3Kγ scaffold activity on airway tone was next investigated by measuring lung resistance in healthy wild-type mice pre-treated with an aerosol of PI3Kγ MP, a control peptide (CP) or saline, before exposure to increasing doses of the contracting agent methacholine (MCh). MCh triggered a dose-dependent increase in airway resistance that was significantly lower in mice treated with PI3Kγ MP than in animals exposed to either CP or vehicle (Fig. 7A). To further explore the therapeutic potential of the PI3Kγ MP, we tested the ability of the peptide to promote airway relaxation in ovalbumin (OVA)-sensitized mice, a well-established model of asthma (Matthey, Roberts et al. 2017). As with healthy animals, single-dose inhalation of the PI3Kγ MP significantly increased cAMP levels in lungs and tracheas (Fig. 7B) and reduced MCh-induced bronchoconstriction in OVA mice, as evidenced by measurements of both tidal volume (Fig. 7C) and lung resistance (Fig. 7D).

Overall, these findings support the potential of the PI3Kγ MP to alleviate the bronchoconstriction associated with chronic respiratory diseases, like asthma, via elevation of cAMP.

The PI3Kγ mimetic peptide limits neutrophilic inflammation in a mouse model of asthma

In addition to their bronchodilator properties, cAMP-elevating agents exhibit anti-inflammatory effects (Barnes 2004). To verify whether the PI3Kγ MP could relieve the inflammation that occurs in chronic airway diseases, OVA mice were administrated with either PI3Kγ MP or CP during the induction of the inflammatory phase (before each OVA challenge). As expected, mice treated with the CP exhibited extensive immune cell infiltration around the bronchi and elevated mucin secretion, as evidenced by staining of lung sections with H&E and PAS, respectively (Fig. 8A and B). Further characterization of immune cells in the bronchoalveolar lavage (BAL) fluid revealed a significant elevation of both neutrophils and eosinophils in allergen-sensitized mice treated with the CP compared to naïve animals (Fig. 8C). Conversely, peribronchial inflammation and mucin production were significantly dampened in mice repeatedly exposed to the PI3Kγ MP (Fig. 8A and B). Moreover, a significantly lower number of neutrophils was detected in the BAL fluid of PI3Kγ MP-treated mice than in control animals (Fig. 8C). These experiments indicated that the PI3Kγ MP is capable of inhibiting the neutrophilic inflammation associated with asthma.

In further support of a role of PI3Kγ scaffold activity in modulating neutrophil function, the PI3Kγ MP inhibited chemoattractant-induced adhesion of

human neutrophils to the intercellular adhesion molecule 1 (ICAM-1) (Fig. 9A), the intercellular adhesion molecule 2 (ICAM-2) (Fig. 9B) and fibrinogen (Fig. 9C), thus explaining the reduced number of this inflammatory cell type in the bronchi of asthmatic mice upon peptide treatment. To confirm that the anti-adhesive properties of the PI3Kγ MP were linked to an upregulation of the cAMP/PKA signaling, neutrophils were pre-treated with a low dose of the PKA inhibitor H89 (200 nM). PKA inhibition completely rescued neutrophil adhesion to ICAM-1, ICAM-2 as well as fibrinogen in the presence of the PI3Kγ MP (Fig. 9A-C), indicating that impaired adhesion depended on PKA hyperactivation. In line with an essential role of ICAM-1 in neutrophil migration in the airways (Chong, Rebeyrol et al. 2021), the PI3Kγ MP was found to significantly dampen neutrophil chemotaxis in response to the chemotactic factor fMLP, an effect that was again abrogated by the PKA inhibitor H89 (Fig. 10A). Because the cAMP/PKA cascade restrains chemoattractant-induced activation of LFA1 by blocking the RhoA small GTPase (Laudanna, Campbell et al. 1997), GTP-bound RhoA was measured. We found that fMLP-induced RhoA activation was fully abrogated in neutrophils treated with the PI3Kγ MP (Fig. 10B).

Taken together, these findings uncover the ability of the PI3Kγ MP to limit neutrophilic airway inflammation by dampening neutrophil adhesion and transmigration, an effect that could be desirable in chronic airway diseases such as asthma, CF or COPD.

The PI3Kγ mimetic peptide affects macrophage activation without altering their bacterial killing capacity

Beside affecting neutrophil adhesion and migration, the cAMP/PKA signaling pathway has also been found to promote macrophage polarization towards an M2-like anti-inflammatory phenotype (Na, Kwon et al. 2020, Negreiros-Lima, Lima et al. 2020). Therefore, we explored the possibility that the PI3Kγ MP affects macrophage polarization via performing flow cytometric analysis of murine peritoneal macrophages either treated with IL-10 to induce M2 polarization, the peptide, or with the combination of the two compounds. As expected, IL-10 significantly induced M2 polarization, while the PI3Kγ MP showed a similar trend, although without reaching statistical significance (Fig. 11A and B). Of note, the peptide did not amplify the effect of IL-10 on macrophage polarization towards the M2 phenotype in cells exposed to both compounds (Fig. 11A and B). We concluded that the compartment-restricted cAMP elevation elicited by the peptide likely does not control macrophage polarization. On the contrary, the peptide reduced the total number of MHC II+ cells (Fig. 11C), suggesting that this PI3Kγ-dependent pool of cAMP could regulate macrophage activation.

Considering the effect of the PI3Kγ MP on macrophage activation, and in general on the anti-inflammatory action of the compound, a potential problem concerning PI3Kγ MP administration in patients affected by chronic airway diseases could be an increased risk of developing local respiratory infection. For this reason, we performed an *in vitro* bacterial killing assay with primary peritoneal macrophages pre-treated with the PI3Kγ MP and then exposed to *E. Coli*. In keeping with published studies, we found that the peptide does not

decrease but rather slightly improves the killing activity of macrophages (Fig. 11D).

Overall, these results imply that the peptide-mediated cAMP elevation reduced activation rather than recruitment of macrophages, without affecting their bacterial killing capacity required to fight infections in patients affected by chronic respiratory disease.

The PI3Kγ mimetic peptide promotes cAMP/PKA-dependent phosphorylation of the CFTR channel in airway epithelial cells

Activation of the cAMP/PKA pathway in airway epithelial cells is well known to induce the secretion of Cl^- via the CFTR channel. Thus, the ability of the PI3Kγ MP to activate CFTR-dependent Cl^- secretion was examined. First, we assessed PKA-mediated phosphorylation of the channel by probing CFTR immunoprecipitates with an antibody recognizing substrate motifs for PKA. CFTR phosphorylation was 5-fold higher in immortalized human bronchial epithelial (16HBE14o-) cells treated with the PI3Kγ MP than in cells exposed to either vehicle or CP (Fig. 12A). Of note, the PI3Kγ MP heightened CFTR phosphorylation at the same level of cells treated with the PDE4 inhibitor rolipram, implying that the PI3Kγ MP likely impacts on PDE4-mediated regulation of CFTR (Fig. 12A) (Blanchard, Zlock et al. 2014). To further support the ability of the PI3Kγ MP to trigger PKA-mediated phosphorylation of the channel, the phospho-occupancy of known PKA sites in the regulatory domain of CFTR was analysed by liquid chromatography (LC)-coupled tandem mass spectrometry (LC-MS/MS) in cystic fibrosis human bronchial epithelial cells (CFBE41o-). These cells expressing His6-

biotin-His6 (HBH)-tagged wt-CFTR variant, containing 3xhemagglutinin (3HA)-tag in the 4th extracellular loop (HBH-CFTR-3HA) (Schnur, Premchandar et al. 2019), were treated with CP, PI3Kγ MP, Fsk or vehicle. CFTR was then affinity-purified from the cell lysates and subjected to LC-MS/MS. While Fsk-mediated adenylyl cyclase activation triggered the phosphorylation of most CFTR phosphorylation sites, the PI3Kγ MP selectively increased the phospho-occupancy of S737 and, to a lesser extent, of S753 (Fig. 12B). To confirm that the PI3Kγ MP primarily acts through S737, CFTR phosphorylation was assessed in human embryonic kidney cells (HEK293T) expressing either wt-CFTR or a non-phosphorylatable mutant where the serine was replaced by alanine (S737A-CFTR). In agreement with mass spectrometry results, the CFTR phosphorylation elicited by the PI3Kγ MP was completely abolished in S737A-CFTR-HEK293T cells (Fig. 12C).

Taken together, these findings indicate that the PI3Kγ MP, through its local elevation of cAMP, induced CFTR phosphorylation selectively at S737 residue.

The PI3Kγ mimetic peptide promotes cAMP-dependent activation chloride efflux and water secretion in airway epithelial cells

Because phosphorylation of S737 can lead to a ~25% increase in the channel open probability (Po), we anticipated that the PI3Kγ MP could activate CFTR conductance (Hegedus, Aleksandrov et al. 2009). The effects of the PI3Kγ MP on Cl^- secretion were validated by I_{SC} measurements in primary human bronchial epithelial (HBE) cells, where the peptide increased CFTR currents in a dose-dependent manner, while no effect was observed after application

of the CP (Fig. 13A). Notably, no further increase in I_{SC} was observed when the PDE4 inhibitor rolipram was applied on top of 30 µM PI3Kγ MP (Fig. 13A), suggesting that the peptide likely inhibits the PDE4 pool associated to CFTR regulation (Hegedus, Aleksandrov et al. 2009). Boosting cAMP production with Fsk produced an additional increment of I_{SC} that was inhibited by the CFTR inhibitor 172 (CFTRinh-172), demonstrating that the current was CFTR-mediated (Fig. 13A). To confirm that the peptide triggers CFTR activation in a cAMP-dependent manner, CFTR currents were measured in HBE cells treated with a non-hydrolysable cAMP analog (CPT-cAMP) before and after exposure to the PI3Kγ MP. While CPT-cAMP further increased I_{SC} on top of the PI3Kγ MP (Fig. 13B), the pre-treatment with the analogue totally abolished the ability of the peptide to elevate CFTR currents (Fig. 13C), demonstrating that the PI3Kγ MP activates the channel through a cAMP/PKA-mediated mechanism.

Primary HBE cells express other ion channels and transporters that can indirectly influence CFTR activity by increasing the electrochemical driving force for luminal Cl^- secretion (Martin, Saint-Criq et al. 2018). Therefore, we next investigated to what extent Cl^- conductance elicited by the PI3Kγ MP depended either on the direct activation of CFTR or on the modulation of other channels. In the presence of the CFTR blocker CFTRinh-172, the PI3Kγ MP retained the ability to induce a transient increase in I_{SC} (Fig. 13D) that is characteristic of the activation of Ca^{2+}-activated Cl^- channels (CaCCs). Of note, the current decreased to baseline after application of clotrimazole (Fig. 13D), an inhibitor of basolateral Ca^{2+}-activated K^+ channels, and bumetanide, an inhibitor of the Na-K-Cl cotransporter NKCC1 (Fig. 13D). These results

suggested that the peptide can promote luminal Cl^- secretion also via CaCCs and can activate ion channels that regulate the driving force for luminal Cl^- exit, like Ca^{2+}-activated K^+ channels (Kunzelmann and Mall 2002). To unmask the direct action of the PI3Kγ MP on CFTR currents, Ca^{2+} stores were first depleted with thapsigargin and then CaCCs were blocked using a general CaCC inhibitor (Fig. 13E). In the absence of functional CaCCs, the peptide elicited a response that was fully abolished by the CFTRinh-172 confirming a direct activation of CFTR by the PI3Kγ MP (Fig. 13E).

The ability of the peptide to directly trigger CFTR-mediated Cl^- currents was finally corroborated in rectal organoids, where CaCCs are not consistently expressed, and organoid size (swelling) in response to Fsk (FIS) is CFTR-dependent (Clancy, Cotton et al. 2019). In agreement with measurement of I_{SC} in polarized epithelial monolayers, the PI3Kγ MP potentiated by 2-fold the swelling of wild-type organoids elicited by a low dose of Fsk (2 μM) priming cAMP production (Fig. 14A). Because the increase in organoid size elicited by Fsk is the result of water influx into the organoid lumen secondary to CFTR activation (Dekkers, Wiegerinck et al. 2013), we anticipated that the PI3Kγ MP could control water secretion from epithelial cells, which is essential for proper mucus hydration and clearance (Mall 2016). Treatment of HEK293T expressing wt-CFTR with the PI3Kγ MP, but not with CP, decreased intracellular water residence time, indicative of rapid water efflux (Fig. 14B).

Altogether, these data demonstrate that the PI3Kγ MP peptide drives Cl^- and consequent water secretion in bronchial epithelial cells through a cAMP-

dependent mechanism, coordinating direct CFTR gating with electrochemical driving force elevation.

The PI3Kγ mimetic peptide increases the amount of plasma membrane CFTR in airway epithelial cells

Beside triggering CFTR phosphorylation and gating, stimulation of the β_2-AR/cAMP signaling has been shown to promote stabilization of the channel at the plasma membrane (PM) (Trotta, Guerra et al. 2015, Lobo, Amaral et al. 2016). Thus, the ability of the PI3Kγ MP to affect CFTR localization at the PM was assessed. In 16HBE14o- cells, CFTR levels at the PM were significantly increased 2 hours after PI3Kγ MP administration compared to CP (Fig. 15A), and this effect was already detectable 5 minutes after treatment, persisting over 30 minutes (Fig. 15B). In line with the previous experiment, the PI3Kγ MP also increased F508del-CFTR exposure at PM in cystic fibrosis human bronchial epithelial cells stably expressing this variant of the CFTR protein (F508del-CFBE41o-) (Fig. 16 A and B). These results clearly suggest that the PI3Kγ MP could be implemented to increase wt-CFTR localization at the PM and to rescue F508del-CFTR trafficking. Intriguingly, upon PI3Kγ MP treatment, we detected an increased signal of the CFTR B band, known as the ER core-glycosylated immature form of the channel (Fig 16A). This indicates that the peptide specifically promotes F508del-CFTR unconventional secretion pathway, through by which proteins bypass the Golgi apparatus to directly target the cell surface (Gee, Noh et al. 2011). To investigate whether CFTR stabilization induced by the PI3Kγ MP was dependent on the activation of the cAMP/PKA signaling, we analysed CFTR levels at the PM in HEK293T-transfected cells after pharmacological

inhibition of PKA. Unexpectedly, upon pre-treatment with H89, the PI3Kγ MP retained the ability to increase CFTR levels at the PM in HEK293T expressing F508del-CFTR (Fig. 17A). The peptide preserved the same effect also in cells treated with PKI, a much more selective PKA-inhibiting compound (Fig 17B). Furthermore, the capability of the PI3Kγ MP to increase CFTR levels at the PM in a PKA-independent manner was also observed in F508del-CFBE41o- cells (Fig. 18A and B). In agreement, cAMP analogues, namely CPT-cAMP and 8-Br-cAMP, and Fsk failed to recapitulate CFTR stabilization at cell surface in F508del-CFBE41o- cells, confirming the involvement of an alternative cAMP/PKA-independent mechanism in the F508del-CFTR stabilization promoted by the PI3Kγ MP (Fig. 19).

Overall, these data validated PI3Kγ as a new pivotal protein regulating CFTR localization at the PM via a cAMP/PKA independent mechanism.

The PI3Kγ mimetic peptide promotes CFTR exocytosis through protein-kinase D1 (PKD1) activation

The finding that the cAMP/PKA signaling pathway was not involved in PI3Kγ MP-mediated CFTR trafficking prompted us to corroborate the mechanism(s) whereby the peptide could induce CFTR stabilization through an unbiased approach. To explore putative candidate targets, we exploited a KinexTM KAM antibody microarray platform using phospho-antibodies to screen for protein phosphorylation events upon treatment with either PI3Kγ MP or CP in CFBE41o- cells. Notably, the Ser910 (S910) residue of protein kinase D1 (PKD1) emerged as the most significantly phosphorylated target

upon PI3Kγ MP treatment (Fig. 20). In further support of this result, we validated by Western blot that the PI3Kγ MP was able to elicit phosphorylation of S910 as well as Ser744-748 (S744-748) residues of PKD1 in human pancreatic epithelial cells (PANC-1) (Fig. 21A), known to express the kinase at high levels. Interestingly, PKD1 has been found to localize in the vicinity of PM to regulate exocytosis and cellular trafficking (di Blasio, Droetto et al. 2010), thus highlighting its potential involvement in CFTR stabilization upon PI3Kγ MP treatment. Putative downstream target of this signaling cascade is the Golgi stacking proteins isoform 55 (GRASP55) (Hausser, Storz et al. 2001), known to play critical roles in the unconventional surface transport of core-glycosylated wt- and F508del-CFTR (Gee, Noh et al. 2011). Therefore, we asked whether silencing of GRASP55 could interfere with CFTR stabilization upon peptide treatment. Notably, upon GRASP55 silencing, the PI3Kγ MP failed to restore CFTR trafficking in HEK293T expressing F508del-CFTR (Fig. 21B). Together, these experiments indicate that the PI3Kγ MP induced CFTR trafficking possibly through PKD1 activation with consequent downstream GRASP55 modulation.

The PI3Kγ mimetic peptide enhances the therapeutic effects of CFTR modulators in cystic fibrosis models

Next, we sought to investigate to what extent the PI3Kγ MP could be exploited for the functional rescue of the most common CF-causing CFTR mutant (F508del-CFTR). F508del-CFTR exhibits multiple molecular defects that require the combined use of correctors (VX-809/lumacaftor or VX-661/tezacaftor or VX-445/elexacaftor) and a potentiator (VX-770/ivacaftor) to restore the PM localization and channel gating, respectively (Gentzsch and

Mall 2018). Because VX-770-mediated potentiation of F508del-CFTR is phosphorylation-dependent (Chin, Hung et al. 2017), we anticipated that the PI3Kγ MP could boost the efficacy of the first-generation drug combination lumacaftor/ivacaftor in primary human bronchial epithelial cells derived from two CF patients homozygous for the F508del mutation (F508del/F508del HBE). Cells were initially exposed to the CFTR corrector VX-809 for 24 hours to restore membrane localization of the channel, and then treated acutely with the potentiator VX-770 and the PI3Kγ MP peptide. While, in the absence of Fsk pre-treatment, VX-770 only slightly increased I_{SC} in F508del/F508del HBE, the PI3Kγ MP potentiated the current by 5 folds when added on top of VX-770 (Fig. 22A and B). Similar results were obtained in VX-809-corrected F508del/F508del HBE cells from a second donor (Fig. 22C).

The synergy between the CFTR potentiator VX-770 and the PI3Kγ MP was further supported by FIS assays in intestinal organoids. The effect of the peptide was first assessed on compound heterozygote CF organoids bearing the F508del mutation with the residual function D1152H mutation. Organoids were corrected with VX-809 for 24 hours before sequential treatment with the potentiator VX-770, a low dose of Fsk and either the PI3Kγ MP or CP. At the end of the treatment, organoid size was 50% higher in the group treated with the active peptide than in those exposed to CP (Fig. 23A). Moreover, pre-incubation with the CFTR inhibitor CFTRinh-172 fully prevented organoid swelling in response to PI3Kγ MP+VX-770+Fsk (Fig. 23A), confirming a specific effect of the peptide on CFTR-mediated Cl^- efflux. Next, we assessed the impact of the PI3Kγ MP on F508del homozygous organoids

that were exposed chronically to both the corrector VX-809 and the potentiator VX-770 for 24 hours before acute application of Fsk plus the PI3Kγ MP, to better mimic chronic CF therapeutic regimens and the consequent destabilizing effects of VX-770 on pharmacologically-corrected F508del-CFTR (Cholon, Quinney et al. 2014). The peptide significantly increased organoid size in a dose-response manner, and up to 6.5-fold the volume of control organoids which were exposed to the combination VX-770+VX-809 only (Fig. 23B). Of note, the maximal synergy between the peptide and CFTR modulators was observed at a low non-saturating dose of Fsk (0.051 µM), which was expected to minimally increase cAMP levels, and which was almost ineffective in inducing swelling in the control VX-770+VX-809 group (Fig. 23B). These results indicate that the interaction between the PI3Kγ MP and CFTR modulators relies on the activation of cAMP/PKA signaling.

Finally, we assessed the ability of the PI3Kγ MP to enhance the therapeutic effects of the recently approved, next-generation triple combination elexacaftor/tezacaftor/ivacaftor (VX-445+VX-661+VX-770). By Western blot, following PI3Kγ MP and Trikafta® treatment, the CFTR amount detected at the PM in F508del-CFBE41o- was significantly higher compared to cells exposed to CFTR modulators alone, indicating that the peptide enhanced the activity of CFTR correctors (Fig. 24A). To confirm these data, we performed electron microscopy in cells receiving VX-445+VX-661+VX-770 alone or in combination with the PI3Kγ MP and we found that the amount of F508del-CFTR at the PM in HEK293T cells was higher when the peptide was used as an add on therapy with the triple combination (Fig. 24B). Next,

to evaluate whether the PI3Kγ MP improves Trikafta® activity on CFTR conductance, 508del/F508del HBE cells from two different CF donors were exposed to VX-661 and VX-445 correctors alone or together with the PI3Kγ MP for 24 hours, and then treated acutely with the CFTR potentiator VX-770. VX-770-mediated Cl^- currents were 40% higher in cells treated chronically with the PI3Kγ MP than in those exposed only to VX-661 and VX-445 (Fig. 25A and B). In both cases, the cAMP analogue CPT-cAMP further increased Cl^- currents which were inhibited by the CFTR blocker CFTRinh-172, demonstrating that Cl^- secretion was CFTR-dependent. Thus, our data support the use of the PI3Kγ MP to increase the efficacy of all combinations of CFTR modulators that include the VX-770 potentiator.

Taken together, these findings demonstrate that the PI3Kγ MP could boost the therapeutic effects of CFTR potentiator and corrector drugs in the presence of physiological submaximal cAMP stimulation, and at the same time ensure bronchodilation and anti-inflammatory effects, for the treatment of chronic airway disease, including asthma and CF.

Discussion

Our results establish that targeting the PKA-anchoring function of PI3Kγ with a mimetic peptide allows therapeutic manipulation of β_2-AR/cAMP signaling in multiple cell types participating to the pathogenesis of chronic obstructive airway diseases. These findings are consistent with a model where PI3Kγ acts as a scaffold protein for PKA (AKAP) in a complex containing the PKA-dependent phosphodiesterase PDE4 (Maurice, Ke et al. 2014), favouring its activation and localized cAMP reduction at the plasma membrane. Accordingly, the pharmacological actions of the PI3Kγ MP stem from its ability to displace PKA from the PI3Kγ complex, thereby preventing PKA-mediated stimulation of a pool of PDE4 that is responsible for lowering cAMP levels close to neighbouring distinct PKA-containing complexes, including that regulating CFTR gating (Blanchard, Zlock et al. 2014).

Although in the last decade numerous inhibitors of the kinase activity of PI3Kγ have been developed, many of which are currently in clinical development for treating neoplastic diseases (De Henau, Rausch et al. 2016, Faia, White et al. 2018), to our knowledge the PI3Kγ MP is the first compound that has been developed to selectively interfere with the anchor protein activity of PI3Kγ, acting as a cAMP-elevating agent. Of note, in the last years peptides have gained increasing interest as therapeutics, thanks to their high selectivity towards the target as well as enhanced efficacy. This specificity is also responsible for excellent safety, tolerability, and efficacy profile of these compounds in humans. However, peptide therapeutics may show some intrinsic weaknesses, including poor chemical and physical stability, and immunogenicity. Our data suggest the possibility to safely use the PI3Kγ MP as an inhaled therapy to target respiratory diseases, since it is

able to efficiently reach the airways but also does not elicit an antibody response. In addition, the presence and, most importantly, the efficacy of the compound 1 day after treatment in mice envisage the possibility of a daily administration of the compound in patients, which eventually may replace the current time-consuming regimens including multiple drugs. Moreover, differently from β_2-AR agonists, the PI3Kγ MP acts through a peculiar mechanism with at least two key advantages. On the one hand, the PI3Kγ MP amplifies β_2-AR/cAMP responses by impinging on cAMP degradation rather than on β_2-AR activation, avoiding receptor desensitization that, in the long-term, is the major cause of reduced efficacy. On the other, being a cell permeable peptide of 5 KDa, the inhaled PI3Kγ MP can be topically delivered to the lungs, where it safely boosts airway cAMP without reaching other tissues where cAMP elevation would not be desirable, such as in the heart (Ghigo, Perino et al. 2012). The local action of the peptide provides a significant added value over other cAMP elevating agents, i.e. classical small molecule PDE4 inhibitors, like roflumilast, that easily diffuse outside the lungs and trigger undesired brain and cardiac effects, especially critical when chronic treatments are needed (Oba 2013). Additionally, small molecule PDE4 inhibitors lead to indiscriminate inhibition of all the different PDE4 subtypes (PDE4A, B, C and D), potentially determining further side effects. Intriguingly, the PI3Kγ MP offers the opportunity of blocking selective PDE4 subtypes with a prominent role in the lung, like PDE4B and PDE4D (Zuo, Cattani-Cavalieri et al. 2019), with an exquisite isoform and compartment selectivity.

Consistent with the pro-relaxing action of cAMP, the PI3Kγ MP demonstrated prominent bronchodilator effects *in vivo* in healthy and asthmatic mice, which could be explained by enhanced β_2-AR/cAMP signaling secondary to PDE4 inhibition in airway smooth muscle cells. These results are in agreement with the reduced airway smooth muscle contractility observed in PDE4D knock-out mice (Mehats, Jin et al. 2003) and with the enhanced β_2-AR-stimulated cAMP levels in human smooth muscle cells following knock down of PDE4D5 (Billington, Le Jeune et al. 2008).

Besides airway smooth muscles, PDE4 is enriched in immune cells and the anti-inflammatory properties of roflumilast, as well as of other PDE4 inhibitors, is well recognized (Zuo, Cattani-Cavalieri et al. 2019). The finding that the PI3Kγ MP peptide specifically inhibits neutrophil recruitment to the lung in a mouse model of asthma makes this molecule particularly attractive for the treatment of chronic airway disease subtypes that are characterized by a prominent neutrophilic inflammation. These conditions, which remain the most difficult to treat (Gernez, Tirouvanziam et al. 2010), include neutrophilic asthma, a severe form of the disease which has been associated to corticosteroid insensitivity (Ray and Kolls 2017), but also COPD and CF lung disease (Butler, Walton et al. 2018, Roesch, Nichols et al. 2018). Another strength related to the possible administration of the PI3Kγ MP in clinics is the fact that, compared to inflammatory drugs for chronic airway diseases like inhaled corticosteroids (Reidl and Monso 2015), the compound is not expected to increase the risk of respiratory infections. Indeed, our results indicate that the PI3Kγ MP might not affect the killing capacity of macrophages, in line with recent studies on pharmacological and genetic

inhibition of PDE4 (Tavares, Garcia et al. 2016, Abou Saleh, Boyd et al. 2021). This is particularly relevant to CF patients who already suffer from devastating infections that are the main cause of lung function decline and, ultimately, of mortality (Shteinberg, Haq et al. 2021).

Another effect of targeting PDE4 is the cAMP/PKA-dependent gating of the CFTR channel, which increases airway surface liquid and facilitates mucus clearance (Turner, Abbott-Banner et al. 2021). Of note, CFTR functional defects and mucus stasis can be observed in patients with COPD and certain forms of asthma (Patel, Bono et al. 2020) but are dramatically critical in CF (Elborn 2016). Previous reports identified PDE4D as a critical negative regulator of the cAMP/PKA-dependent activation of wild-type CFTR in bronchial epithelial cells, highlighting the potential of PDE4 inhibitors to stimulate the channel (Blanchard, Zlock et al. 2014, Turner, Luo et al. 2020). Our study extends this view by pinpointing PI3Kγ as a key AKAP orchestrating cAMP-mediated signal transduction in a microdomain known to involve β_2-ARs and PDE4D, together with the CFTR channel (Naren, Cobb et al. 2003).

While a generalized cAMP elevation, as that induced by forskolin, correlated with the phosphorylation of most of CFTR phospho-sites, the local cAMP elevation triggered by the PI3Kγ MP resulted in the selective phosphorylation of S737. Although S737 phosphorylation is known to play a key role in the modulation of the open probability of the channel, a few reports indicate opposite actions (Rich, Gregory et al. 1993, Vais, Zhang et al. 2004, Hegedus, Aleksandrov et al. 2009) that likely depend on contextual modifications of other phosphorylation sites (Baldursson, Berger et al. 2000, Kongsuphol,

Cassidy et al. 2009). Despite this controversy, our observations indicate that selective phosphorylation of S737 upon PI3Kγ MP treatment leads to increased CFTR currents, in line with previous findings in CFTR mutants where the reintroduction of S737 in a PKA-insensitive CFTR variant increases the open probability of the channel to an extent comparable to that reached by the peptide (Hegedus, Aleksandrov et al. 2009).

Intriguingly, the PI3Kγ MP contributes to Cl^- secretion not only through a direct action on the CFTR, but also by engaging Ca^{2+}-activated Cl^- channels (CaCCs) as well as basolateral, clotrimazole-sensitive Ca^{2+}-activated K^+ channels, the latter providing the electrochemical driving force for luminal Cl^- exit (Martin, Saint-Criq et al. 2018). The activation of these Ca^{2+}-dependent channels is consistent with the potential of cAMP/PKA signaling to increase cytoplasmic Ca^{2+} levels in airway epithelial cells (Ahuja, Jha et al. 2014). Whether the effects mediated by the activation of these CaCCs are either beneficial or detrimental it is still debated. For example, TMEM16A activity on one hand causes excessive mucus secretion during inflammation, on the other hand supports ciliated airway epithelial cells-mediated fluid secretion and airway smooth muscle contraction (Kunzelmann, Ousingsawat et al. 2019). However, benzimidazolones activity (Mall, Gonska et al. 2003, Roth, Hirtz et al. 2011) tend to support the notion of CaCCs synergizing with the direct activation of the CFTR, indicating that the PI3Kγ MP can coordinate different mechanisms culminating in Cl^- secretion, provided that sufficient CFTR is appropriately located at the plasma membrane. Of note, this increased efflux of Cl^- upon administration of the peptide was paralleled by a significant water efflux from epithelial cells, corroborating the ability of

the PI3Kγ MP to potentially restore mucus hydration and clearance in obstructive airway disease.

Consistent with a key role of cAMP not only in regulating the gating of the CFTR but also its stabilization at the plasma membrane (Lobo, Amaral et al. 2016), we found that the PI3Kγ MP stabilize both wild-type and the most prevalent CF mutant (F508del-CFTR) on the cell surface, highlighting the possibility of using the PI3Kγ MP to simultaneously target different aspects of CFTR dysfunction. More specifically, the PI3K MP increases at the plasma membrane the amount of F508del-CFTR in its ER core-glycosylated immature form, indicating that the peptide induces F508del-CFTR stabilization likely through the "unconventional trafficking" route (Gee, Noh et al. 2011) from the endoplasmic reticulum to the plasma membrane, bypassing Golgi-mediated conventional exocytosis. Although the physiological relevance of such process remain obscure, our results identify PI3Kγ as a potential key regulator of this trafficking route. Unexpectedly, this PI3Kγ MP-mediated effect on CFTR trafficking occurred in a cAMP/PKA independent manner, suggesting the involvement of alternative mechanism(s). Our unbiased protein kinase array approach identified PKD1 as a possible molecular mediator of the effect of the peptide. We observed that the PI3Kγ MP elicits phosphorylation of PKD1 on S910 as well as S744-748, two phospho-sites that can be used to monitor the activation status of the protein (Zhang, Connelly et al. 2021). Recent research has identified PKD1 as a serine/threonine kinase with a crucial role in protein trafficking, regulating fission of transport vesicles that are on their way to the plasma membrane and promoting recycling of integrins to different membrane

regions (di Blasio, Droetto et al. 2010, Steinberg 2012, Zhang, Connelly et al. 2021). Such evidence corroborates our hypothesis that PKD1 is possibly involved in mediating CFTR trafficking, despite the mechanism underlying PKD1 phosphorylation following peptide administration remains to be investigated. Among downstream effectors of PKD1 signaling is GRASP55, a protein that has been previously involved in Golgi membrane stacking (Hausser, Storz et al. 2001) as well as F508del-CFTR unconventional trafficking (Gee, Noh et al. 2011). Our observation that the PI3Kγ MP fails to rescue F508del-CFTR trafficking upon GRASP55 silencing, points to a role of the GRASP55/PKD1 axis in this mechanism activated by the PI3Kγ MP. GRASP55 has been demonstrated to interact with the C-terminus of the CFTR via PDZ-domain and that this interaction is crucial to achieve CFTR unconventional trafficking (Gee, Noh et al. 2011). PKD1 similarly presents a PDZ domain near the C-terminus that could potentially interact with the PDZ binding domain of the CFTR, driving channel trafficking. Noteworthy, the substitution of S910 with alanine (S910A) alters the dynamics of PKD1-dependent cellular responses, such as trafficking (Gee, Noh et al. 2011), by disrupting docking interactions with scaffold proteins containing PDZ domains. In this direction, further investigations are required to understand the exact mechanism through which the peptide induces PKD1 phosphorylation as well as to examine whether this protein co-localizes or interacts with the CFTR.

The ability of the PI3Kγ MP to promote CFTR stabilization and gating encouraged us to explore whether the peptide can be exploited to increase the therapeutic efficacy of approved CFTR modulators used in clinics. This

implies the use for therapy of CFTR correctors to enable the plasma membrane exposure of the mutant CFTR, combined with potentiators inducing a conformational shift that facilitate PKA-dependent gating of the channel (Ahuja, Jha et al. 2014). In line with this view, the efficacy of VX-770, the gold-standard CFTR potentiator found in all the approved combinations of CFTR modulators, depends on concomitant cAMP/PKA phosphorylation of the channel (Eckford, Li et al. 2012). While forskolin has been extensively used to increase cAMP in the preclinical testing of all CFTR modulators, the PI3Kγ MP ensures a more physiological and compartment-restricted increase in cAMP in the vicinity of CFTR that maximized the action of all combinations, including both lumacaftor/ivacaftor (LUMA/IVA) and elexacaftor/tezacaftor/ivacaftor (ETI). While the first drug combination LUMA/IVA failed to prove significant clinical benefit (Elborn, Ramsey et al. 2016), the latest released ETI is highly effective, with lung function improvements in CF F508del patients up to 14% change in predicted forced expiratory volume in 1 second [ppFEV1]) (Heijerman, McKone et al. 2019, Middleton, Mall et al. 2019). Despite the marked efficacy, the rescue of CFTR activity and lung function by ETI is still incomplete, only reaching 60% of physiological values (Veit, Roldan et al. 2020), which leaves CF as a still lethal disease. Our observation that the peptide can triplicate the amount of plasma membrane CFTR and almost double the gating of the F508del-CFTR mutant after correction and potentiation with ETI suggests that targeting the AKAP function of PI3Kγ might represent an avenue for reinstating F508del-CFTR activity close to 100% of the wild-type function. As such, the model where CFTR activity measured *in vitro* in HBE cells is linearly proportional to patients' clinical improvement (change in ppFEV1) (Mall, Mayer-

Hamblett et al. 2020) predicts that the addition of the PI3Kγ MP on top of existing treatments can maximize CFTR opening, at least matching what measured in healthy carriers of CFTR mutations. In addition, the PI3Kγ mimetic peptide may also be effective on rare, not yet treatable mutations, such as some class III-IV rare mutations which have been reported to be responsive to β2-adrenergic receptor agonists (Vijftigschild, Berkers et al. 2016, Turner, Luo et al. 2020).

While initial preclinical toxicology studies in non-rodents have shown that the inhaled PI3Kγ MP remains confined in the lungs and is tolerable, additional investigations are awaited to corroborate the ability of the aerosolized peptide to overcome the biological barriers imposed by the disease, such as the mucus (d'Angelo, Conte et al. 2014). Nonetheless, our results demonstrate that the amount of the compound that penetrates the mucus layer of the ovalbumin model of inflammatory lung disease is sufficient to elicit clinically meaningful responses. In addition, the treatment with ETI is associated with a substantial improvement in mucus mobilization (Morrison, Shaffer et al. 2021) and could thus facilitate the combined action of the PI3Kγ MP.

Taken together, this study highlights the therapeutic potential of increasing cAMP concentration close to the plasma membrane with an unprecedented subcellular compartmentalization. With its pharmacological properties, the PI3Kγ MP might be useful for the treatment of airway diseases including asthma and COPD where cAMP-elevating agents with broncho-relaxant properties are highly desirable. In addition, by inhibiting PDE4, the PI3Kγ MP may exert a selective activity on neutrophil adhesion and pulmonary

recruitment. Furthermore, the PI3Kγ MP might find an application in CF that, despite the success of currently approved modulators, is still in high demand for treatments allowing CF patients a normal lifespan (Bell, Mall et al. 2020). As such, future clinical testing of PI3Kγ MP is expected to clarify the therapeutic potential of a compound combining bronchodilator, anti-inflammatory activity and CFTR modulation in patients with CF, COPD and asthma.

Figures

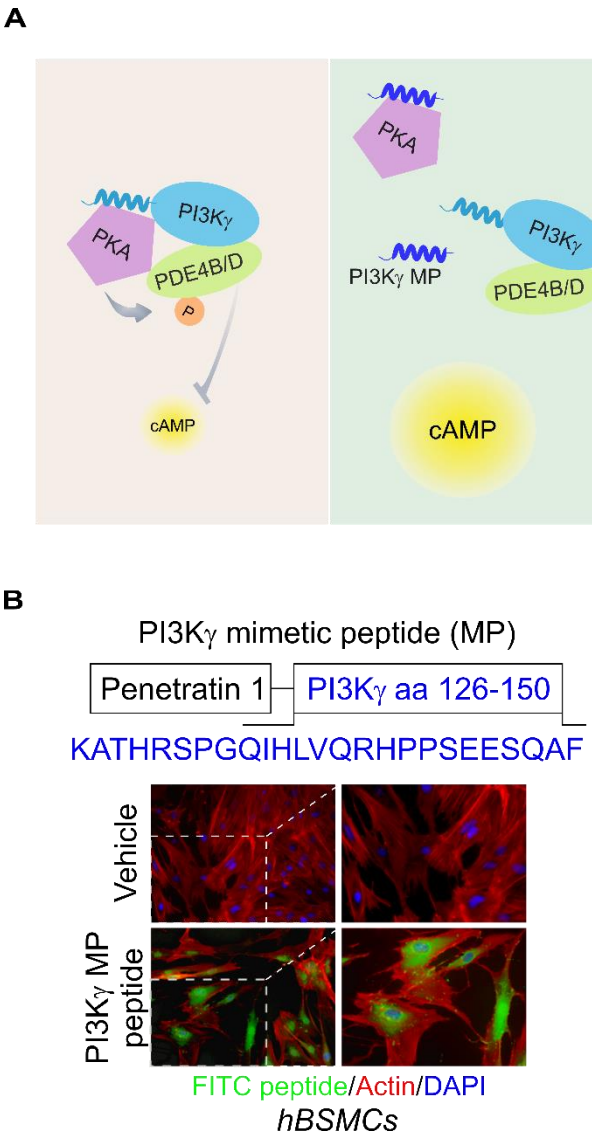


Figure 1. Design of the cell permeable PI3Kγ mimetic peptide. (**A**) Graphical representation of the molecular mechanism by which the PI3Kγ mimetic peptide increases cAMP levels . (**B**) Top, schematic representation of the cell-permeable PI3Kγ mimetic peptide (PI3Kγ MP). The 126-150 region of PI3Kγ was fused to the cell-penetrating peptide Penetratin 1 (P1). Bottom, intracellular fluorescence of human bronchial smooth muscle cells (hBSMCs) following 1 hour incubation with a FITC-labeled version of the PI3Kγ MP (50 µM) or vehicle.

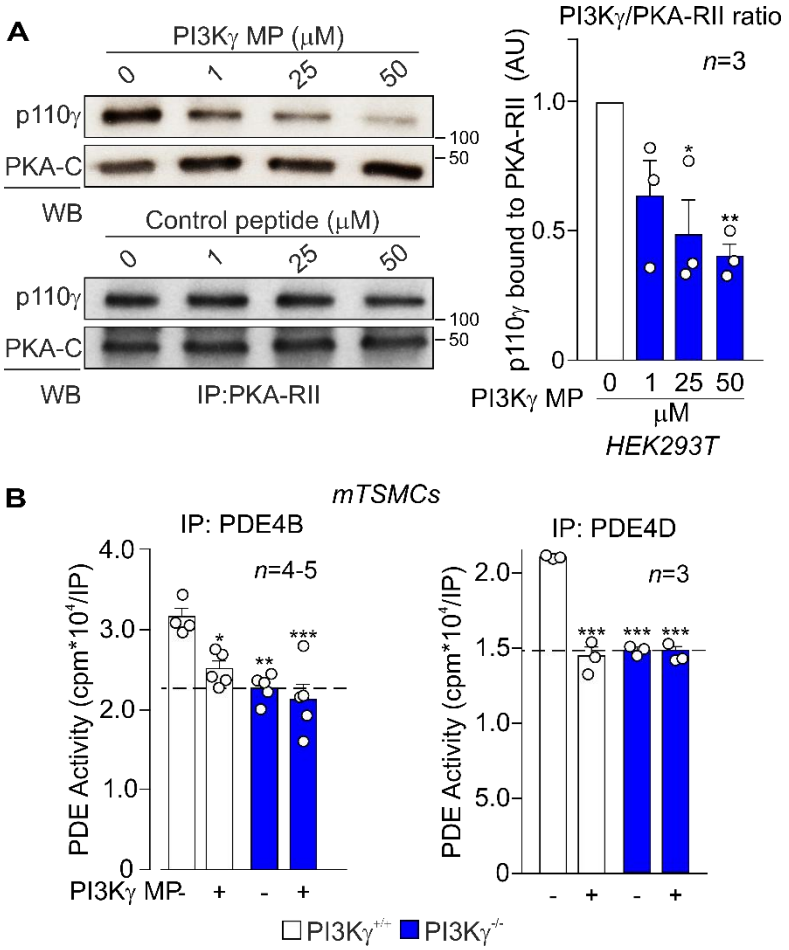


Figure 2. The PI3Kγ MP reduces the activity of PDE4B/D via disrupting the interaction between p110γ and PKA. (**A**) Co-immunoprecipitation of the catalytic subunit of PI3Kγ (p110γ) and PKA-RII from human embryonic kidney cells (HEK293T) expressing p110γ and exposed to increasing doses of PI3Kγ MP for 2 hours. Representative immunoblots (left) and relative quantification (right) of n=3 independent experiments are shown. (**B**) PDE4B and PDE4D activity in PI3Kγ$^{+/+}$ and PI3Kγ$^{-/-}$ tracheal smooth muscle cells (mTSMCs) treated with either vehicle (Veh) or PI3Kγ MP (50 μM) for 30 min. For PDE4B IP: PI3Kγ$^{+/+}$+Veh n=4; PI3Kγ$^{+/+}$+PI3Kγ MP n=5; PI3Kγ$^{-/-}$+Veh n=5 and PI3Kγ$^{-/-}$+ PI3Kγ MP n=5 independent cultures. For PDE4D IP: n=3 independent cultures in all groups. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

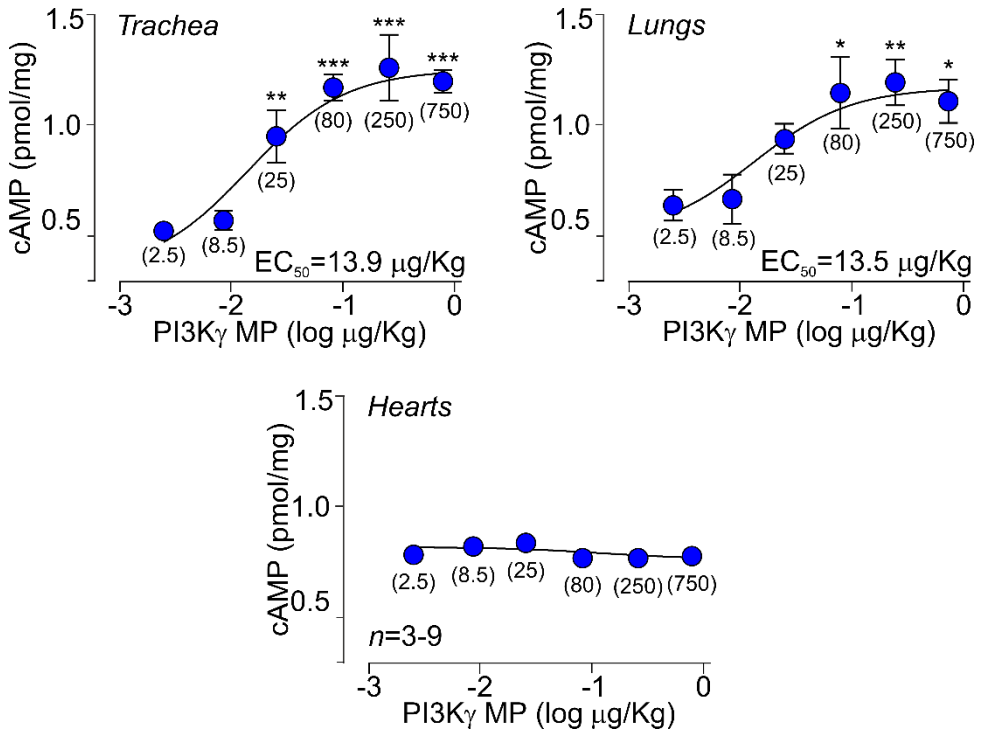


Figure 3. The PI3Kγ MP increases airway cAMP levels *in vivo* in a dose-dependent manner. cAMP levels in tissues from BALB/c mice at 24 hours after intratracheal instillation of different doses of PI3Kγ MP (0-750 µg/kg). Values in brackets indicate the PI3Kγ MP dose expressed as µg/kg. In each group, $3 \leq n \leq 9$ (n is the number of mice). *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

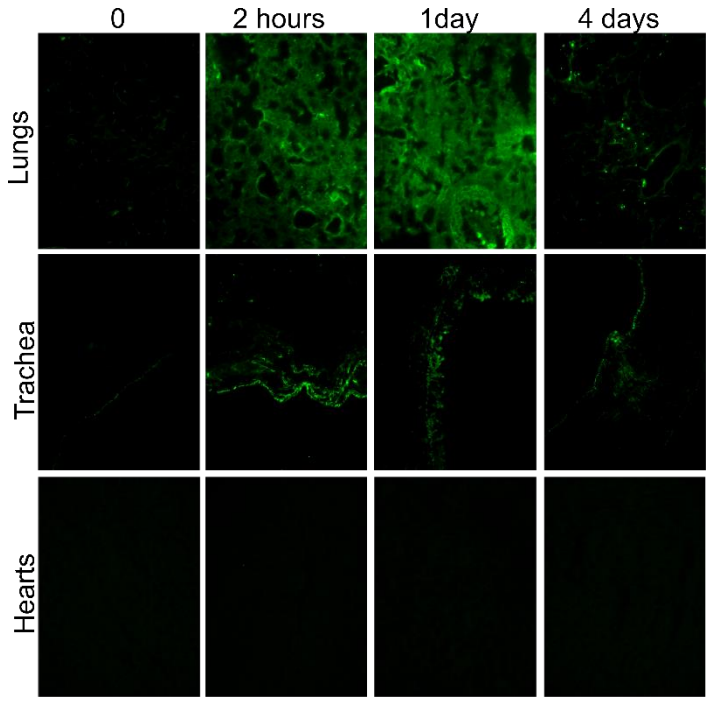


Figure 4. The PI3Kγ MP persists in the airways of mice up to 24 hours after a single-dose intratracheal instillation. Tissue distribution of a FITC-labeled version of the PI3Kγ MP at indicated time points after intratracheal instillation of 0.08 mg/kg (1.5 µg) in BALB/c mice. Representative images of n=3 experiments are shown.

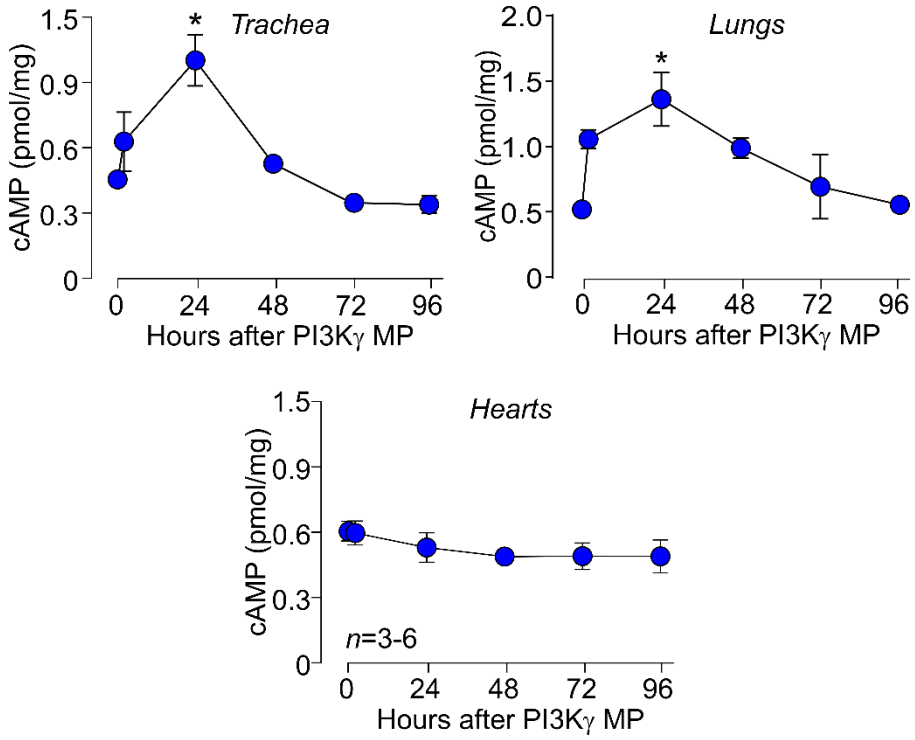


Figure 5. The PI3Kγ MP significantly increases cAMP levels in the airways of mice up to 24 hours after a single-dose intratracheal instillation. cAMP levels in tissues from mice treated as in Figure 4. In each group, 3≤n≤6 (n is the number of mice). *P<0.05 by Kruskal Wallis test followed by Dunn's multiple comparison test. Throughout, data are mean ± s.e.m.

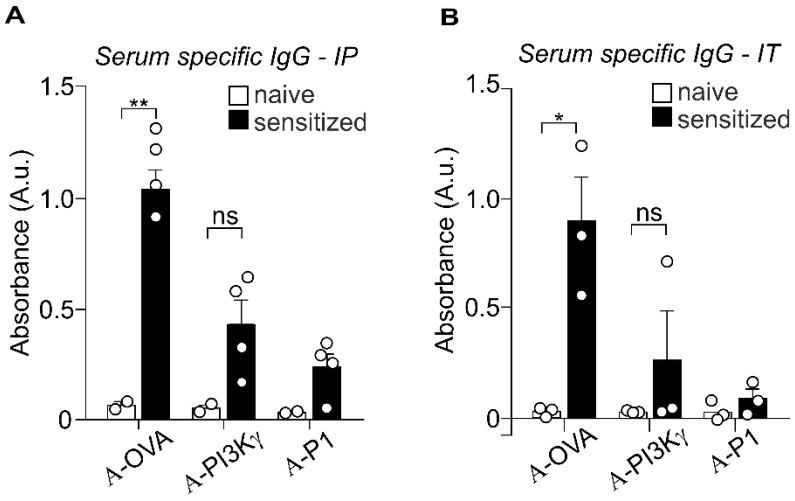


Figure 6. The PI3Kγ MP does not elicit a significant antibody response *in vivo*. (**A**) Anti-OVA, anti-PI3Kγ and anti-P1 antibodies detected by ELISA in the serum of naïve (n=2) mice and animals injected intraperitoneally (IP) with 100 µg of OVA with PI3Kγ MP (0.75 mg/kg; n=4) twice a week for 2 weeks. (**B**) Anti-PI3Kγ and anti-P1 antibodies detected by ELISA in the serum of naïve mice (n=3) and in OVA-sensitized animals treated intratracheally (IT) with PI3Kγ MP (0.75 mg/kg; n=3) on days 14, 25, 26 and 27 of the OVA sensitization protocol. Anti-OVA antibodies were quantified for confirming the generation of antigen-specific antibodies. *P<0.05 and **P<0.01 by unpaired Student's t test. Throughout, data are the mean ± s.e.m.

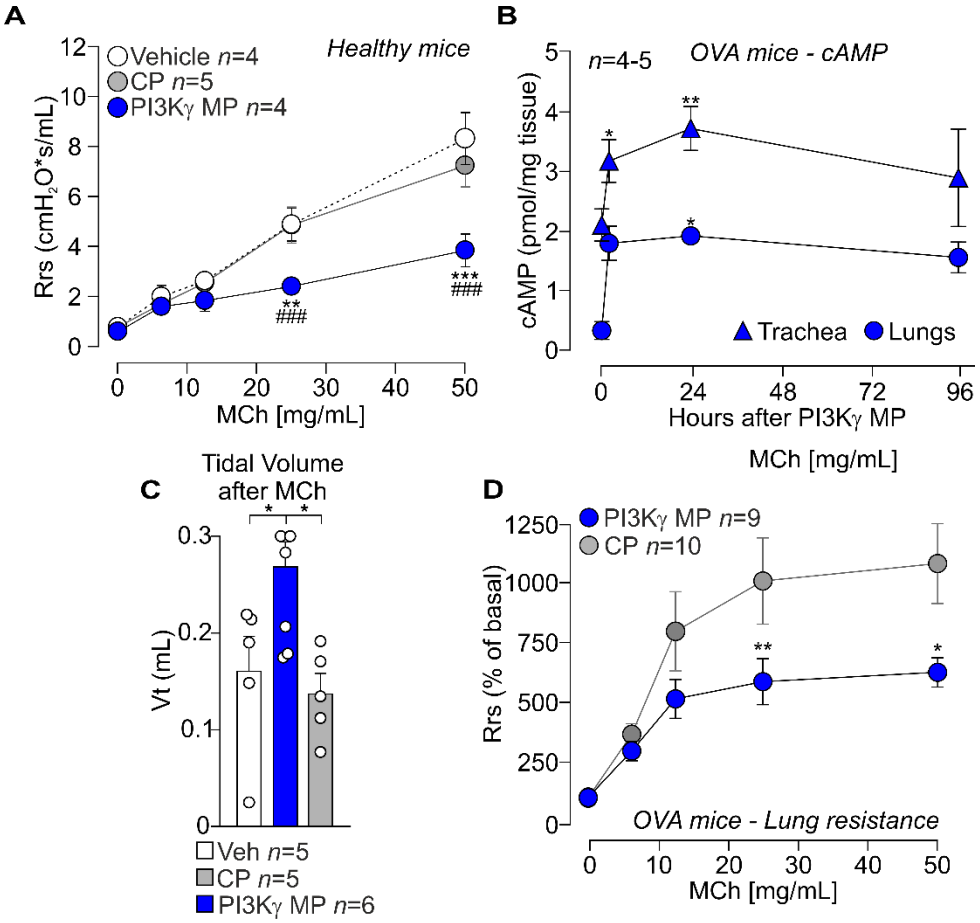


Figure 7. The PI3Kγ MP promotes airway relaxation in a mouse model of asthma. (**A**) Average lung resistance in healthy mice treated with vehicle (n=4), 1.5 µg PI3Kγ MP (n=4) or equimolar amount of control peptide (CP; n=5) directly before exposure to increasing doses of the bronchoconstrictor methacholine (MCh). (**B**) cAMP levels in lungs and tracheas of ovalbumin (OVA)-sensitized mice at the indicated time points after intra-tracheal administration of PI3Kγ MP (15 µg). In each group, 3≤n≤8 (n is the number of mice). (**C**) Tidal volume of OVA-sensitized mice pre-treated with vehicle (n=5), PI3Kγ MP (15 µg; n=6) and CP (equimolar amounts; n=5) and exposed to MCh (500 µg/kg). (**D**) Average lung resistance (expressed as % of basal) in OVA-sensitized mice treated with 15 µg of PI3Kγ MP (n=9) or equimolar amount of CP (n=10) 30 min before MCh challenge. In panel (**A**), **P<0.01 and ***P<0.001 versus vehicle and ### P<0.001 versus CP by two-way ANOVA followed by Bonferroni's post-hoc test. In panel (**B**) and (**C**), *P<0.05 and **P<0.01 by one-way ANOVA followed by Bonferroni's post-hoc test. In panel (**D**), *P<0.05 and **P<0.01 between groups by two-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

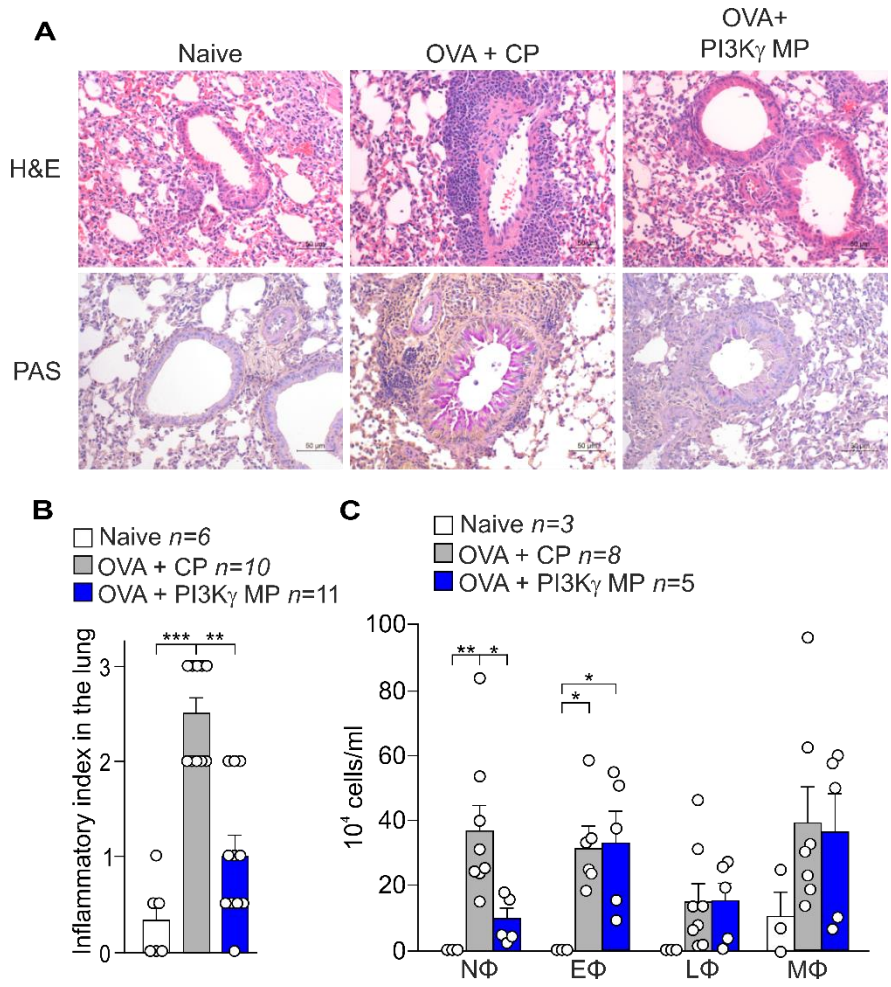


Figure 8. The PI3Kγ MP limits neutrophilic lung inflammation in asthmatic mice. (**A**) Representative images of hematoxylin-eosin (top) and periodic acid-Schiff's reagent (bottom) staining of lung sections of naïve and ovalbumin (OVA)-sensitized mice, pre-treated with PI3Kγ MP (25 µg) or CP (equimolar amount), before each intranasal OVA administration (days 14, 25, 26 and 27 of the OVA sensitization protocol). (**B**) Semi-quantitative analysis of peri-bronchial inflammation in lung sections as shown in (**A**). Naïve n=6, OVA+CP n=8 and OVA+PI3Kγ MP n=5 animals. (**C**) Number of neutrophils (NΦ), eosinophils (EΦ), lymphocytes (LΦ) and macrophages (MΦ) in the bronchoalveolar lavage (BAL) of mice treated as in (**A**). Naïve n=3, OVA+CP n=8 and OVA+PI3Kγ MP n=5 animals. In panel (**B**), **P<0.01 and ***P <0.001 by Kruskal Wallis test followed by Dunn's multiple comparison test. In panel (**C**), *P <0.05 and **P<0.01 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

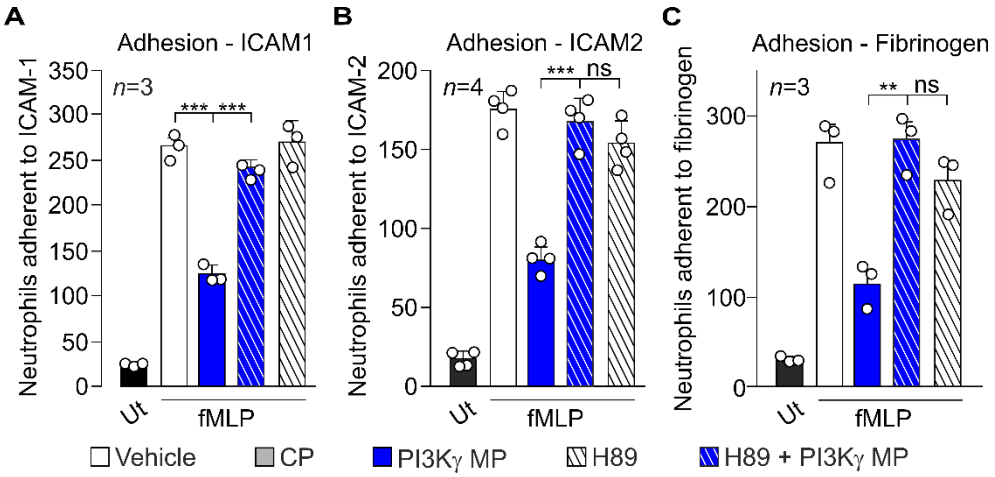


Figure 9. The PI3Kγ MP limits PKA-mediated adhesion of neutrophils to different substrates. fMLP-induced adhesion to ICAM-1 (**A**), ICAM-2 (**B**) and fibrinogen (**C**) of human neutrophils pre-treated with the PKA inhibitor H89 (200 nM, 30 min) before exposure to PI3Kγ MP (50 μM, 1 hour). Static adhesion was induced with 25 nM fMLP for 1 min. Average numbers of adherent cells/0.2 mm2 is shown. In panels (**A**) and (**C**), n=3 in all groups; in panel (**B**), n=4 in all groups. **P<0.01 and ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

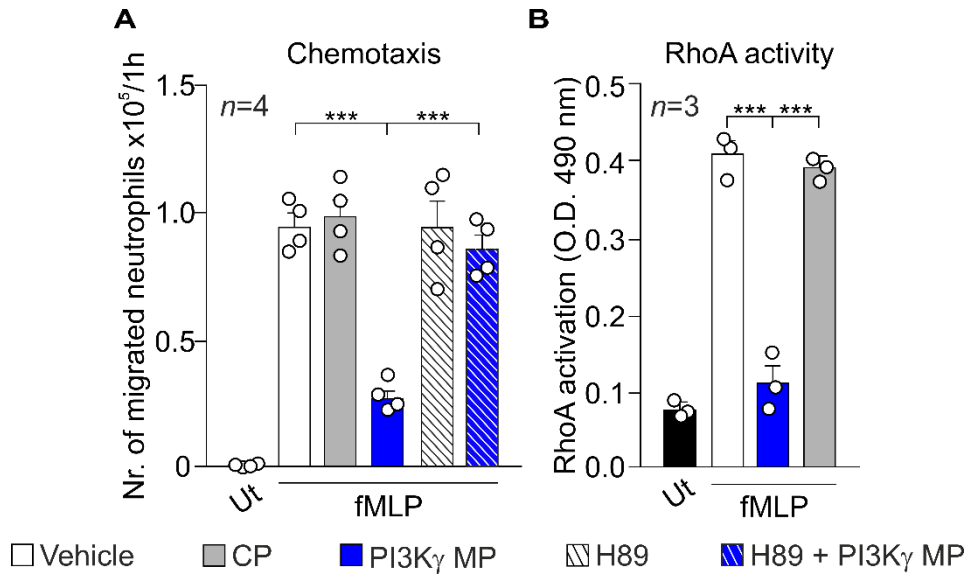


Figure 10. The PI3Kγ MP significantly dampens neutrophil chemotaxis and LFA1 activation. (**A**) fMLP-triggered chemotaxis of human neutrophils pre-treated with the PKA inhibitor H89 (200 nM, 30 min) before exposure to PI3Kγ MP (50 μM). n=4 in all groups. (**B**) fMLP-induced RhoA activity in human neutrophils treated with vehicle, CP (50 μM) or PI3Kγ MP (50 μM). n=3 in all groups. ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

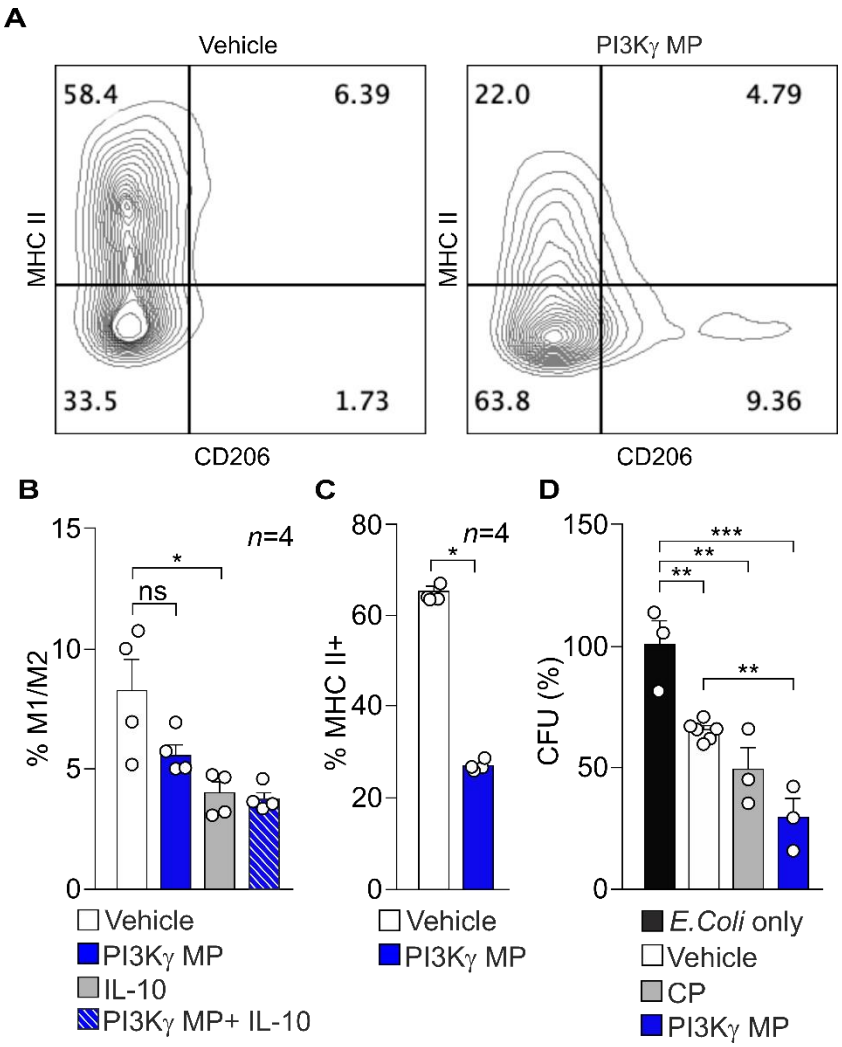


Figure 11. The PI3Kγ MP inhibits macrophage activation without affecting bacterial killing. (**A**) Representative flow cytometry analysis of MHC II and CD206 expression in primary mouse peritoneal macrophages (F4/80+ cells) treated with vehicle or PI3Kγ MP (25 μM) for 24 hours. (**B**) Quantification of M1 (CD206-) and M2 (CD206+) macrophages from FACS analysis of cells treated with vehicle, PI3Kγ MP (25 μM), IL-10 (20 ng/mL) or PI3Kγ MP+IL-10 for 24 hours. (**C**) Quantification of MHC II+ cells from FACS analysis represented in (**A**). (**D**) Peritoneal macrophages from C57BL/6 mice were pre-treated with vehicle (PBS), control peptide (CP; 25 μM) or PI3Kγ MP (25 μM) for 30 minutes, before incubation with mouse serum-opsonized E. coli for 1 hour at 37°C. Afterwards, macrophages were lysed, and the number of live bacteria (combined extra- and intracellular) was determined by plating serial dilution of the samples on LB agar plates. Results are expressed as % of colony forming units (CFU). In panels (**B**) and (**C**), *P<0.05 by unpaired Kruskal-Wallis test. In panel (**D**), **P<0.01 and ***P<0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

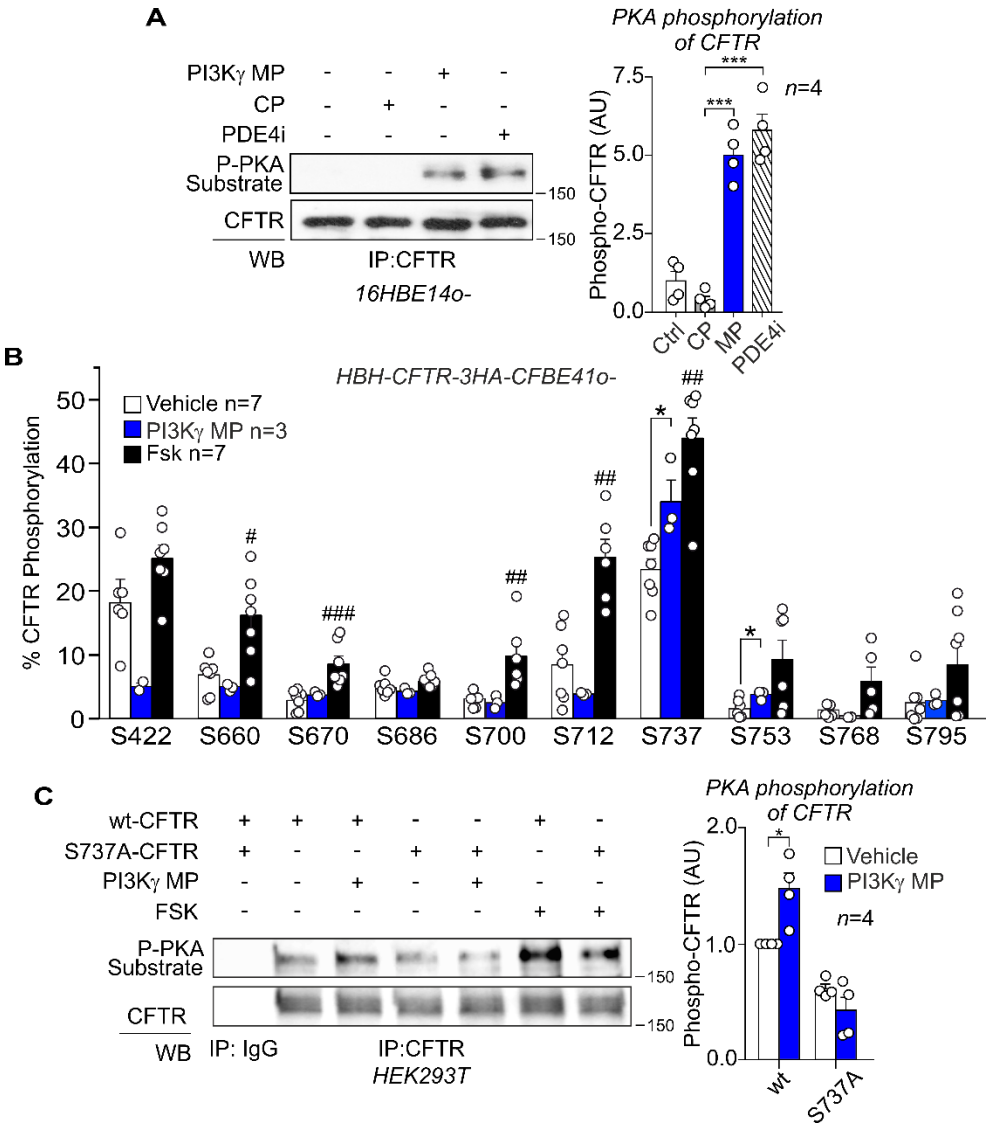


Figure 12. The PI3Kγ MP induces CFTR phosphorylation at serine 737. (**A**) Representative Western blot (left) and relative quantification (right) of PKA-mediated phosphorylation of CFTR in human bronchial epithelial cells (16HBE14o-) treated with vehicle, CP (25 µM), PI3Kγ MP (25 µM) and the PDE4 inhibitor Rolipram (PDE4i; 10 µM) for 30 min. CFTR was immunoprecipitated (IP) and PKA-dependent phosphorylation was detected in IP pellets by immunoblotting with a PKA substrate antibody. n=4 independent experiments. (**B**) Relative phosphorylation (%) or phospho-occupancy of identified PKA sites in CFTR in cystic fibrosis bronchial epithelial cells (CFBE41o-) expressing HBH-CFTR-3HA treated with vehicle (DMSO; n=7), PI3Kγ MP (25 µM, 1h, n=3) and Fsk (10 µM, 10 min, n=7). n is the number of biological replicates from n=3 independent experiments. The phospho-occupancy or the percent of relative phosphorylation of each site was calculated as a ratio of all phosphorylated and unphosphorylated peptides that contained a given phosphosite, i.e., % phosphorylation of site A = [area of peptides phosphorylated at site A /sum of areas of all peptides carrying site A] as described in Methods. (**C**) Representative Western blot (left) and relative quantification (right) of PKA-mediated phosphorylation of CFTR in HEK293T cells expressing either wt- or S737A-CFTR and exposed to vehicle, PI3Kγ MP (25 µM, 1

h) or Fsk (10 µM, 10 min). CFTR was immunoprecipitated (IP) and PKA-dependent phosphorylation was detected in IP pellets by immunoblotting with a PKA substrate antibody. n=4 independent experiments. In panels (**A**) and (**C**), *P<0.05 and ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. In panel (**B**), unpaired t-tests followed by Holm-Sidak's multiple comparisons test were performed on each phosphosite between two different treatment conditions. #P<0.05, ##P <0.01 and ###P<0.001 Fsk versus vehicle, *P<0,05 PI3Kγ MP versus vehicle. Throughout, data are mean ± s.e.m.

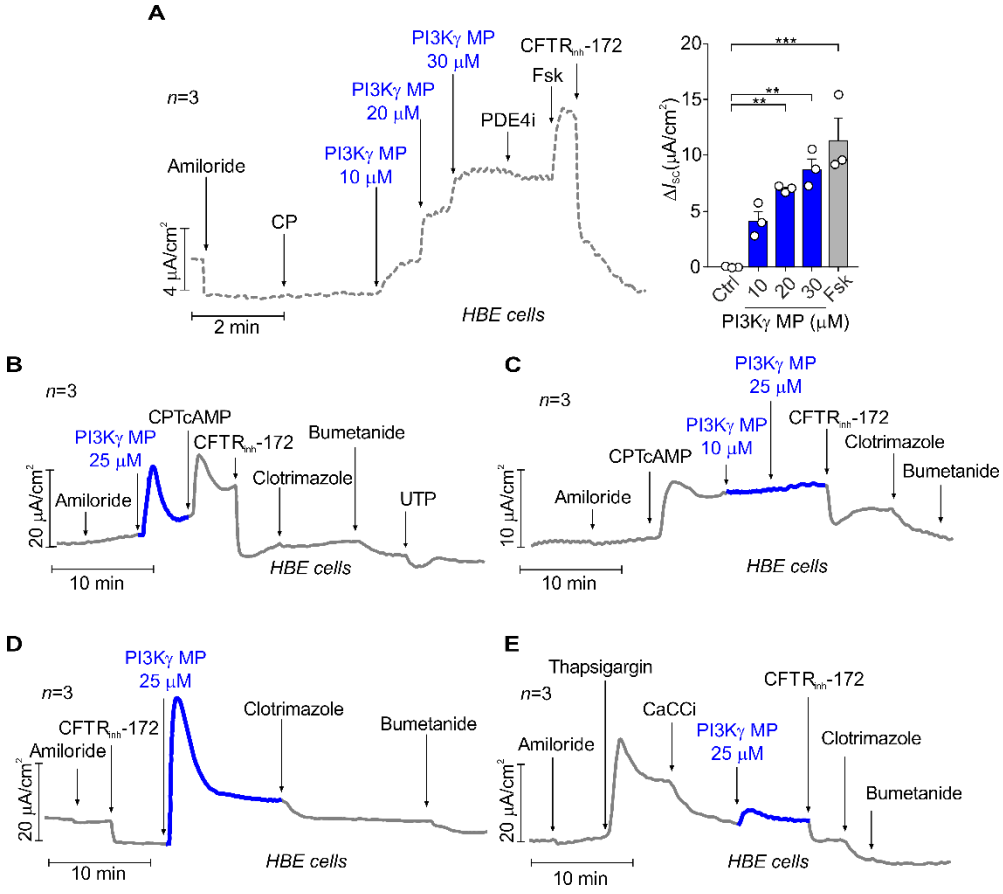


Figure 13. The PI3Kγ mimetic peptide triggers cAMP-dependent Cl^- efflux in HBE cells through coordinate activation of CFTR, CaCCs and KCa. (**A**) Left, Representative trace of short-circuit currents (I_{SC}) measured in Ussing chambers in primary human normal bronchial epithelial (pHBE) cells cultured at the air-liquid interface (ALI). Right, average current variations in response to the indicated treatments. The following treatments were applied at the indicated times: ENaC inhibitor amiloride (10 μM), CP (30 μM), PI3Kγ MP (10-30 μM), PDE4 inhibitor Rolipram (PDE4i; 10 μM), forskolin (Fsk, 10 μM) and CFTR inhibitor 172 (CFTRinh-172; 20 μM). n=3 biological replicates from the same donor. (**B-E**) I_{SC} of primary human normal bronchial epithelial (HBE) cells grown at the air-liquid interface (ALI). The following drugs were applied at the indicated times: amiloride (ENaC inhibitor; 10 μM), PI3Kγ MP (10 μM or 25 μM), CPT-cAMP (non-hydrolysable cAMP analog; 100 μM), CFTR inhibitor (CFTRinh-172; 10 μM), clotrimazole (Ca^{2+}-dependent K^+ channels inhibitor; 10 μM), bumetanide (Na-K-Cl cotransporter NKCC1 inhibitor; 100 μM), and UTP (100 μM). (**B**) I_{SC} elicited by the PI3Kγ MP (25 μM). CPT-cAMP further increased I_{SC} on top of the PI3Kγ MP. (**C**) I_{SC} induced by increasing concentrations of the PI3Kγ MP (10 and 25 μM) in HBE cells pre-treated with CPT-cAMP. The pre-treatment fully prevented the PI3Kγ MP from further increasing I_{SC}. (**D**) I_{SC} stimulated by 25 μM PI3Kγ MP in HBE cells pre-treated with CFTRinh-172. The peptide elicited a transient increase of I_{SC} (solid blue line) that was indicative of activation of Ca^{2+}-activated Cl^- channels (CaCCs). Baseline I_{SC} was restored by application of clotrimazole, an inhibitor of basolateral Ca^{2+}-activated K^+ channels (KCa), and bumetanide, an inhibitor of the Na-K-Cl cotransporter NKCC1. (**E**) I_{SC} stimulated by 25 μM PI3Kγ MP after depletion of Ca^{2+} stores with thapsigargin (1 μM) and inhibition of CaCCs (CaCCi, 10 μM). Of note, the I_{SC} induced by the PI3Kγ MP was completely inhibited by CFTRinh-172

(solid blue line). *P<0.05, **P <0.01 and ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

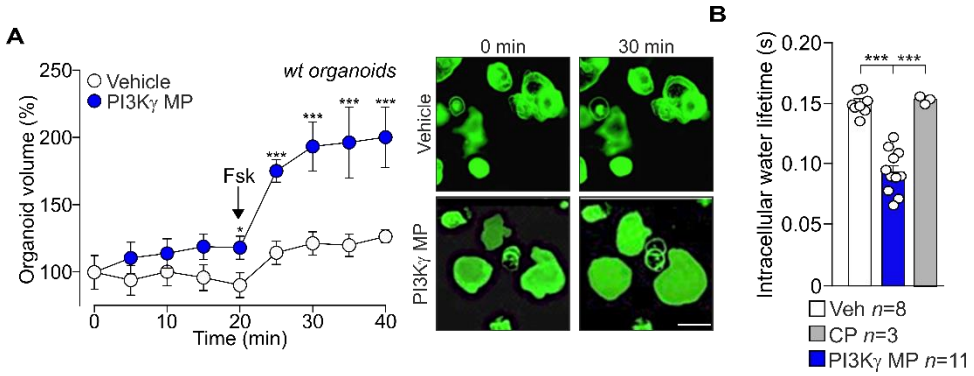


Figure 14. The PI3Kγ MP increases CFTR channel efflux and water transport across the plasma membrane. (**A**) Normalized swelling curves (left) and representative confocal images (right) of Fsk-stimulated calcein green-labeled wild-type (wt) organoids pre-incubated with PI3Kγ MP (25 μM) or vehicle (Veh) for 20 min. Fsk was used at 2 μM. Scale bar, 100 μm. Veh n=25 and PI3Kγ MP n=28 organoids from n=3 independent experiments. (**B**) Water residence time (τ_{in}) determined by 1H NMR relaxometry (as described in Supplementary Material) in HEK293T cells transfected with wt-CFTR and treated with vehicle (DMSO; n=8), CP (25 μM; n=3) and PI3Kγ MP (25 μM; n=11). n indicates the number of biological replicates in n=3 independent experiments. In panel (**A**), *P<0.05 and ***P<0.001 by two-way ANOVA followed by Bonferroni's multiple comparisons test. In panel (**B**), *P<0.05, **P <0.01 and ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

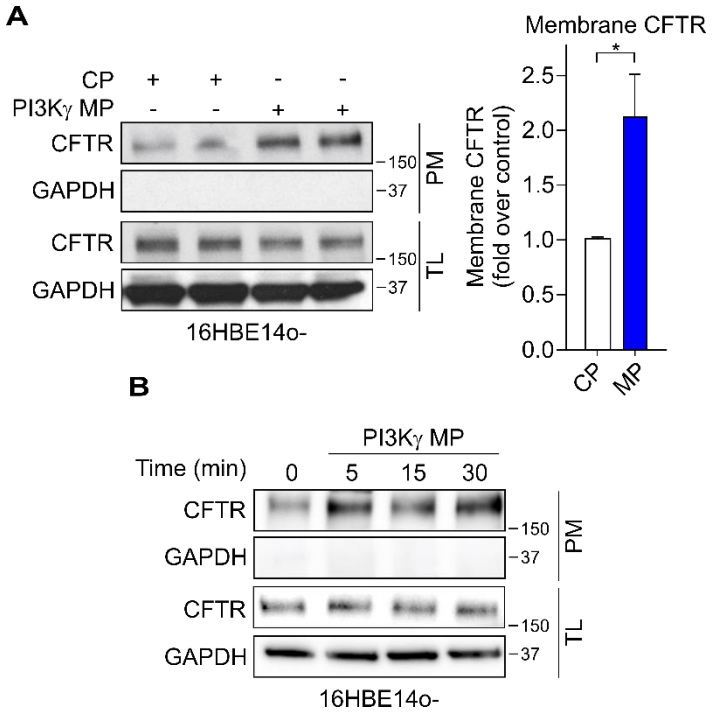


Figure 15. The PI3Kγ MP increases the amount of wt-CFTR channel at the plasma membrane in airway epithelial cells. (**A**) 16HBE14o- cells were exposed to either CP (25 μM) or to the PI3Kγ MP (25 μM) for 2 hours and subjected to cell surface biotinylation. (**B**) 16HBE14o- cells were exposed to the PI3Kγ MP 25 μM at indicated times (0, 5, 15, 30 minutes) and subjected to cell surface biotinylation. After streptavidin pull down, CFTR was detected by Western Blot. The cytosolic marker GADPH was not detected in the biotinylated fraction. CFTR and GADPH were detected in the total lysate (TL) as a control. PM, plasma membrane. *P<0.05 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

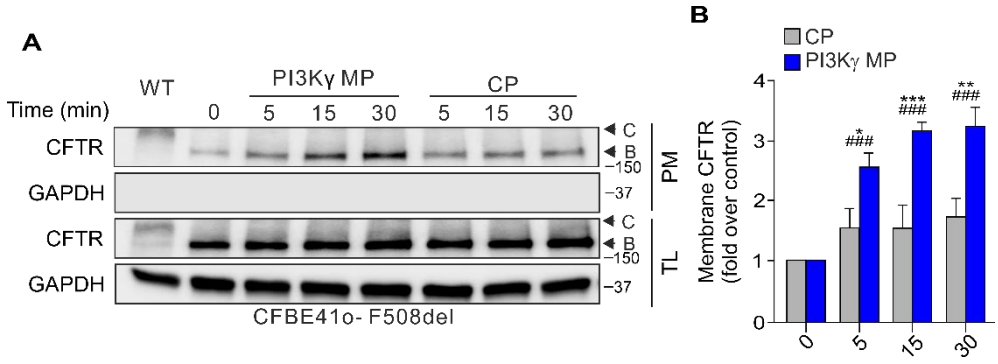


Figure 16. The PI3Kγ MP increases the amount of F508del-CFTR channel at the plasma membrane in airway epithelial cells. Representative Western Blot (**A**) and relative quantification (**B**) of CFTR amount at the PM in untreated wt- and F508del-CFTR-CFBE41o- cells previously treated with the PI3Kγ MP 25 µM and equimolar control peptide (CP) at different time points as indicated (0, 5, 15, 30 minutes). Cells, after surface biotinylation, were lysed and subjected to streptavidin pull down, as previously described. CFTR Band B (immature ER core-glycosylated CFTR) and band C (mature complex-glycosylated CFTR) were detected in both the total lysate (TL) and the plasma membrane (PM). The cytosolic marker GADPH was not detected in the biotinylated fraction as a control. ###P<0.001 PI3Kγ MP at time points 5, 15 and 30 min versus PI3Kγ MP at time point 0 min by one-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, **P <0.01 and ***P <0.001 PI3Kγ MP versus CP by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

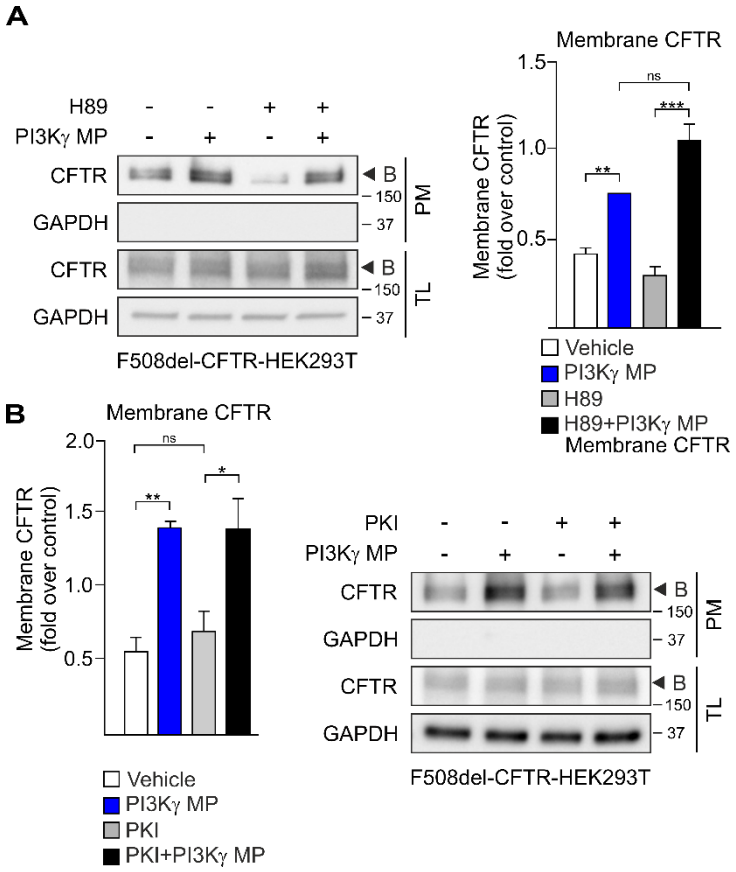


Figure 17. The PI3Kγ MP increases the plasma membrane CFTR levels in a PKA-independent manner in HEK293T cells. (**A**) Representative Western Blot (left) and relative quantification (right) of CFTR amount at the cell surface in HEK293T cells expressing F508del-CFTR treated with vehicle (DMSO), PI3Kγ MP (25 μM), H89 15 μM) or PI3Kγ MP (25 μM) together with H89 (1 μM) for 30 minutes. After surface biotinylation, cells were lysed and subjected to streptavidin pull down. CFTR was detected by Western Blot. (**B**) Relative quantification (left) and representative Western Blot (right) of CFTR amount at the cell surface in HEK293T cells expressing F508del-CFTR treated with vehicle (DMSO), PI3Kγ MP (25 μM), PKI (5 μM) or PI3Kγ MP (25 μM) together with PKI (5 μM) for 30 minutes. After surface biotinylation, cells were lysed and subjected to streptavidin pull down. CFTR was detected by Western Blot. P<0.05, **P <0.01 and ***P <0.001 PI3Kγ MP versus CP by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

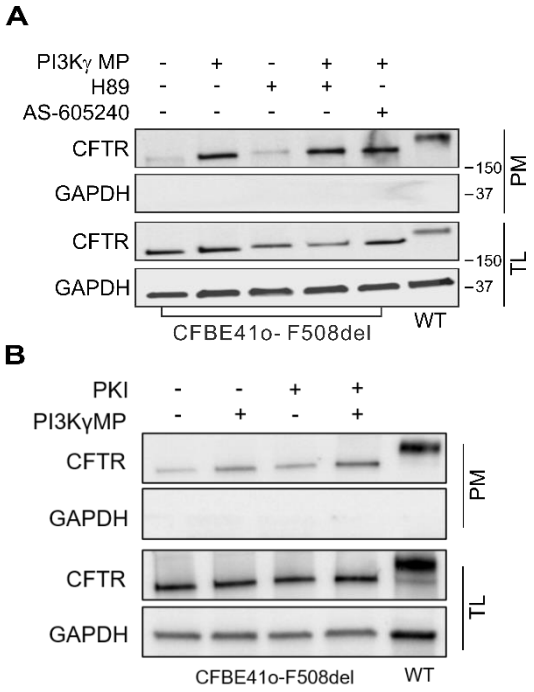


Figure 18. The PI3Kγ MP increases the plasma membrane CFTR levels in a PKA-independent manner in F508del-CFBE41o- cells. (**A**) Representative Western Blot of CFTR amount at the cell surface in F508del-CFBE41o- cells treated with vehicle (DMSO), PI3Kγ MP (25 μM), H89 (1 μM) or PI3Kγ MP (25 μM) together with H89 (1 μM) for 30 minutes. After surface biotinylation, cells were lysed and subjected to streptavidin pull down. CFTR was detected by Western Blot. (**B**) Representative Western Blot of CFTR amount at the cell surface in F508del-CFBE41o- cells treated with vehicle (DMSO), PI3Kγ MP (25 μM), PKI (5 μM) or PI3Kγ MP (25 μM) together with PKI (5 μM) for 30 minutes. After surface biotinylation, cells were lysed and subjected to streptavidin pull down. CFTR was detected by Western Blot.

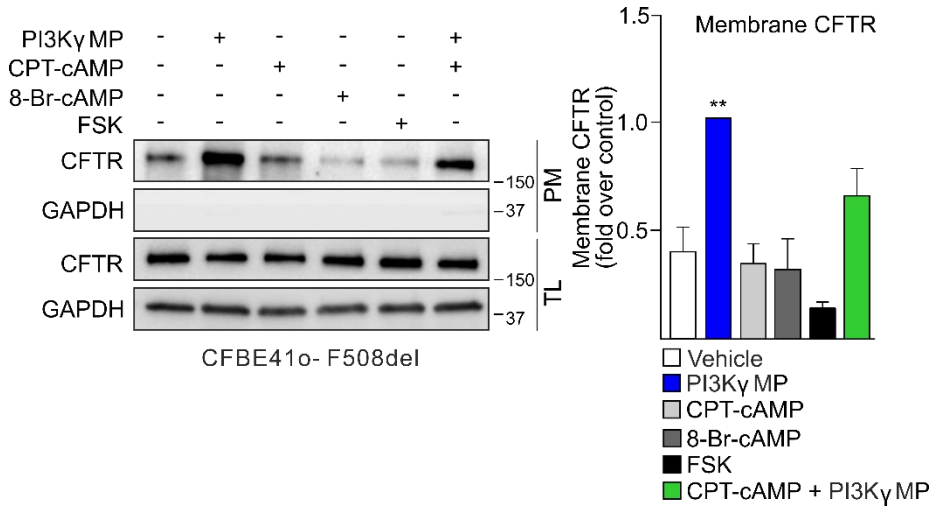


Figure 19. cAMP analogues do not recapitulate the increased CFTR levels on the plasma membrane mediated by the PI3Kγ MP. Representative Western Blot (left) and relative quantification (right) of CFTR amount at the cell surface in F508del-CFBE41o- cells treated with vehicle (DMSO), PI3Kγ MP (25 μM), CPT-cAMP and 8-Br-cAMP (100 μM), Fsk (2 μM) or PI3Kγ MP plus CPT-cAMP. Cells were biotinylated, lysed and subjected to streptavidin pull down, as described above. CFTR was detected by Western Blot. The cytosolic marker GADPH was not detected in the biotinylated fraction. CFTR and GADPH were detected in the total lysate (TL) as a control. PM, plasma membrane. PM, plasma membrane. **P <0.01 by one-way ANOVA followed by Bonferroni's post-test. Throughout, data are mean ± s.e.m.

Protein	Phosphosite	%CFC
PKD1	S910	1313
PCTK2	Pan-specific	112
PFN1	Y129	98
CDK2	Pan-specific	88
PAK5	Pan-specific	85
eIF4B	S422	77
PCYT1B	S315+S319	73
MAPK13	T180+Y182	71
P70S6K	S434	56
PDGFRa	Y768	54
LATS2	Pan-specific	50
HDAC5	S498	49
CaMK2a	Pan-specific	-70
STAT5	Y694	-77

Figure 20. Protein Kinase D1 (PKD1) is phosphorylated upon PI3Kγ MP treatment in epithelial cells. Report of phosphorylated targets of phosphor-proteomics analysis performed in F508del-CFBE41o- upon phosphor-proteomics analysis versus CP treatment. Data are presented as change from control (CFC%). Value of 100% corresponds to a 2-fold increase in signal intensity following PI3Kγ MP treatment as compared to CP. A negative value indicates the degree of reduction in signal intensity from the selected control.

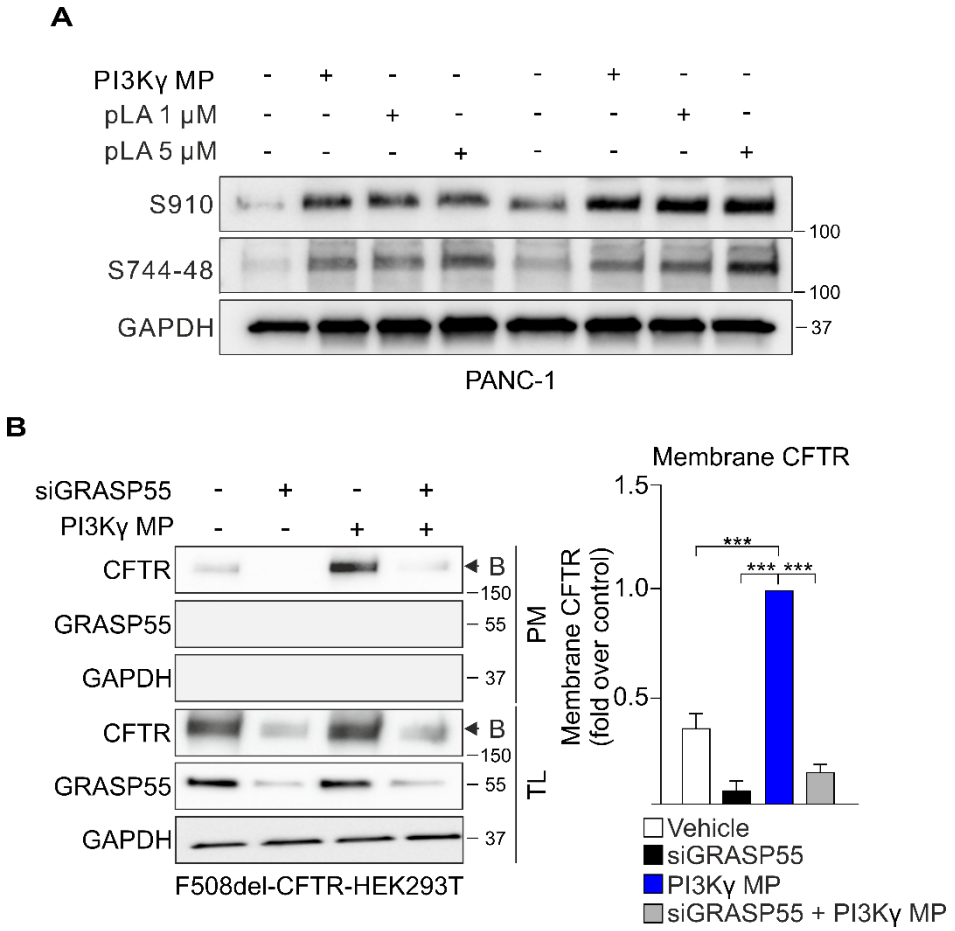


Figure 21. The PI3Kγ MP induces CFTR trafficking possibly through PKD1 phosphorylation and consequent downstream activation of GRASP 55. (**A**) Representative Western Blot of PKD1 phosphorylation on residues S910 and S744-748. Human pancreatic cancer cells (PANC-1) were lysed after administration of the PI3Kγ MP (25μM, 30 min) or poly-L-arginine (pLA) at indicated concentrations for (10 min) as a positive control. (**B**) Representative Western Blot (left) and relative quantification (right) of CFTR amount at the cell surface in HEK293T cells expressing F508del-CFTR treated with vehicle (DMSO), PI3Kγ or transfected with siRNA targeting GRASP55. After surface biotinylation, cells were lysed and subjected to streptavidin pull down. Upon GRASP 55 silencing, the protein expression was markedly reduced in the total lysates. The cytosolic marker GADPH was not detected in the biotinylated fraction. CFTR and GADPH were detected in the total lysate (TL) as a control. PM, plasma membrane. ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

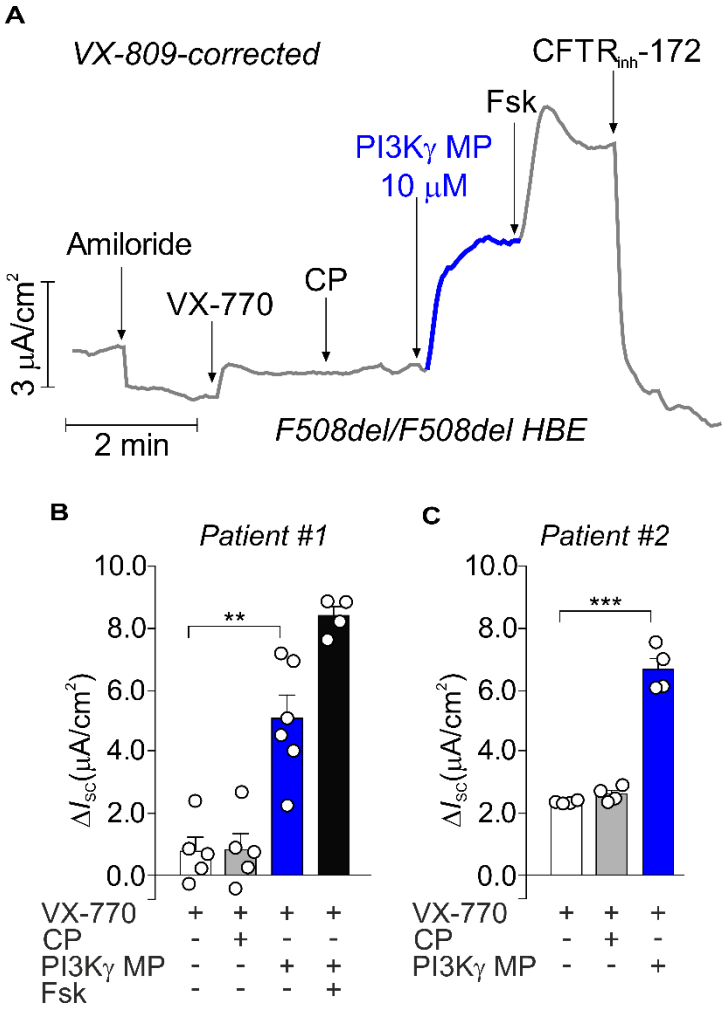


Figure 22. The PI3Kγ MP enhances the effect mediated by the potentiator VX-770 on CFTR-mediated currents.(**A**) Representative trace of short-circuit currents (I_{SC}) measured in Ussing chambers in primary human CF bronchial epithelial cells from a CF donor (Patient #1) homozygous for the F508del mutation (F508del/F508del HBE) and grown at the air-liquid interface (ALI). Cells were corrected with VX-809 for 48 hours (5 μM) and then exposed to the following drugs at the indicated times: Amiloride (Amil, 100 μM), CP (10 μM), PI3Kγ MP (10 μM), forskolin (Fsk, 10 μM), VX-770 (1 μM) and the CFTR inhibitor 172 (CFTRinh-172; 10 μM). (**B**) Average total current variation in response to VX-770 (1 μM), CP (10 μM), PI3Kγ MP (10 μM) and forskolin (Fsk, 10 μM) of n=4 technical replicates of the same donor. (**C**) Average total current variation in response to VX-770 (1 μM), CP (25 μM) and PI3Kγ MP (25 μM) in F508del/F508del HBE cells from a second CF donor (Patient #2) grown at ALI and pre-corrected with VX-809 for 48 hours (5 μM). n=4 technical replicates of the same donor. **P <0.01 and ***P <0.001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

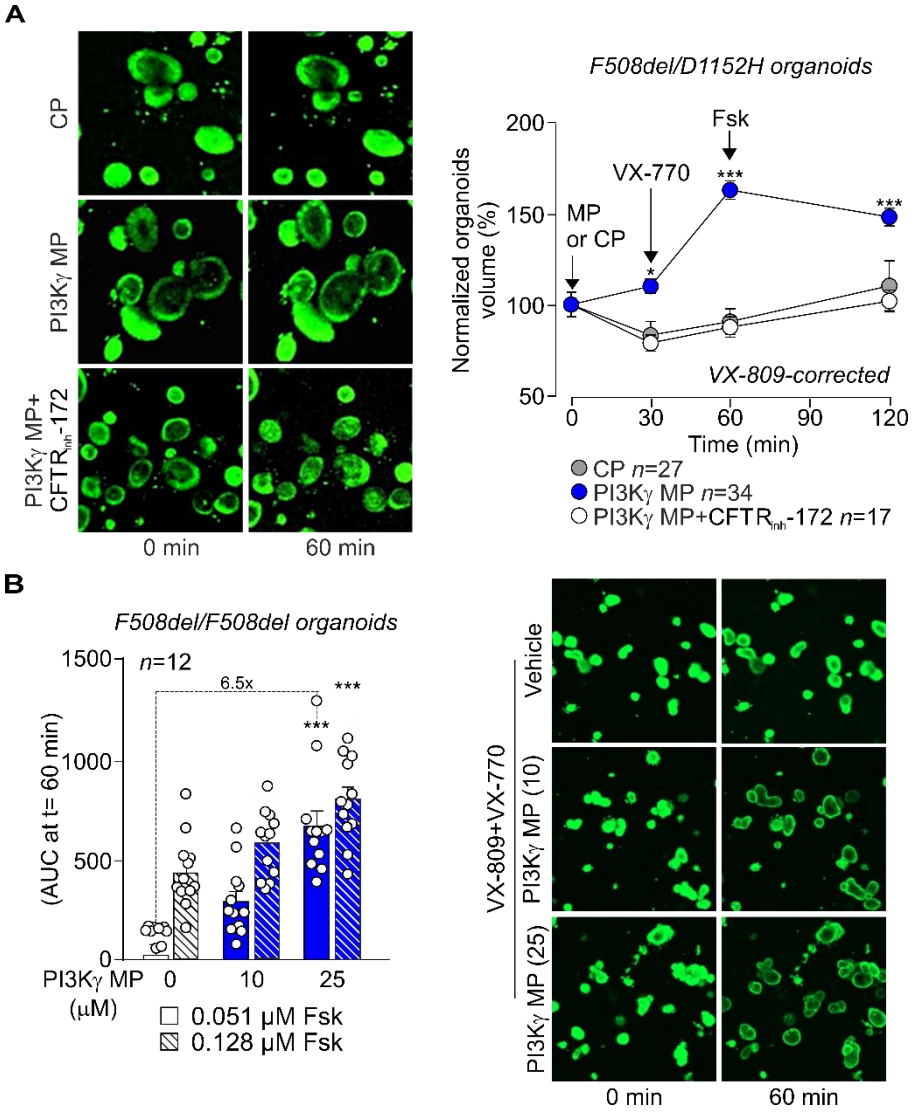


Figure 23. The PI3Kγ MP induces forskolin-induced swelling of CF organoids. (**A**) Representative confocal images and forskolin-induced swelling (FIS) of calcein green-labeled rectal organoids from a compound CF patient carrying F508del and D1152H mutations (F508del/D1152H). Organoids were corrected with VX-809 (3 μM) for 24h, incubated with Calcein-green (3 μM) for 30 min and exposed to either PI3Kγ MP or CP (both 25 μM) for 30 min before stimulation with Fsk (2 μM). Organoid response was measured as percentage change in volume at different time points after addition of Fsk (t=30, t=60, and t=120 min) compared to the volume at t=0. 15<n<34 organoids from 1 donor in n=2 independent experiments. (**B**) FIS responses (left) and representative confocal images (right) of calcein green-labeled rectal organoids from a CF patient homozygous for the F508del mutation (F508del/F508del organoids). Organoids were pre-incubated with the CFTR corrector VX-809 (3 μM) and the CFTR potentiator VX-770 (3 μM) for 24 hours before exposure to two different concentrations of Fsk (0.51 μM; 0.128 μM) and of PI3Kγ MP (10 μM; 25 μM). The peptide was acutely added to the organoids together with Fsk. Organoid response was measured as area under the curve of relative size increase of organoids after 60 min Fsk stimulation, t = 0 min: baseline of 100%.

n=12 organoids/group analyzed in n=2 independent cultures from n=2 different donorsIn panel (**A**), *P<0.05 and ***P<0.001 by two-way ANOVA followed by Bonferroni's post-hoc test. In panel (**B**), ***P <0.001 by Kruskal Wallis test followed by Dunn's multiple comparison test. Throughout, data are mean ± s.e.m.

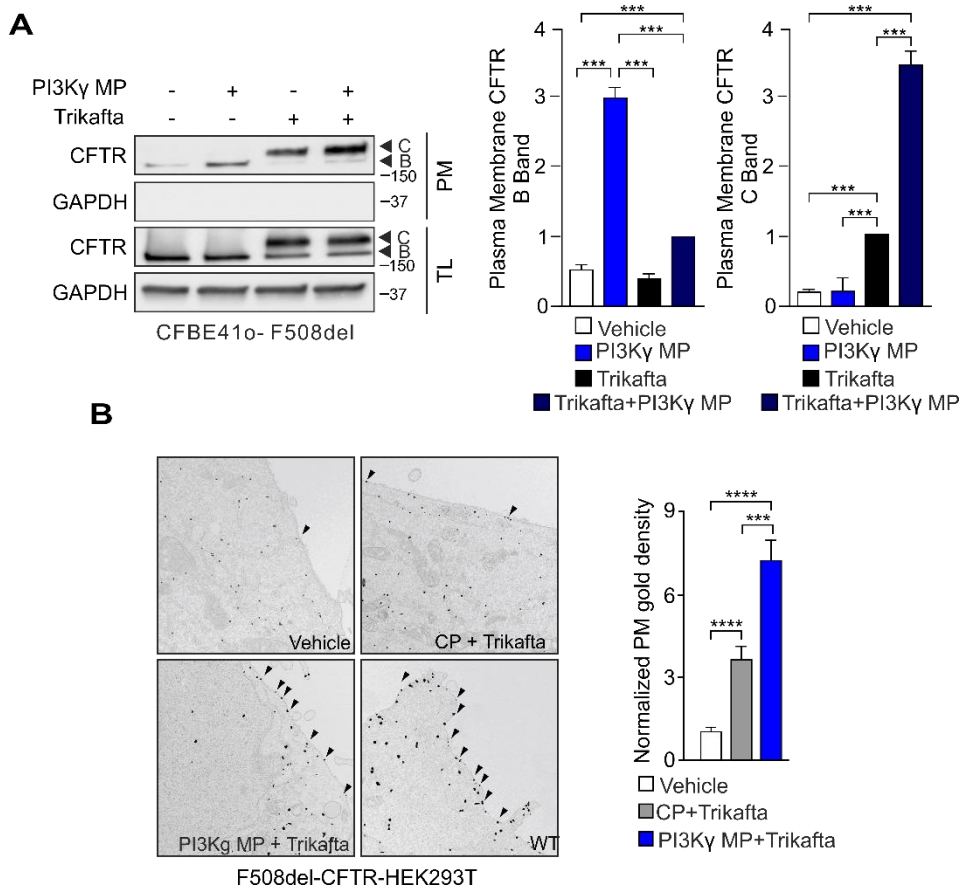


Figure 24. The PI3Kγ MP enhances the effect mediated by Trikafta on CFTR localization at the plasma membrane. (**A**) Left. Plasma membrane (biotinylated) CFTR in F508del-CFBE cells treated chronically with the PI3Kγ MP (25 μM, 24 hours) and the correctors VX-661 and the potentiator VX-770 (10μM+3μM+1 μM, 24 hours) alone or together with PI3Kγ MP (25 μM, 24 hours). CFBE cells were treated with the compounds and after 24 hours cell surface biotinylation was performed. After The cytosolic marker GAPDH was not detected in the biotinylated fraction. CFTR and GAPDH were detected in the total lysate as a control. Right. Quantification of cell surface biotinylation and Western blot experiments as shown in the left panel. (**B**) Representative image of immunogold electron microscopy (left) performed on HEK293T cells expressing F508del-CFTR and WT-CFTR with relative quantification (right). HEK293T cells expressing F508del-CFTR were analyzed following administration of vehicle (DMSO), CP plus the correctors VX-661 and the potentiator VX-770 (10μM+3μM+1 μM, 24 hours) or PI3Kγ MP plus plus the correctors VX-661 and the potentiator VX-770 (10μM+3μM+1 μM, 24 hours). HEK293T expressing WT-CFTR were intended as a positive control and so not treatedused as positive control. After the treatment, cells were stained with primary antibodies and nanogold-labeled secondary antibodies.antibodies in blocking buffer. Cells were then fixed and nanogold was enlarged with gold enhancement solution. After being processed into ultrathin slices, images were acquired and analyzed as described in the Materials and Methods. ***P <0.001 and ****P< 0.0001 by one-way ANOVA followed by Bonferroni's post-hoc test. Throughout, data are mean ± s.e.m.

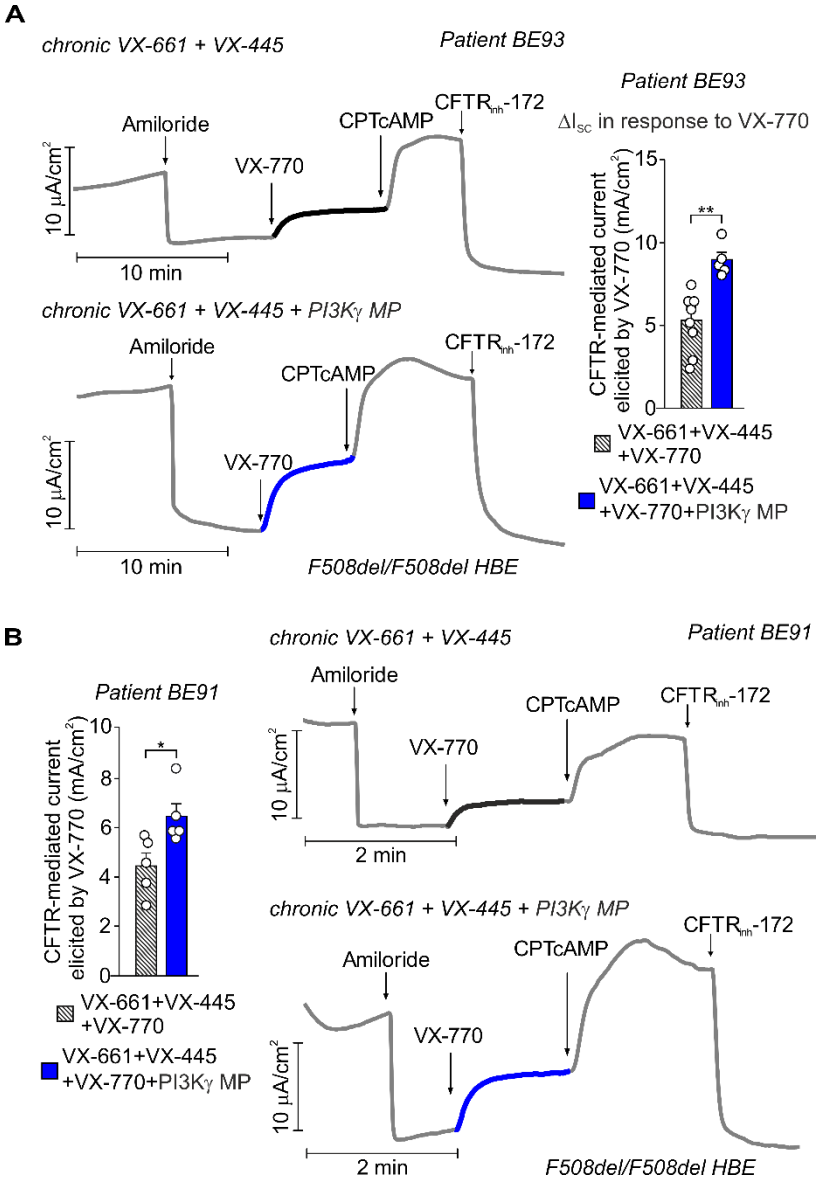


Figure 25. The PI3Kγ MP enhances the effect mediated by Trikafta on CFTR-mediated currents in F508del/F508del HBE cells. (**A**) Left, Representative traces of I_{SC} in primary human CF bronchial epithelial cells from a CF donor (Patient BE93) homozygous for the F508del mutation (F508del/F508del HBE) and grown at the air-liquid interface (ALI). Cells were corrected for 24 hours with VX-661 and VX-445 alone (10µM+3µM) or together with PI3Kγ MP (10 µM), before acute exposure at the indicated time to the following drugs: Amiloride (100 µM), VX-770 (1 µM), CPT-cAMP (100 µM), and $CFTR_{inh}$-172 (10 µM). Right, Average total current variation in response to VX-770 (1 µM) from 5≤n≤8 technical replicates of donor BE93. (**B**) Left, Average total current variation in response to VX-770 (1 µM) from n=5 technical replicates of a second F508del/F508del donor (patient BE91). Right, Representative traces of I_{SC} of F508del/F508del HBE cells from patient BE91 grown at the air-liquid interface (ALI) and treated chronically with the correctors VX-661 and VX-445 (10µM+3µM, 24 hours) alone, or together with PI3Kγ MP (25 10 µM). Cells were then exposed acutely to the following drugs at the indicated times: Amiloride (100 µM), VX-770 (1 µM), CPT-cAMP (100 µM), and CFTRinh-172 (10 µM). *P<0.05 and **P<0.001 and by Student's t test. Throughout, data are mean ± s.e.m.

Materials and Methods

Antibodies, plasmids, siRNA, peptides and reagents. The CFTR antibody A2-570 was kindly provided by Dr. J. R. Riordan (University of North Carolina at Chapel Hill, Chapel Hill, NC) via the CFTR Antibody Distribution Program of the Cystic Fibrosis Foundation. The CFTR antibody M3A7 (MAB3480) was from Millipore (Billerica, MA). Phospho-Ser/Thr-PKA substrate (9621S) antibody was from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies against PDE4B and PDE4D were described previously (Ghigo, Perino et al. 2012). Monoclonal antibody against p110γ was described previously (Perino, Ghigo et al. 2011). Antibodies against PKA-C (sc-903), PKA-RII (sc-908), GAPDH (sc-32233) and control IgG (sc-2025) for immunoprecipitation were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). GRASP55 antibody (ab211531) was purchased from Abcam (Cambridge, UK). Antibodies against phospho-PKC mu (Ser910; PA538388) and (Ser744, Ser748; PA5-110148) were purchased from Invitrogen (Waltham, MA).

The pEGFP-C1 plasmids expressing wild-type (wt-CFTR-GFP) and the F508del mutant CFTR (F508del-CFTR-GFP) were kind gifts from Dr. Peter Haggie (University of California San Francisco). The S737A-CFTR-GFP mutant plasmid was generated by site directed mutagenesis (Agilent Quikchange Lightning Site-Directed Mutagenesis kit, Agilent Technologies, Santa Clara, CA) starting from the wt-CFTR-GFP using the following primers: 5'CAGAATCTGGTACTAAGGCCAGCCTTCTCTCTAAAGG-3' and 5'CCTTTAGAGAGAAGGCTGGCCTTAGTACCAGATTCTG-3'. The mutant sequence was verified by standard Sanger sequencing using the following primer: 5'AGATGCTCCTGTCTCCTGG-3'.

siRNAs targeting the expression of GRASP55 (siGENOME Human GORASP2, siRNA SMARTpool) were purchased from Dharmacon (Horizon Discovery Ltd, Waterbeach, UK).

Penentratin-1 (CP: RQIKIWFQNRRMKWKK) and the PI3Kγ-mimetic peptide (PI3Kγ MP: RQIKIWFQNRRMKWKKGKATHRSPGQIHLVQRHPPSEESQAF) were synthesized by GenScript (Piscataway, NJ).

Methacholine, ovalbumin, amiloride, clotrimazole, pCPT-cAMP, 8-Br-cAMP, poly-L-arginine (mol wt 15,000-70,000), bumetanide, CFTRinh-172, ionomycin, thapsigargin, CaCCs inhibitor and UTP were all purchased from Sigma-Aldrich, (Saint Louis, MO). H89 dihydrochloride, forskolin, H89, PKI (14-22 amide, myristoylated) and the PDE4 inhibitor Ro 20-1724 were all from Biotechne (Minneapolis, MN). VX-809 (Lumacaftor), VX-770 (Ivacaftor), VX-661 (Tezacaftor) and VX-445 (Elexacaftor) were purchased from MedChemExpress LLC (New Jersey, NJ).

Animals. PI3Kγ-deficient mice ($PI3K\gamma^{-/-}$) were described previously (Hirsch, Katanaev et al. 2000, Patrucco, Notte et al. 2004). Mutant mice were back-crossed with C57Bl/6j mice for 15 generations to inbreed the genetic background and C57Bl/6j were used as controls ($PI3K\gamma^{+/+}$). For asthma studies, wild-type BALB/C females were used. Mice used in all experiments were 8–12 weeks of age. Mice were group-housed, provided free-access to standard chow and water in a controlled facility providing a 12-hour light/dark cycle and were used according to institutional animal welfare guidelines and legislation, approved by the local Animal Ethics Committee.

All animal experiments were approved by the animal ethical committee of the University of Torino and by the Italian Ministry of Health (Authorization n°757/2016-PR).

Human material. Approval for primary bronchial epithelial cells and organoids cultures was obtained by the different local ethics committees (University of California San Francisco, Istituto Giannina Gaslini, the University of North Carolina at Chapel Hill, University of Verona and University Medical Center Utrecht) and informed consent was obtained from all participating subjects.

Cell lines and transfection. Normal human bronchial smooth muscle cells (hBSMCs) were purchased from Lonza (CC-2576, Lonza Walkersville, Inc. USA), cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA). Cells were used for experiments up to passage 15. Immortalized normal human bronchial epithelial (16HBE14o−) and cystic fibrosis human bronchial epithelial (CFBE41o–) cell lines were kindly provided by Dr. Dieter Gruenert (University of California San Francisco, San Francisco, CA). CFBE41o– stably expressing either wt-CFTR (wt-CFTR-CFBE41o-) or F508del-CFTR (F508del-CFTR-CFBE41o-) were kindly provided by Dr. Lianwu Fu from the UAB Research Foundation (Birmingham, AL). These cell lines were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA) on culture dishes pre-coated with human fibronectin (1 mg/ml; Sigma-Aldrich, Saint

Louis, MO), bovine collagen I (3 mg/ml; Sigma-Aldrich, Saint Louis, MO) and bovine serum albumin (0.1%; Sigma-Aldrich, Saint Louis, MO). The CFBE410- cell line expressing an inducible wt-CFTR with a 3HA-tag (CFTR-3HA) in the fourth extracellular loop and/or an additional HBH tag at the N-terminus (HBH-CFTR-3HA) was described previously (Schnur, Premchandar et al. 2019). In this cell line, CFTR expression was induced with 50-250 ng/ml doxycycline exposure for at least four days. These cells were cultured in MEM supplemented with 10% FBS, 5 mM L-Glutamine, 10 mM HEPES (Invitrogen, Waltham, MA), G418 (200 μg/ml; InvivoGen, San Diego, CA), and puromycin (3 μg/ml, InvivoGen, San Diego, CA) and grown on plates or filter inserts pre-coated with 10 μg/ml human fibronectin (EMD, San Diego, CA), 30 μg/ml PureCol collagen preparation (Advanced BioMatrix, San Diego, CA), and 100 μg/ml bovine serum albumin (Sigma-Aldrich, Saint Louis, MO) diluted in LHC-8 basal medium (Invitrogen, Waltham, MA). For Ussing chamber experiments performed in wt-CFTR-CFBE41o-, cells were seeded and differentiated for at least four days on fibronectin-collagen-coated cell culture dishes or polyester-permeable supports (Snapwell filters, Corning, Corning, NY). Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA). Cells were transfected with the indicated plasmids (up to 10 μg of total cDNA) with calcium phosphate and used for experiments 24h after transfection. All cells were cultured at 37°C and under a 5% CO_2 atmosphere.

Protein extraction and immunoprecipitation. mTSMCs, 16HBE14o−, CFBE41o–, HEK293T and PANC-1 cells were scraped in 120 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0) and 1% Triton X-100, supplemented with protease and phosphatase inhibitors, as described above. Detergent-insoluble material was precipitated by centrifugation at 13000 rpm for 10 min at 4°C. Supernatants were subjected to immunoprecipitation or used directly for Western blotting. Liquid nitrogen-frozen tissues were homogenized in 120 mmol/L NaCl, 50 mmol/LTris-HCl (pH 8.0), protease inhibitor Complete (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and 10 mmol/L sodium pyrophosphate). After 30 min incubation on ice, lysates were centrifuged at 13000 rpm for 10 min at 4°C and either used for Western blotting or subjected to immunoprecipitation/PDE activity measurements. For immunoprecipitation assays, pre-cleared extracts were incubated with 20 µl of a 1:1 slurry of protein A- or G-Sepharose (Amersham Biosciences, Buckinghamshire, UK) and 1-3 ug of antibody/mg of protein for 2 h at 4°C. Immunocomplexes were then extensively washed with lysis buffer and either used for Western blotting or for PDE activity quantification.

Western blotting. Proteins concentration from tissue or cell extracts was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Proteins were analysed in 7.5% or 4-20% gradient SDS-PAGE gels, as appropriate, and transferred to PVDF membranes. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline with Tween 20 (TBST) at RT for 1 h. Subsequently, membranes were incubated at 4°C overnight with primary antibodies diluted in TBST at the indicated dilution

ratio. Primary antibody binding was detected using a horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Saint Louis, MO) and developed using a chemiluminescence ECL assay kit (Millipore Corporation, Billerica, MA). Quantification of protein bands was performed with Quantity One analysis software (Bio-Rad, Hercules, CA).

PDE assay. PDE activity in immunoprecipitates was measured according to the two-step method of Thompson and Appleman (Thompson and Appleman 1971) as previously described (Ghigo, Perino et al. 2012), with minor modifications. Briefly, immunoprecipitations were assayed in a total volume of 200 µl of reaction mixture containing 40 mmol/L Tris-HCl (pH 8.0), 1 mmol/L $MgCl_2$, 1.4 mmol/L 2-mercapto-ethanol and 0.1 µCi of [3H]cAMP (Amersham Bioscience, Buckinghamshire, UK) for 40 min at 33°C. To stop the reaction samples were boiled at 95°C for 3 min. The PDE reaction product 5'-AMP was then hydrolysed by incubation of the assay mixture with 50 µg of Crotalus Atrox snake venom for 15 min at 37°C (Sigma-Aldrich, Saint Louis, MO). The resulting adenosine was separated by anion exchange chromatography using 400 µl of a 30% (w/v) suspension of Dowex AG1-X8 resin (Bio-Rad, Segrate, Milano, Italy). The amount of radiolabelled adenosine in the supernatant was quantitated by scintillation counting (Ultima Gold scintillation liquid from Perkin Elmer, Waltham, MA).

Peptide competition assay. HEK293T cells were transfected with pcDNA3.1-PI3Kγ using the calcium phosphate method and 48 h post-transfection cells were treated with different doses of the PI3Kγ MP or control peptide for 2 h. Cells were lysed in cold lysis buffer as described above, and the regulatory subunit of PKA (PKA-RII) was immunoprecipitated with the

appropriate antibody. The presence of the PI3Kγ catalytic subunit (p110γ) in immunoprecipitates was evaluated through SDS−PAGE and immunoblotting analysis.

FITC labelling of the PI3Kγ mimetic peptide. The PI3Kγ MP was labelled with FITC fluorochrome according to the method of Holmes (Holmes, Lantz et al. 2001) with minor modifications. Briefly, 1-2 mg/ml of peptide was dialyzed against 500 ml FITC labelling buffer (0,05M H_3BO_3, 0,2M NaCl, adjust pH to 9.2 with 5M NaOH) at 4°C with two or three changes over 2 days. 2 µl of 5 mg/ml FITC in DMSO were added for each milligram of peptide and incubated for 2 h at RT. Unbound FITC was removed by dialysis against 500 ml final dialysis buffer (0,1M Tris Cl pH 7.4, 0,1% NaN_3, 0,2M NaCl, adjust pH to 7.4 with 5M NaOH) at 4°C with two or three changes over 2 days and the FITC-peptide complex was then diluted 1:1 in Hanks' balanced salt solution (HBSS) without phenol red, 0,1% NaN_3, 5% BSA.

Isolation of peritoneal macrophages. Peritoneal macrophages were prepared from 8- to 12-week-old wild-type (WT) and PI3Kγ$^{-/-}$ mice, as described previously (Hirsch, Katanaev et al. 2000). Briefly, cells were collected from euthanized animals by peritoneal lavage with 5 mL of PBS, supplemented with 5 mM EDTA. Cells were centrifuged for 3 min at 300 g and the pellet was resuspended in culture media including Roswell Park Memorial Institute (RPMI) media, 100 U/ml penicillin and 100 µg/ml streptomycin, and 10% heat inactivated FBS (Thermo Fisher Scientific, Waltham, MA). Macrophages were seeded in 96-well plates ($1*10^6$ cells/ well) and incubated for at least 16/18 h at 37°C with 5% CO_2 before experiments.

cAMP measurements. Lungs, tracheas and hearts were collected from euthanized mice, powdered in liquid nitrogen and extracted with cold 6% trichloroacetic acid. Samples were sonicated for 10 sec, incubated at 4°C under gentle agitation for 10 min and then centrifuged at 13000 rpm at 4°C for 10 min. Supernatants were washed four times with five volumes of water saturated diethyl ether and lyophilized. cAMP content was detected with Amersham cAMP Biotrak Enzyme immunoassay System (GE Healthcare Life Sciences, Pittsburgh, PA), according to the manufacturer's protocol.

Isolation of mouse tracheal smooth muscle cells. Murine tracheal smooth muscle cells (mTSMCs) were cultured from explants of excised tracheas using modifications of previously described methods (McGraw, Forbes et al. 1999). The whole trachea between the larynx and the main stem bronchi was removed and placed in a sterile Petri dish containing RT Hanks' balanced saline solution, supplemented with a 2x concentration of antibiotic-antimycotic (Gibco, Carlsbad, CA). With the aid of a dissecting microscope, additional surrounding tissue was removed, the tracheal segment was split longitudinally and dissected into 2-3 mm squares. All the segments from a single trachea were then placed intima side down in a sterile 60-mm dish. After allowing the explants to adhere, 2.5 mL of Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA), supplemented with 20% FBS and 2x concentration of antibiotic-antimycotic (Gibco, Carlsbad, CA) was added to cover the explants. Explants were incubated at 37°C in a humidified environment of 95% air, 5% CO_2. Three days after plating, the concentration of FBS and antibiotic-antimycotic were reduced to 10% and 1x, respectively. Tracheal segments were removed when the outgrowing cells became locally

confluent. Once the 60-mm dish became confluent, cells were harvested by trypsinization and passed to a single 60-mm dish. Tracheal smooth muscle cells were subsequently passaged at a 1:2 ratio. Greater than 90% of these cells from each donor mouse were smooth muscle cells, as determined by immunofluorescence performed by an antibody raised against smooth muscle actin (Sigma-Aldrich, Saint Louis, MO). All experiments were performed on confluent cells at passage 3.

Ovalbumin immunization. To investigate anti-inflammatory effects: Ovalbumin (100 µg OVA, Sigma-Aldrich, Saint Louis, MO) complexed with aluminum potassium sulfate (1 mg, alum) was administered intraperitoneally (i.p.) on days 1 and 14, and intranasally (i.n.) (50 µg OVA in 50 µL PBS) on days 14, 25, 26, and 27. Control mice received i.p. injections of alum alone and intranasal PBS. To assess the anti-inflammatory action of the PI3Kγ MP, wild-type BALB/C females were treated by i.t. instillation with the PI3Kγ MP (25 µg in a final volume of 50 µL of PBS) or equimolar amounts of control peptide before each i.n. administration of OVA (days 14, 25, 26, and 27). To investigate lung function: Balb/c mice were sensitized with OVA (20 µg) and aluminium hydroxide (2 mg) i.p. on days 0 and 14. Then, animals were challenged on days 21-23 by i.h. application of 1% OVA for 30 min. 24 h after the last OVA inhalation mice were treated with i.t. application of PI3Kγ MP (15 µg) or equimolar concentrations of control peptide 30 min before assessing airway hyperreactivity to inhaled methacholine.

Airway inflammation analysis. 24 h after the final OVA injection (day 28), mice were anesthetized (sodium pentobarbital, 70-90 mg/kg, i.p.) and the tracheas incised and cannulated. The airways were washed with 2.5 ml of

PBS and the total number of cells in the bronchoalveolar lavage (BAL) was determined with a Neubauer hemocytometer. A volume of 50 µL of BAL was centrifuged onto cytospin glass slides at 400 rpm at RT for 5 min and stained with a Diff-Quick system (LabAids, Ronkonkoma, NY). A total of 100 cells per slide were counted and classified as neutrophils, macrophages, lymphocytes and eosinophils on the basis of morphological criteria. Erythrocytes and epithelial cells were ignored, and the results were expressed as cells/ml. To assess the peribronchial inflammation, the lungs of a group of animals that had not been subjected to bronchoalveolar lavage were explanted, fixed in a solution of 4% paraformaldehyde (PFA) for 24h at 4°C and embedded in paraffin. 5 µm-thick slices were cut, deparaffinized, stained with a hematoxylin-eosin solution (Bio-Optica, Milano, Italy), dehydrated and mounted with glass coverslips. The extent of peribronchial inflammation was classified as follows: 0- normal; 1- few inflammatory cells; 3- a thick ring of inflammatory cells. To evaluate the presence of goblet cells, lung slices were stained with periodic acid-Schiff's reagent (PAS) (Bio-Optica, Milano, Italy) and the percentage of PAS-positive cells was calculated by counting the number of PAS-positive epithelial cells and total epithelial cells.

Airway responsiveness measurements. For tidal volume measurements: OVA-sensitized mice were treated by intratracheal instillation (i.t.) of 70 µL of phosphate-buffered saline (PBS) alone or containing the PI3Kγ MP (15 µg) or equimolar amounts of CP. Airway responsiveness was evaluated 30 min after treatment. Airway responsiveness measurements were performed as described previously, with minor modifications (Fanelli, Puntorieri et al. 2010). Mice were anaesthetized (pentobarbital sodium, 70–90 mg/kg, i.p.),

tracheostomized and ventilated with a positive end inspiratory pressure of 10 cmH_2O, positive end expiratory pressure (PEEP) of 3 cmH_2O, respiratory rate of 90 breaths/min on room air. Airway opening pressure (Pao) proximal to the endotracheal tube, and the pressure inside the chamber were measured with pressure transducers (Special Instruments, Digima Clic; Nordlingen, Germany). Gas flow was measured with a pneumotachograph (Special Instruments, Digima Clic; Nordlingen, Germany). Tidal volume was calculated as the integral of the flow signal. Mechanical ventilation variables were recorded using ICU-Lab software (KleisTEK Advanced Electronic Systems; Bari, Italy). Airway hyperresponsiveness was assessed as a change in tidal volume after i.v. challenge with 500 µg/kg methacholine. For resistance measurements: Experiments were performed with the flexiVent system (Scireq, Montreal, Quebec, Canada). After anesthetization (fentanyl 50 µg/kg, medetomidine 0.5 mg/kg, midazolam 5 mg/kg, i.p, 100 µl per 10 g BW), mice were tracheotomized, and then ventilated with a tidal volume of 10 ml/kg at 150 breaths/min and a positive end-expiratory pressure of 2.5 cmH_2O. Two deep inflation maneuvers were performed for airway recruitment before the measurements were started. Resistance was determined by "snapshot" perturbations based on the single-compartment model. Healthy mice received PI3Kγ MP or the CP (1.5 µg or equimolar amounts of CP) as an aerosol via the nebulizer directly before the measurement, OVA mice had been treated 30 min before (15 µg or equimolar amount of CP). To induce airway constriction, mice were exposed to increasing concentrations of methacholine (MCh, 0; 6.25; 12.5; 25; 50 mg/ml) that was applied as an aerosol via the Aeroneb Lab nebulizer (AG-AL1100,

Aerogen, Galway, Ireland), producing a standard volumetric mean diameter of the particles between 2.5 and 4 mm.

Neutrophil adhesion assay. Twelve-well glass slides were coated with human fibrinogen (20 μg/well in endotoxin-free PBS) or with purified human ICAM-1 (3000 sites/μm2) for 16h at 4°C. Neutrophils (105/well; 5×106/mL in PBS containing 10% heat-inactivated fetal calf serum (FCS), 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2) were treated with vehicle, CP (50 μM) or different doses of PI3Kγ MP for 1 hour, with or without pre-treatment with the PKA inhibitor H89 (200 nM, 30 min) and then added to the plate. After 1 min incubation at 37°C, cells were stimulated by the addition of 25 nM fMLP for 1 min before washing, fixation on ice in 1.5% glutaraldehyde for 60 min, and computer-assisted enumeration of cells bound in 0.2 mm^2, as described previously (Laudanna, Campbell et al. 1996).

Chemotaxis assay. Human neutrophil migration assays were performed using 3 μm pores transwell filters (Corning, Corning, NY), inserted in 24-well plates. The bottom chamber was filled with 700 μl of RPMI + 10% FBS containing 25 nM fMLP, and the top chamber was filled with 0.15×10^6 cells in 200 μl of RPMI + 10% FBS, after treatments as indicated. Plates were incubated for 60 min at 37°C. At the end of the incubation, the inserts were removed, and the total number of cells migrated in the lower chamber was counted by flow cytometer (MACSQuant Analyzer 10; Miltenyi Biotec, Bergisch Gladbach, Germany).

RhoA activation assay. RhoA activation was determined using G-LISA activation assay kits (Cytoskeleton, Denver, CO), by adaptation of previously

reported protocols (Bolomini-Vittori, Montresor et al. 2009). Neutrophils pre-treated with vehicle, CP (50 μM) or PI3Kγ MP (50 μM) were stimulated or not with 25 nM fMLP for 1 min and then lysed for 15 min in the lysis buffer indicated in the manufacturer's protocol. Protein concentrations were quantified by Bradford assay. Equal amounts of proteins were added in triplicate to a 96-well plate coated with the RhoA-binding domain of rhotekin and incubated at 4°C for 30 min. Wells containing only lysis buffer were used as blank samples. After washing, the amount of RhoA-GTP bound was revealed by an anti-RhoA antibody, followed by a secondary HRP-labeled antibody and detection of HRP. Signals were measured with a microplate spectrophotometer (Victor X5 multilabel plate reader, PerkinElmer, Waltham, MA) by quantifying absorbance at 490 nm.

Flow cytometry analysis. After extraction, peritoneal macrophages were seeded in 12-well plates ($0.5*10^6$ cells/ well). The day after, cells were treated with the selected compounds for 24 hours. Afterwards, cells were detached using 1x Versene solution (Invitrogen, Waltham, MA) and aliquoted in flow cytometry tubes ($0.1*10^6$ cells/ tube). Macrophages were then blocked with an anti-mouse IgG Fc Antibody (1:1000) for 5 min at RT, and then stained with antibodies recognizing M1 and M2 macrophage polarization markers (PE/Cyanine7 anti-mouse F4/80, PE anti-mouse CD206, APC anti-mouse Qa-2, FITC anti-mouse CD86, APC/Cyanine7 anti-mouse CD11c; BioLegend, San Diego, CA, USA) for 30 min at 4°C. Cell viability was assessed by evaluating propidium iodide (Sigma-Aldrich, Saint Louis, MO) incorporation. All data were acquired using the FACSVerse™ instrument (BD bioscience, Franklin Lakes, NJ).

Macrophage killing assay. The ability of peritoneal macrophages to kill bacteria *in vitro* was assayed by incubating serum-opsonized E. coli bacteria (E13231; Invitrogen, Waltham, MA) with macrophages (ratio of 10:1 bacteria/ macrophage) in PBS for 1 hour at 37°C. Afterwards, cells were centrifuged at 5000g for 5 min and resuspended with deionized water to lyse macrophages. Serial dilutions of the lysate were then plated on Luria-Bertani plates and incubated overnight at 37°C. Colony-forming units were then counted.

Biodistribution and immunogenicity of the PI3Kγ MP. To assess the biodistribution of the PI3Kγ MP, wild-type BALB/c mice were injected i.t. with a FITC-conjugated PI3Kγ MP (1.5 μg in a final volume of 50 μL PBS) or vehicle. 30 min, 2 h, 1 day and 4 days after the injection, animals were anesthetized, trachea, lungs and hearts were extracted and frozen in OCT compound. 10 μm-thick cryosections were obtained with a cryostat Leica CM1850 (Leica Microsystems GmbH, Wetzlar, Germany) and bright field and FITC images were acquired with a Zeiss Observer-Z1 microscope, equipped with the Apotome (Carl Zeiss, Oberkochen, Germany). To evaluate immunogenicity, antigen-specific IgG were examined by ELISA in the serum of OVA-sensitized mice treated with the PI3Kγ MP or CP before each intra-nasal administration of OVA (days 14, 25, 26, and 27). Peptides (25 μg of PI3Kγ MP or equimolar amount of CP in a final volume of 50 μL of PBS) were administered either i.t. or intraperitoneally. Naïve mice were used as negative controls. For IgG quantification by ELISA, OVA, CP and PI3Kγ MP (15 μg/ml final concentration) were coated to a 96-well microtiter plate in 100 μL of PBS buffer overnight at 4 °C. The day after, washing and blocking

steps were performed using PBS and 3% BSA in PBS, respectively, at RT. Blood from naïve and OVA-sensitized mice was centrifuged at 13000 rpm for 10 min and 100 µL of supernatant were first diluted in PBS 1:1000 and then added to the pre-coated plate. After 1-hour incubation at RT, the plate was washed, and peroxidase-conjugated anti-mouse secondary antibody was incubated for 1 hour at RT. The binding was revealed by standard colorimetric reaction adding 70 µl of chromogenic TMB substrate (3,3′,5,5′-tetramethylbenzidine). The reaction was stopped with 70 µl of 0.5 M HCl and absorbance at 450 nM was measured using ELISA plate reader.

Sample purification for tandem mass spectrometry analysis. MS/MS experiments were performed as described previously (Premchandar, Kupniewska et al. 2017). Briefly, HBH-CFTR-3HA-expressing CFBE (HBH-CFTR-3HA-CFBE41o-) monolayers were grown in fibronectin-coated 6-cm dishes at least four days post-confluence. CFTR expression was induced with doxycycline (250 ng/ml) for four days. On the day of the experiment, the cells were treated with the indicated concentration with the following: DMSO (10 min); 10 µM forskolin (10 min); 25 µM PI3Kγ MP (60 min) and 10 µM CP (60 min) in Krebs-bicarbonate Ringer (KBR) buffer (140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 25 mM HCO_3^-, 2.4 mM HPO_4, 0.4 mM H_2PO_4, 1.2 mM $Ca^{2+,}$ 1.2 mM Mg^{2+}, 5 mM glucose, pH 7.4) at 37 C. Afterwards, cells were washed with ice-cold PBS (1X PBS, 1mM $MgCl_2$, $CaCl_2$ 0.1mM) supplemented with phosphatase inhibitor cocktail (2 mM sodium fluoride, 2 mM imidazole, 1.15 mM sodium-molybdate, 4 mM sodium orthovanadate, 4 mM sodium tartrate, 1 mM sodium pyrophosphate, 1 mM β-Glycerophosphate) for 1-2 min. Cells were solubilized with lysis

buffer (20 mM Tris pH 8.0, 300 mM NaCl, 0.4% Triton X-100, 1 mM DTT) supplemented with protease inhibitors (10 µM Leupeptin, 10 µM Pepstatin A, 100 µM PMSF) and kinase inhibitors (4 mM EDTA and 4 mM EGTA). Complete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO) and PhosSTOP™ (Sigma-Aldrich, Saint Louis, MO) phosphatase inhibitors were added to the lysis buffer. Lysates were centrifuged at 14000 rpm for 10 min in pre-chilled Eppendorf tubes, and the supernatant was then incubated with Dynabeads® MyOne™ Streptavidin C1 (Thermo Fisher Scientific, Waltham, MA) with end-to-end rotation at 4 °C for 1 hour. To remove non-specifically bound proteins, the beads were washed nine times: 1x with lysis buffer, 1x high salt buffer (20 mM Tris-HCL pH 8.0, 500 mM NaCl, 0.4% Triton X-100, 1 mM DTT), 2x 6 M Urea (20 mM Tris pH 8.0, 300 mM NaCl, 0.4% Triton X-100, 1 mM DTT, 6 M Urea), 2x with dodecyldimethylglycin (DMNG)-containing solution (300 mM NaCl, 20 mM Tris pH 8.0, 0.01% DMNG) to eliminate Triton X-100 and 3x with 50 mM ammonium bicarbonate supplemented with 0.01% DMNG. The washed beads were stored on ice in 50 mM ammonium bicarbonate until digestion.

On-bead digestion and LC-MS/MS. The on-bead trypsin digestion was performed after the beads were resuspended in 2 M Urea/50 mM ammonium bicarbonate, and trypsin digestion was done overnight at 37°C. The samples were then reduced with 13 mM DTT at 37°C and cooled for 10 min at room temperature. The samples were alkylated with 23 mM iodoacetamide at RT for 20 min in the dark. The supernatants were acidified with trifluoroacetic acid and cleaned from residual detergents and reagents with MCX cartridges

(Oasis MCX 96-well Elution Plate; Waters, Milford, MA) according to the manufacturer's instructions. After elution in 10% ammonium hydroxide/90% methanol (v/v), samples were dried with a Speed-vac. The samples were then reconstituted under agitation for 15 min in 12 µL of 2%ACN-1%FA and loaded into a 75 µm i.d. × 150 mm, Self-Pack C18 column. In the end, the samples were installed in the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark). Peptides were eluted with a two-slope gradient at a flow rate of 250 nl/min. Solvent B first increased from 1 to 36% in 66 min and then from 36 to 90% B in 14 min. The HPLC system was coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, MA) through a Nanospray Flex Ion Source (Thermo Fisher Scientific, Waltham, MA). Nanospray and S-lens voltages were set to 1.3–1.8 kV and 50 V, respectively. The capillary temperature was set to 225 °C. Full scan MS survey spectra (m/z 360–1560) in profile mode were acquired in the Orbitrap with a resolution of 120,000 with a target value at 1e6. The most intense peptide ions were fragmented by both HCD and EThcD and analyzed in the linear ion trap with a target value at 2e4 and normalized collision energy at 28 V. An MS3 scanning was performed upon detection of a neutral loss of phosphoric acid (48.99, 32.66 or 24.5 Th) in HCD MS2 scans. The duty cycle was set to 3 sec, and target ions selected for fragmentation were dynamically excluded for 30 sec after three MS/MS events.

CFTR peptide identification and phosphorylation quantification. The peak list was generated with Proteome Discoverer (version 2.1) using the following parameters: minimum mass - 500 Da, maximum mass - 6000 Da, no grouping of MS/MS spectra, precursor charge-auto, and the minimum

number of fragment ions - 5. Protein database searching was performed with Mascot 2.6 (Matrix Science) against the UniProt human protein database and a user-defined CFTR database. The mass tolerance for precursor and fragment ions were set to 10 ppm and 0.6 Da respectively. Trypsin was used as the enzyme allowing up to 1 missed cleavage. Cysteine carbamidomethylation was set as a fixed modification and methionine oxidation as variable modifications. Data interpretation was performed with Scaffold (version 4.8). Phosphorylated and non-phosphorylated CFTR peptides were quantified using Pinnacle (Optys Technologies, Philadelphia, PA). The protein/peptide results from Mascot (*.dat files) were combined with the raw data files (*.raw files) from the mass spectrometer using this software. The sites under consideration are PKA sites with mono- and dibasic residues in their vicinity, so there may be missed cleavages during trypsin digestion. To account for this, we included all the peptides containing the putative phosphosite for quantification. Each peptide's relative intensity or abundance was obtained from the extracted ion chromatograms (XICs) and integrating the area under each XIC using Pinnacle. The counts from each peptide were then normalized to the total ion current (TIC) or the total CFTR abundance in the respective treatment groups. Counts were then normalized for total ion current. Each peptide's file area was normalized between treatments to the total CFTR peptide count injected in the respective treatment group. The percent of relative phosphorylation (or phospho-occupancy) of each site was calculated as the ratio of all phosphorylated peptides that contained a given phosphosite divided by the integrated areas of all peptides carrying the same site and multiplied by 100 according to the formula: %

phosphorylation of site A = 100 x(sum of the area of peptides phosphorylated at site A)/(sum of areas of all peptides carrying site A).

Intracellular water residence time determination by 1H-NMR relaxometry. Intracellular water residence time was measured as described previously (Terreno, Geninatti Crich et al. 2006, Ruggiero, Baroni et al. 2018, Montiel, Bella et al. 2020). In a cell suspension, water molecules distribute in the extracellular space and in the intracellular (in) cytoplasm and exchange between the two compartments. From the relaxometric point of view, when a contrast agent (i.e. Gd-HPDO3A) is added to the extracellular space, the water exchange modulates the observed relaxation behaviour, and the intracellular water residence time (τ_{in}) may be determined by analysing the data in terms of the two-Site eXchange (2SX) model (Terreno, Geninatti Crich et al. 2006, Ruggiero, Baroni et al. 2018). Briefly, HEK-293T cells expressing the wt-CFTR were detached with 0,1% trypsin and 0.1% EDTA (Termofisher Scientific, Waltham, MA) and re-suspended in the presence of Gd-HPDO3A (10–20 mM in PBS) kindly provided by Bracco S.p.A. (Milan, Italy) with/without the PI3Kγ MP, previously dissolved in DMSO. Relaxometric measurements were carried out within 15 min. Cells were transferred in 5 mm NMR tubes and centrifuged 5 min at 0.1 rcf at RT. Measurements of the cellular pellet were carried out at 0.5 T and 25°C on a Stelar SPINMASTER spectrometer (Stelar, Mede, Italy) by means of the inversion-recovery (IR) pulse sequence with 32 τ increments distributed in the time range 0.005-4 s, relaxation delay 5 s, 2 averages. The intracellular water residence time (τ_{in}) was determined by analysing the IR data in terms of the two-Site eXchange (2SX) model.

Primary bronchial epithelial cell culture. Experiments with primary bronchial epithelial (HBE) cells were performed in three different laboratories.

Istituto Giannina Gaslini: HBE cells were isolated, cultured, and differentiated as described (Scudieri, Musante et al. 2018). Briefly, epithelial cells were obtained from mainstem bronchi of CF individuals undergoing lung transplant. For this study, cells were obtained from two CF donors homozygous for the F508del mutation and one non-CF patient. Bronchi were set overnight at 4°C in a solution containing protease XIV to detach cells. Epithelial cells were then cultured in a serum-free medium (LHC9 mixed with RPMI 1640, 1:1) supplemented with hormones and supplements that favor cell number amplification. For cells deriving from patients with CF, the culture medium contained a complex mixture of antibiotics (usually colistin, piperacillin, and tazobactam) to eradicate bacteria in the first days. To obtain differentiated epithelia, cells were seeded at high density on porous membranes 500,000 cells for 1-cm2 Snapwell inserts (Corning, Corning, NY). After 24 h, the serum-free medium was replaced with Dulbecco's modified Eagle's medium/Ham's F12 containing 2% FBS plus hormones and supplements. Differentiation of cells into a tight epithelium was monitored by measuring TEER and PD with an epithelial voltohmmeter (EVOM1; World Precision Instruments, Sarasota, FL). The medium was replaced daily on both sides of the permeable supports up to 8 to 10 days (liquid-liquid culture). Subsequently, the apical medium was totally removed, and the cells received nutrients only from the basolateral side [air-liquid culture (ALC)]. This

condition favoured a further differentiation of the epithelium. Cells were maintained under ALC for 2 to 3 weeks before experiments.

University of California San Francisco: HBE cells were isolated from tracheas and mainstem bronchi obtained from autopsies of patients homozygous for F508del-CFTR (F508del/F508del HBE patient #1). Cells were cultured as described previously (Zhang, Gallup et al. 2013, Blanchard, Zlock et al. 2014). Briefly, human tracheal tissue strips were washed in PBS. The bronchial epithelium was separated from underlying stroma using enzymatic digestion followed by vigorous agitation to dislodge the epithelial sheets. Single cells were isolated from epithelial sheets after a short incubation in 0.25% trypsin/EDTA. Primary HBE cells were plated 1×10^5 cells/cm2 onto 0.4-μm-pore Transwell polycarbonate membrane (Corning, Corning, NY) and grown in defined air-liquid interface medium for 2 to 3 weeks to produce differentiated, polarized HBE cultures resembling natural airway epithelium.

Charles River Laboratories: HBE cell monolayers obtained from donors homozygous for the F508del CFTR missense mutation were grown on Snapwell™ polyester or polycarbonate filter inserts. To allow proper differentiation, epithelial cells were cultured at least 21 days on permeable supports following switch to ALC 4 days after plating by removal of media from the apical surface.

Short-circuit current (I_{SC}) measurements. Short-circuit current (I_{SC}) measurements in primary human bronchial epithelial cells were carried out in three independent laboratories.

Istituto Giannina Gaslini: snapwell inserts carrying differentiated bronchial epithelia were mounted in a vertical diffusion chamber, resembling an Ussing chamber with internal fluid circulation. Both apical and basolateral hemichambers were filled with 5 ml of a solution containing 126 mM NaCl, 0.38 mM KH_2PO_4, 2.13 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$, and 10 mM glucose. Both sides were continuously bubbled with a gas mixture containing 5% CO_2:95% air, and the temperature of the solution was kept at 37°C. The transepithelial voltage was short-circuited with a voltage clamp (DVC-1000; World Precision Instruments, Sarasota, FL) connected to the apical and basolateral chambers via Ag/AgCl electrodes and agar bridges (1 M KCl in 1% agar). The offset between voltage electrodes and the fluid electrical resistance were cancelled before each set of experiments. I_{SC} was recorded with a PowerLab 4/25 (ADInstruments, Dunedin, New Zealand) analog-to-digital converter connected to a PC.

University of California San Francisco: I_{SC} was measured as described previously (Xie, Rich et al. 2011). In short, cells were grown on 1.12 cm^2 Snapwell inserts. The filters were mounted into Ussing chambers, and a chloride gradient was applied by bathing the cells in high-chloride basolateral buffer (140 mM NaCl, 5 mM KCl, 0.36 mM K_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 10 mM HEPES, and 10 mM glucose, pH 7.4) and low-chloride apical buffer (133.3 mM Na-gluconate, 5 mM K-gluconate, 2.5 mM NaCl, 0.36 mM K_2HPO_4, 0.44 mM KH_2PO_4, 5.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 10 mM Hepes, and 10 mM mannitol, pH 7.4). The buffers were aerated with a mix of 95% O2 and 5% CO_2 and temperature was kept at 37°C throughout the experiment. The

cultures were voltage-clamped at 0 mV using an EVC4000 MultiChannel V/I Clamp (World Precision Instruments, Sarasota, FL). After a 30-min stabilization period, drugs were added at specified times, while I_{SC} was continuously recorded.

Charles River Laboratories: filters were mounted in Ussing recording chambers (Physiologic Instruments, Inc., San Diego, CA) and maintained in both the apical and basolateral chambers with a HEPES buffered physiological saline (137 mM NaCl, 4.0 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES; 10 mM glucose, pH adjusted to 7.4 with NaOH). No ion concentration gradient was applied across the epithelium under these symmetric solution conditions and with the transepithelial potential voltage clamped to 0 mV (short circuit conditions), no electrochemical driving force was present for ion movement. Epithelia were continuously voltage clamped at 0 mV in symmetric solutions to measure the I_{SC} and maintained at a temperature of 35±2°C. Epithelial channel modulators prepared as DMSO stocks were added sequentially and cumulatively directly to Ussing chambers without intermediate dilution. Amiloride was added to the mucosal (apical) side of Snapwell™ filter inserts to block epithelial Na channels (ENaC) which overlap chloride channel currents. Transport modulators were applied sequentially and cumulatively to the apical (mucosal) and/or basolateral (serosal) epithelial surfaces. Transepithelial resistance was monitored periodically with small command voltage steps during the measurement unless these interfere with subsequent data analysis.

Human intestinal organoids and forskolin-induced swelling (FIS) assay.

Experiments on human intestinal organoids have been carried out in two independent laboratories.

University Medical Center Utrecht: crypts were isolated from human intestinal tissues derived from two different donors harboring the F508del mutation in homozygosity. Biopsies were washed with cold complete chelation solution and incubated with 10 mM EDTA for 1 h at 4 °C. The supernatant was harvested, and EDTA was washed away. Crypts were isolated by centrifugation and embedded in Matrigel (growth factor reduced, phenol free; BD bioscience, Franklin Lakes, NJ) and seeded in 24-well plates. The Matrigel was polymerized for 10 min at 37 °C and immersed in complete culture medium containing Wnt conditioned-media (50%, produced using stably transfected L cells), TGF-ß type I Receptor inhibitor A83-01 (Tocris Bioscience, Bristol, UK), Nicotinamide (Sigma-Aldrich, Saint Louis, MO) and P38 inhibitor SB202190 (Sigma-Aldrich, Saint Louis, MO). Confluent organoids, after mechanical dissociation by pipetting, were centrifuged and resuspended in cold Matrigel. Organoid swelling was measured in triplicate at two independent culture time points with 2 different concentrations of Fsk (0.51 μM and 0.128 μM), as previously described (Dekkers, Wiegerinck et al. 2013), and two concentrations of PI3Kγ MP (10 μM and 25 μM). The CFTR modulators (3 μM VX-770 and 3 μM VX-809) were per-incubated for 24h while the PI3Kγ MP was acutely added to the organoids together with Fsk. In short, intestinal organoids were seeded in 96-well culture plates in 4 μL of 50% Matrigel (+50% culture media) containing 20 to 40 organoids and immersed in 50 μL of media. The day after, organoids were incubated for 30

min with 3 μM calcein green (Invitrogen, Waltham, MA) to fluorescently label the organoids and stimulated with Fsk. The total calcein green labeled area per well was monitored by a Zeiss LSM800 confocal microscope (Carl Zeiss, Oberkochen, Germany) every 10 min for 60 min, while the environment was maintained at 37°C and 5% CO_2. A Zen Image analysis software module (Carl Zeiss, Oberkochen, Germany) was used to quantify the organoid response (area under the curve measurements of relative size increase of organoids after 60 minutes forskolin stimulation, t = 0 min baseline of 100%).

University of Verona: organoids were generated from rectal biopsies after intestinal current measurements obtained during standard care or from voluntary participation in studies. Human intestinal biopsies (3.5mm) from healthy donors and F508del/D1152H patients were washed with cold complete chelation solution and incubated with 10 mM EDTA for 30-60 min at 4°C. The supernatant was harvested and EDTA was washed away. Crypts were isolated by centrifugation and embedded in Matrigel (growth factor reduced, phenol-free, BD bioscience, Franklin Lakes, NJ) and seeded (50-200 crypts per 50 μl Matrigel per well) in 24-well plates. Matrigel was polymerized for 10 min at 37°C and immersed in complete culture medium supplemented with 1% penicillin/streptomycin, 10 mM HEPES, Glutamax, N2, B27 (all from Invitrogen, Waltham, MA), 1 μM N-acetylcysteine (Sigma-Aldrich, Saint Louis, MO) and growth factors: 50 ng/ml mEGF, 50% Wnt3a-conditioned medium and 10% Noggin-conditioned medium, 20% Rspo1-conditioned medium, 10 μM Nicotinamide (Sigma-Aldrich, Saint Louis, MO), 10 nM Gastrin (Sigma-Aldrich, Saint Louis, MO), 500 nM A83-01 (Tocris

Bioscience, Bristol, UK) and 10 μM SB202190 (Sigma-Aldrich, Saint Louis, MO). To perform FIS assays, rectal human organoids at second split were seeded on 8 well chamber slide (Ibidi, Gräfelfing, Germany) in 5 μl 50% Matrigel containing about 20 organoids in 200μL culture medium composed by Advanced Dmem-F12 (Invitrogen, Waltham, MA), 1% GlutaMAX-1 (Invitrogen, Waltham, MA), 1% HEPES (Invitrogen, Waltham, MA), 1% penicillin/streptomycin (Lonza) 1% Primocin (Invitrogen, Waltham, MA). Two days after seeding, organoids were incubated for 30 min with 100μL standard culture medium containing 3 μM calcein-green (Invitrogen, Waltham, MA), and stimulated with Fsk (2 μM) with either PI3Kγ MP or CP (25 μM) as indicated. Images were collected in a temperature-controlled stage mounted on a Confocal Live Cell Microscopy (inverted microscope TCS-SP5, Leica Microsystems Inc, Buffalo Grove, IL). The increase in organoids volume (xyz plane) relative to t=0 of treatment was quantified using IMMARIS 7.2.1 software (Bitplane, Zurich, Switzerland); organoid volume was normalized with respect to the baseline (= 100%). Cell debris and not viable structures were manually excluded from image analysis using pre-defined settings.

Cell surface biotinylation assay. Cells were washed twice with ice-cold PBS (pH=8.0) and incubated with 0.5 mg/ml Sulfo NHS-SS-biotin (Thermo Fisher Scientific, Waltham, MA) in PBS (pH=8.0) for 1 h at 4°C under gentle agitation. Cells were washed once with 25 mM Tris (pH=8.0) to quench non-reacted biotinylation reagent and twice with ice-cold PBS to remove non-reacted biotinylation reagent. Protein extraction was performed as described above. An aliquot of supernatants corresponding to 600 μg of proteins was

incubated with high-capacity streptavidin agarose resin (Thermo Fisher Scientific, Waltham, MA), for 2 h at 4°C with gentle mixing, following the manufacturer's recommendation. Streptavidin bound complexes were pelleted at 1000 g and washed four times with lysis buffer. Biotinylated proteins were eluted from the resin with reducing Sample Buffer 2X and directly used for Western blotting.

Targeted phosphoproteomics by antibody microarray. Following administration of PI3Kγ MP and control peptide (CP) at equimolar concentration (25 μM, 30 min) CFBE41o- were lysed in lysis buffer as described above, and protein samples were frozen at-80 C before being subjected to an antibody microarray (KAM-1325880 array) and data analysis, which was performed at Kinexus (System proteomic company, Vancouver, Canada). The array monitors changes in the expression levels and phosphorylation states of signaling proteins which includes 1150518 pan-specific antibodies (for protein expression) and 875359 phospho- site-specific antibodies (for phosphorylation). The resultant changes are expressed as percentages of change with respect to the control (CFC) and as Z-factors. Changes above R50%CFC and Z ratioR+1.00 (hyper phosphorylation) or% 1.00 (dephosphorylation) we were re considered as real significant changes significant ones. Only proteins whose phosphorylation status differed after the folding pulse were considered.

Immunogold Electron Microscopy. Cells were plated on alcian blue-coated glass coverslips and fixed 10 min with 0.05% glutaraldehyde in 4% paraformaldehyde (PFA) electron microscopy (EM) grade and 0.2 M HEPES buffer and 50 min in 4% PFA EM grade in 0.2 M HEPES buffer. After three

washes in PBS, cells were incubated 10 min with 50 mM glycine and blocked 1 h in blocking buffer (0.2% bovine serum albumin, 5% goat serum, 50 mM NH_4Cl, 0.1% saponin, 20 mM PO_4 buffer, 150 mM NaCl). Staining with primary antibodies and nanogold-labeled secondary antibodies (Nanoprobes, Yaphank, NY) were performed in blocking buffer at room temperature. Cells were fixed 30 min in 1% glutaraldehyde and nanogold was enlarged with gold enhancement solution (Nanoprobes, Yaphank, NY) according to the manufacturer's instructions. Cells were post fixed with osmium tetroxide, embedded in epon, and processed into ultrathin slices. After contrasting with uranyl acetate and lead citrate, the sections were analyzed with Zeiss LEO 512 electron microscope. Images were acquired by 2k × 2k bottom-mounted slow-scan Proscan camera controlled by the EsivisionPro 3.2 software.

Statistical analysis. Prism software (GraphPad software Inc., La Jolla, CA) was used for statistical analysis. Data are presented as scatter plots with bars (means ± standard error of the mean s.e.m.). Raw data were first analysed to confirm their normal distribution via the Shapiro-Wilk test and then analysed by unpaired Student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA. Bonferroni correction (one-way and two-way ANOVA) was applied to correct for multiple comparisons. In the absence of a normal distribution, a nonparametric Krukal-Wallis or Mann-Whitney test were used, followed by Dunn's correction for multiple comparisons if appropriate. $p< 0.05$ was considered significant (*), $p< 0.01$ was considered very significant (**), and $p< 0.001$ was considered extremely significant (***).

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Therapeutic peptides for the treatment of cystic fibrosis: Challenges and perspectives

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ABSTRACT

Cystic fibrosis (CF) is the most common amongst rare genetic diseases, affecting more than 70.000 people worldwide. CF is characterized by a dysfunctional chloride channel, termed cystic fibrosis conductance regulator (CFTR), which leads to the production of a thick and viscous mucus layer that clogs the lungs of CF patients and traps pathogens, leading to chronic infections and inflammation and, ultimately, lung damage.

In recent years, the use of peptides for the treatment of respiratory diseases, including CF, has gained growing interest. Therapeutic peptides for CF include antimicrobial peptides, inhibitors of proteases, and modulators of ion channels, among others. Peptides display unique features that make them appealing candidates for clinical translation, like specificity of action, high efficacy, and low toxicity. Nevertheless, the intrinsic properties of peptides, together with the need of delivering these compounds locally, e.g. by inhalation, raise a number of concerns in the development of peptide therapeutics for CF lung disease.

In this review, we discuss the challenges related to the use of peptides for the treatment of CF lung disease through inhalation, which include retention within mucus, proteolysis, immunogenicity and aggregation. Strategies for overcoming major shortcomings of peptide therapeutics will be presented, together with recent developments in peptide design and optimization, including computational analysis and high-throughput screening.

1. Introduction

Cystic fibrosis (CF) is the most common life-threatening recessive genetic disease of the Caucasian population. The pathology stems from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP (cAMP)-stimulated chloride channel expressed in exocrine epithelia, which are responsible for maladaptive chloride transport and the ensuing dehydration of secretions. In turn, mucus hyperviscosity may affect multiple organs, by causing chronic airways obstruction, pancreatic insufficiency and intestinal malabsorption. Despite being classified as a multi-organ disease, the major cause of mortality and morbidity in CF patients is pulmonary manifestation, ensuing from a vicious circle of airways obstruction, infection and inflammation [1].

Inhalation is the preferred route of administration for drugs targeting respiratory dysfunction in CF, given the possibility to locally target lung cells while concomitantly reducing side effects in off-target organs, compared to oral delivery [2]. Several peptides have been proposed as therapy for respiratory diseases, including CF, for examples derivatives of Short Palate Lung and Nasal Epithelium clone 1 (SPLUNC1) [3,4], Alpha-1-Antitrypsin (AAT) [5,6] or antimicrobial peptides, such as Esculentin [7,8], M33 [9,10] and Colistin [11,12]. Peptides offer many advantages over classical small molecules, such as high specificity and efficacy, as well as low toxicity [2]. However, CF lungs represent a challenging environment for peptide-based drugs because of the presence of a thick and viscous mucus, enriched in proteolytic enzymes, which may limit the penetration and reduce the stability of the drug.

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The aim of this review is to provide an overview of recent developments in peptide design and optimization, and to discuss how these strategies have been exploited for the identification and development of new peptide therapeutics for CF. Chemical optimization strategies for ameliorating physical-chemical properties, formulation and delivery of peptide therapeutics will be also described, along with relevant examples of optimized peptides for the treatment of CF lung disease.

2. Strategies for new peptide design and generation

Protein-protein interactions (PPIs) are the molecular engine of many fundamental cellular functions and therefore have served as a preferential drug target over the last two decades. However, interfering intracellular PPIs with small molecules is difficult in the case of large or flat binding sites. In recent years, the small size and the balance of conformational rigidity and flexibility made peptides promising candidates to target challenging PPI interfaces as well as catalytic or regulatory sites in protein targets. Accordingly, the last two decades have seen a significant renaissance of peptides in drug discovery [13] and over 60 peptides have been approved for clinical use so far [14]. Therefore, characterizing the mechanisms of peptide-protein recognition is pivotal for the design of new peptide-based strategies that are able to target endogenous proteins and interfere with PPIs, as well as for the improvement of the affinity and specificity of the peptide-protein binding in existing approaches [14].

To this purpose, a variety of computation-aided rational designs have been developed for the selection of peptide leads, depending on both the target of interest and what is known about its associated ligands and molecular pharmacology.

The most common and historically productive strategy in peptide discovery is the manipulation of the parent peptide's structure and properties to optimize half-life, potency, selectivity, or other properties, in order to attain the desired compound profile. However, this approach can only be used when targeting a known signaling peptide and alternative strategies (e.g. high-throughput screening, computational docking and sequence-structure-based predictions) are needed when it is not possible to optimize an existing cognate ligand for the target of interest. In the context of CF therapy, such biological targets for optimized or new peptide-based drugs may include ion channels, like ENaC and the macromolecular complex of CFTR, as well as microbial membranes.

2.1. High-throughput screening (HTS) for new peptide leads

De novo peptide ligand discovery has traditionally been done by screening large libraries of peptides, produced either synthetically or biologically [15]. Biological library methods include phage, ribosomal, and mRNA display and have undoubtedly become the standard for peptide discovery. Among these, phage display, in which the genome of each phage is engineered to display a different peptide on the surface, plays the most prominent role to date. By definition, the peptides produced in phage display libraries are comprised of the proteinogenic amino acids, therefore the obtained peptide leads usually must be optimized *via* medicinal chemistry to generate compounds with the pharmaceutical properties required by a drug. Several ingenious techniques have emerged, however, that could lead to much more "drug-like" peptide leads directly arising from phage libraries [13].

The original approach involved sequential rounds of affinity enrichment and expansion, leading to enriched phages identification. However, the high number of biopanning rounds involved can cause selection bias, dropouts and enrichment of false positives. Recently, these issues have been significantly reduced by the application of next generation sequencing (NGS) to phage display experiments [16]. NGS is quantitative and sensitive enough to minimize the number of biopanning rounds needed, minimizing the bias caused by multi-cycle screening. Traditionally, phage-displayed libraries have been constrained by the need to use only linear display of non-modified naturally-occurring amino acids. This limitation has been overcome by the development of strategies for on-phage chemical modifications, including the introduction of chemical entities (e.g. cyclization linkers [17], fluorophores [18], small molecules), or post-translational modifications like glycosylation [19]. These advances in modern biopanning approaches support the notion that peptide leads with high affinity and efficacy can be identified and subsequently optimized for clinical development [14].

Genomic or peptidomic/proteomic approaches are also being employed for the discovery of new peptides with novel biological functions [20]. Peptidomics combines peptide sequence identification with the profiling of peptides in various tissues and fluids and aims to systematically catalogue genetically encoded polypeptides [21]. This is facilitated by spectacular advances in mass spectrometry and bioinformatics that permit the identification (and sequencing) of peptides present in the tissue of interest with excellent sensitivity.

Other powerful methods, including oriented peptide array library and SPOT synthesis, have been successfully used to characterize peptide-protein recognition specificities [22].

The peptide array library integrates the oriented peptide library and array technologies: hundreds of individual pools, each of them consisting of an oriented peptide library, are synthesized on solid supports, and the preferred amino acids at every position are read directly from arrays, without protein sequencing. A disadvantage of this method is that the binding peptides are analyzed in a pool, making it impossible to obtain actual sequences of peptides and quantitatively compare their specific affinities for a defined target [23].

SPOT synthesis (Fig. 1) permits parallel synthesis and screening of thousands of cellulose membrane-bound peptides to study PPIs a proteomic context [22]. Unfortunately, peptides synthesized according to the standard SPOT protocol lack free C-termini due to their C-terminal fixation to the cellulose support. In principle though this issue can be solved, by reversing the peptide orientation (inverted peptides), creating N-terminally fixed inverted peptide arrays, enabling free C-terminal display on planar cellulose supports [24]. This improved method for generating cellulose membrane-bound inverted peptides may be a powerful tool to screen proteomic databases on a large scale and to find new ligands [25]. This strategy was used on one particular type of peptide binding domain, PDZ [23].

PDZ is one of the most abundant protein interaction modules and is involved in a variety of important cellular functions. The PDZ domain family is surprisingly complex and diverse, recognizing up to seven C-terminal ligand residues and forming at least 16 unique specificity classes across human and worm [23,26]. Several PDZ proteins are known to interact with the CFTR channel, including the CFTR-associated ligand (CAL), a negative regulator of the F508Δ mutant protein [27]. CAL, with is one PDZ domain (CALP), competes for CFTR binding with its antagonists NHERF1 and NHERF2 (Na+/H+ Exchanger Regulatory Factor 1/2), proteins containing two PDZ domains (N1P1, N1P2, N2P1 and N2P2), which control both the activity and the cell surface abundance of CFTR.

To target this mechanism, a series of five PDZ domains known to interact with CFTR were exploited to obtain a selective inhibitor of CFTR:CAL interaction that does not affect the biologically relevant PDZ competitors NHERF1 and NHERF2. A variety of different cellulose-bound peptide libraries was synthesized with the method

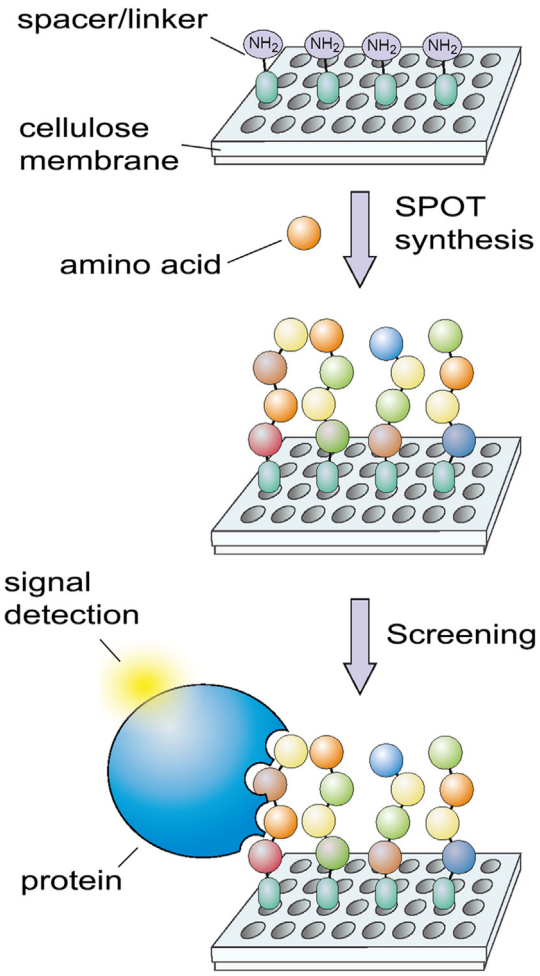

Fig. 1. Schematic representation of SPOT-based generation of peptide arrays and their applications.

of inverted peptides, based on SPOT technology. Arrays encoding a human C-terminal peptide library (6223HumLib) were then probed with each of the five PDZ domains [28]. These data provide proof-of-principle for selective PDZ inhibition and establish CAL inhibitors as founding members of a class of CFTR "stabilizers", specifically designed to reduce ΔF508-CFTR post-endocytic breakdown [27]. On the one hand, these peptides represent powerful tools to analyze the effects of CAL inhibition on the cell-surface abundance of CFTR in bronchial epithelial cells [28]. On the other hand, these molecules could be exploited in combination with correctors of the primary folding defect of the most common mutants, ΔF508-CFTR to cooperatively rescue its trafficking defect [28].

Other screenings have been conducted to identify antimicrobial peptides. Antimicrobial cationic peptides (AMPs) are a key component of the innate immune system, acting as the first line of defense against infectious agents. The properties of these peptides make them extremely attractive candidates for development as therapeutics for CF [29,30] Although lung epithelial cells secrete antimicrobial peptides and proteins [31], most endogenous peptides, such as β-defensins and LL-37, are low concentrated in the lung. Moreover, they are salt sensitive *in vitro,* thus presumably ineffective in the high-salt environment of the apical side of CF epithelial cells [32]. In this regard it has to be noted that the "high salt" hypothesis and whether the ionic composition of the airway surface fluid is different between CF and healthy individuals are still matter of debate [33]. A promising approach to overcome the limited efficacy of endogenous peptides is represented by the exogenous application of antimicrobial cationic peptides, with combined antimicrobial and anti-inflammatory activity, directly to the lungs, through aerosol formulations. In a study of Zhang et al. [29], 155 antimicrobial peptides, consisting of three distinct structural classes, were screened against mucoid and multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Staphylococcus aureus*, that are the microorganisms more frequently isolated from CF lungs [29,30]. Four peptides, HBCM2, HBCM3, HBCPα2, and HB71, demonstrated significant reduction in *Pseudomonas* bioburden in the lung of rats. In another study by Pompilio et al., three α-helical antimicrobial peptides, namely BMAP-27 and BMAP-28, cathelicidin-derived peptides of bovine origin, and the artificial P19(9/B) peptide have been tested for their *in vitro* antibacterial and anti-biofilm activity against selected *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains collected from CF patients [30]. The efficacy of these AMPs was compared to the reference antibiotic Tobramycin, which is used in chronic suppressive therapy in CF patients. BMAP-27 and BMAP-28 are cationic (charge: +11 and + 8, respectively) and both adopt an α-helical structure on interaction with the negatively charged bacterial surface. Recent results have suggested that AMPs with these characteristics may be the most effective against strains that produce exogenous polysaccharides, known to inhibit the activity of other types of AMPs. On this basis, the P19(9/B) peptide has been rationally designed with the aim of optimizing its propensity to assume α-helical conformation, making use also of non-proteinogenic amino acids [30]. The activity shown by α-helical peptides against planktonic and biofilm cells makes them promising "lead compounds" for the development of novel treatments of CF lung disease.

2.2. Computational docking of peptides

Computational docking procedures have proven to be an important tool of computer-aided drug design of small-molecule drugs. Similar efforts are being made in the field of peptide therapeutics [34]. Although the docking methods designed for small-molecule interactions are usually not well suited for the modelling of significantly more flexible and larger peptide molecules, the interest in peptide therapeutics has triggered the rapid development of new docking techniques. Peptide-protein docking strategies are usually categorized into local or global docking, based on the extent of the structural information that is provided as an input (Fig. 2).

Local docking is the most commonly used strategy, as it searches for a potential binding pose for peptide at a user-defined binding site in the resolved structure of its target receptor. DynaDock [35], Rosetta FlexPepDock [36] and PepCrawler [37] are the most popular methods that provide different approaches of defining peptide-binding sites. Whilst the local docking approach searches only for the peptide-binding pose, global docking searches for both the peptide-binding pose and site at the target protein. Therefore, global docking is often preferred when no prior information is available on binding sites. Several global docking methods are capable of predicting peptide conformation from a given query sequence, for example ClusPro (ClusPro PeptiDock [38]) and pepATTRACT [39]. Other global docking methods such as PeptiMap [40], AnchorDock [41] and CABS-Dock [42] also provide automatic docking simulation with varying algorithms such as small molecule binding adaption, in-solvent simulation, flexibility of query peptide or target protein at predicted binding proximity [14].

Generally, different level of peptides flexibility can be

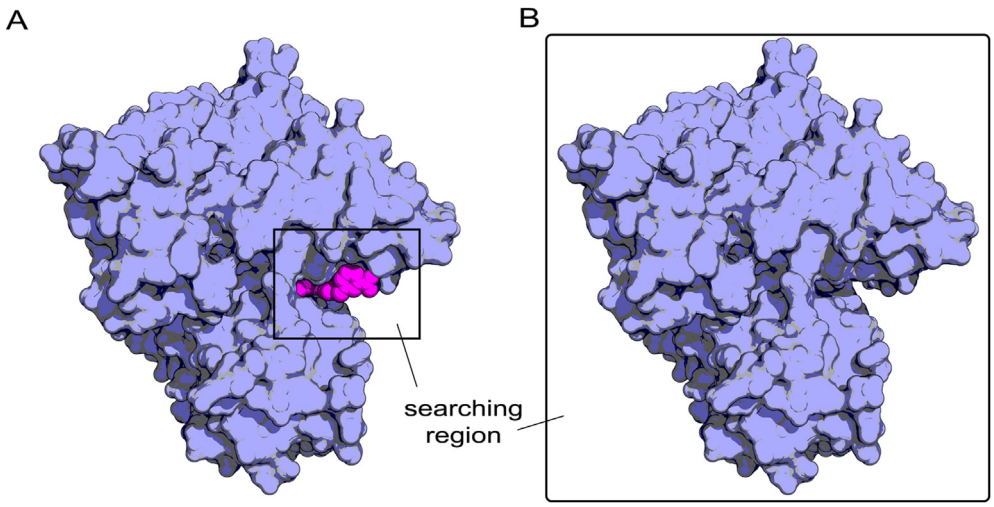

Fig. 2. Graphical representation of local docking versus global docking methods. Shown on the left, local docking for binding pose within a limited region (A), while on the right, global docking for both binding site and binding pose (B).

considered in docking calculations. For instance, in rigid-body docking strategies, two interacting proteins are both considered absolutely rigid. Among these strategies is Z-DOCK [43], which generates accurate predictions of PPI interaction when proper scoring scaffolds are provided [43].

In the most recent docking studies, ligands are typically represented as partially or fully flexible, while protein flexibility still remains a challenge. Attempts have been made to introduce protein flexibility *via* soft docking, multiple copies, unbound dynamics of the protein, and side-chain flexibility [44]. An example of a flexible docking procedure was the exploration of peptide binding selectivity to homology models of PDZ domains, using the PDZ-DocScheme. It is a family of docking protocols for PDZ domains that is based on simulated annealing molecular dynamics and rotamer (discrete conformations) optimization and is applicable to the docking of long peptides to both known PDZ structures and their homology models [44].

2.3. Sequence- or structure-based predictions

To generate predictions of peptide-protein or protein-protein binding affinity, sequence-based strategies rely on the sequence and functional information available in many public databases. PPA-Pred [45], for example, developed a model based on sequence features by classifying protein-protein complexes according to their biological functions and percentage of binding residues for binding affinity prediction [14]. Despite offering less confident prediction on binding affinity and an inability to predict conformational binding poses, sequence-based models can be refined with dataset updates in experimental and functional scaffolds.

Many studies utilize existing information from structural databases such as the Protein Data Bank (PDB) to identify sequence-binding motifs for peptide designs. For example database PepX [46] is comprised of more than 500 experimentally studied peptide interactions with high-resolution structures and allows simple inputs of user-defined peptide templates [14]. Furthermore, sequence- and structural-based strategies can also utilize learning machines to enhance their prediction confidence over time [47].

3. Major challenges and possible solutions for the development of peptides for inhalation therapy in cystic fibrosis

3.1. The mucus barrier

Impaired chloride secretion through the CFTR in epithelial cells triggers an imbalanced water content of the periciliary liquid layer (PCL). PCL dehydration is further aggravated by increased sodium absorption through the Epithelial Sodium Channel (ENaC), a channel expressed on the apical surface of epithelial cells and whose activity is CFTR-dependent [48]. CFTR dysfunction drives ENaC hyperactivity, resulting in excessive Na^+ absorption and a depletion of the Airway Surface Liquid (ASL) volume [2]. The ensuing formation of an adherent mucus layer as well as the impaired beating of cilia affect mucus clearance and cause the consequent formation of thick mucus plaques and plugs on airway surfaces [49]. In healthy patients, the mucus is mostly composed of mucins, glycoproteins which constitute a physiological barrier to toxins and pathogens. In CF lungs, however, mucins are overproduced and oversecreted in response to inflammation and pathogens in the respiratory tract and are not cleared by the damaged ciliated epithelium. In addition, the CF mucus shows an increased content of DNA, released by pathogens and necrotic neutrophils, and actin released from necrotic cells, which further enhance viscosity and adhesiveness and decrease mucociliary clearance [2,50,51].

Due to increased thickness and viscosity, mucus plaques are depleted in O_2, constituting the ideal environment for bacterial infections and the consequent formation of a biofilm [49], which is colonized by pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenza,* which acquire selective advantage at the expenses of the physiological microbiota [52–54]. Moreover, pathogens are less accessible to neutrophils and antibiotics, failing to penetrate into the thickened mucus plaques. The evasion of defense mechanisms, coupled with the competitive advantage of bacteria growing in the biofilm form, allows the development of antibiotic resistance and the ensuing persistence of infections, which represent a relevant challenge in CF treatment.

3.1.1. Strategies to reduce retention in the mucus

The thick and viscous layer of mucus together with biofilms may

represent a challenge for effective delivery of drugs for inhalation to the lungs of CF patients, especially for big-sized and positively charged peptides. A strategy to improve penetration and reduce retention in the mucus is to exploit carriers like nanoparticles (NPs) [55]. Drugs, including peptides, can be encapsulated within the NP or can be attached onto its surface [56]. Moreover, NPs can be optimized in size and surface charge to improve the penetration of the NP-drug complex in the mucus. NPs should be small enough to diffuse through the 300-100 nm mesh sized network made by mucin fibers, DNA and other macromolecules in CF mucus, since bigger particles would be trapped by physical exclusion [55]. Moreover, the interaction between NPs and the mucus can be decreased by making the surface charges of NPs neutral or negative, give that mucins and other components of the thick layer, such as actin and free DNA, are negatively charged. Hence, while positively charged NPs would tend to interact electrostatically with these components of the mucus, neutral or negatively charged NPs would be repelled.

Another possibility to reduce both electrostatic and hydrophobic interactions is to coat the surface of NPs with electrostatically neutral and muco-inert polymers [55,57]. Among the most used is the low molecular weight polyethylene glycol (PEG). OF note, PEG dimension is indeed crucial in determining mucus penetrative or adhesive properties. Accordingly, it has been demonstrated that a five-fold reduction in PEG dimensions results in a 1000-fold increase in the motility of NPs inside mucus [58,59]. Suk et al. were among the first to attempt the use of PEG-coated NPs to improve penetrance in CF mucus and demonstrated that medium-sized PEG-coated NPs (200 nm) move in the mesh 90-fold faster than similarly-sized uncoated particles [60]. Moreover, PEGylation facilitates the mobility of NPs across biofilms generated by bacteria resident in the airways of CF patients, such as *Burkholderia multivorans* and *Pseudomonas aeruginosa* [61].

Intriguingly, PEGylation on NPs was also used by Craparo et al. to improve CF mucus penetration of the anti-inflammatory drug Ibuprofen. In this study, rhodamine B (RhB) was covalently bound to polyactic acid (PLA) using α,β-Poly(N-2-hydroxyethyl)-d,L-aspartamide (PHEA), to form fluorescent labelled particles smaller than 200 nm, that were coated with 0%, 2%, or 8.5% PEG. The penetrating ability of NPs increased with the density of PEG, and the disposition of PEG onto the NPs surface was shown to be critical for their motility into the CF mucus. Moreover, NPs coated with a dense brush-like PEG corona could more easily pass through a CF-mimicking mucus in comparison to PEG disposed in a mushroom conformation, since hydrophobic interactions with mucins are halted [62].

Another possibility is to directly PEGylate drugs, and this approach has already been exploited for the treatment of CF. Du et al. conjugated Tobramycin (Tob) with PEG (Tob-PEG), and found that this formulation has a 3.2-fold higher antibacterial activity against *Pseudomonas aeruginosa* and its biofilm than Tob alone [63]. Moreover, Tob-PEG was found to be more effective than Tob in an *in vitro* CF-like mucus, underlying its improved ability to diffuse through the mesh [64]. Similarly, PEGylation of peptides and proteins has already been exploited with the aim of reducing mucosal viscoelasticity in CF. For example, a PEGylated version of the recombinant human deoxyribonuclease I (rhDNase) has been developed. This protein retains the same mucolytic activity in CF sputa than the non-PEGylated form, independently of the purulence of sputum samples but shows increase stability [65,66]. In addition, PEGylation at the N-terminal region of an antimicrobial peptide, namely CaLL, comprising fragments of LL-37 and cecropin A peptides, was tested. PEG-CaLL derivatives retained significant antimicrobial activity against bacterial lung pathogens, even in the presence of lung lining fluid, showing increased stability [67]. Therefore, these data suggest that PEGylation of drugs, and in particular of peptide therapeutics, can not only reduce their electrostatic and hydrophobic interactions with CF mucus, but also increase their stability in the milieu of CF lungs.

Although PEGylation represents the standard approach for reducing drug and/or carrier retention in mucus, the use of other hydrophilic polymers is under investigation. This is the case of poly(vinyl alcohol) (PVA), which has been successfully employed to achieve mucus- and biofilm-penetrating PLGA NPs for prolonged release and efficacy of antimicrobial peptides to treat lung infections in CF patients [68,69].

The synthetic mucus-crossing NPs described so far are uniform in size and charge. However, these features do not match those of naturally occurring particles, such as viruses and macromolecules, that are efficiently transported across the mucus thanks to their complex coat proteins and asymmetric charges [70]. On these grounds, Leal et al. exploited phage libraries to identify novel mucus-penetrating peptides for diffusive transport through a model of CF mucus [71]. 2.0×10^{10} random heptapeptides with a flexible linker (GGGS) were genetically engineered into the genome of the phages in order to be displayed on their surfaces, with each phage having a different peptide sequence. These phages were mixed with CF mucus, added on the donor side of the transwell system, and the number of phages that diffused to the receiver side was quantified. Thirty phages resulting from this selection were isolated and the corresponding peptides were sequenced. Intriguingly, compared to the original library, these peptides were enriched in Pro, Ser, and Thr amino acids, which form the backbone of mucin proteins, suggesting that these peptides may diffuse in mucus, potentially due to unhindered weak intermolecular interactions with mucins. Moreover, most of the identified sequences were mostly hydrophilic and characterized by neutral charges, further explaining their improved penetrance in CF mucus [71].

Overall, optimization of the physical-chemical properties and the use of NPs to improve mucus-penetrance are promising tools to allow the efficient inhaled delivery of drugs that would be otherwise not suitable for CF treatment, including peptides and proteins [71].

3.1.2. Strategies to increase intracellular uptake

The thick and sticky mucus that characterizes the airways of CF patients represents only one of the barriers that a drug should cross to exert its biological activity. Cell membranes, with their low permeability, hinder the distribution of pharmaceuticals, severely limiting their therapeutic value. This issue is even more relevant for biologicals, and in particular peptides and proteins, which are often characterized by a hydrophilic nature. One possible strategy to overcome this complication is to exploit cell-penetrating peptides (CPPs) that allow intracellular internalization of a wide range of drugs, especially biologicals [72]. One of the first CPPs to be identified is derived from the HIV-1 transcription trans-activation (TAT) protein, a transcription factor of 86 amino acids, which was reported already in the 80ies to be efficiently internalized by cells *via* endocytosis [72,73]. From this evidence, structure-function studies identified the portion of the protein responsible for cellular uptake, which is a region of 13 amino acids extending from residues 48 to 60 (GRKKRRQRRRPPQ) that can be linked to peptides through a disulfide bond [72]. The positive charge of TAT sequence, which is rich in arginine, explains the electrostatic binding to cell membrane anionic components, such as proteins, lipid head groups and proteoglycans [74].

Another frequently used CPP is Penetratin, which derives from the homeodomain of Antennapedia, a homeoprotein of Drosophila melanogaster [72,75]. From structure-function studies, 16 amino acids (RQIKIWFQNRRMKWKK) corresponding to the third helix of

the Antennapedia homeodomain were identified to be responsible for intracellular uptake [72,76]. Its amphipathic nature allows Penetratin to adopt a random coil structure, which enables cellular internalization *via* interactions between the hydrophobic components of Penetratin and the apolar lipid membrane [77].

Besides TAT and Penetratin, several other CPPs have been identified, being typically characterized by sequences composed by 5–30 amino acids that can cross biological membranes through energy-dependent or -independent mechanisms [72]. These peptide sequences have now been used for the delivery of proteins, peptides, DNAs, siRNAs and small drugs, which can be covalently conjugated to CPPs by chemical linkage, for example *via* disulfide or thioester bonds, or by cloning and subsequent expression of fusion proteins. However, these approaches may alter or limit the biological activities of the conjugated pharmaceuticals. Another strategy is to link CPPs to the drugs through non-covalent electrostatic and/or hydrophobic interactions, this approach being effective also in protecting drugs from protease or nuclease degradation [78,79].

Thanks to their properties, CPPs are already efficiently used in the pre- and clinical setting and some attempts have been made also for CF-related applications. Porsio et al. developed PEGylated and TAT-decorated fluorescent nanoparticles (FNPs) in order to deliver Ivacaftor to the airways of CF patients. This is one of the main clinical modulators of CFTR which is administered orally, causing common (headache, stomach pain and nausea, rash, diarrhea, pain) and serious (liver problems) systemic side effects [80]. Using CF-artificial mucus, it was shown that PEG mainly improves the permeation of FNPs through the mucus, while the TAT motif potently enhances the uptake of FNPs by lung epithelial cells. Moreover, these particles were demonstrated to be efficiently delivered through dry powder inhalers (DPIs), suggesting the possible and effective use of these formulations for the treatment of CF lung disease [80]. Notably, McNeer et al. demonstrated the possibility to conjugate to CPPs also NPs, such as those containing triplex-forming peptide nucleic acids (PNAs), synthetic oligonucleotide analogs that can induce DNA repair upon sequence-specific triplex formation at targeted genomic sites [81]. This genome engineering approach was exploited to correct the gene and restore the activity of the most common CFTR mutant, ΔF508-CFTR, both *in vitro* in human bronchial epithelial cells and *in vivo*. To this purpose, NPs were formulated using poly(lactic-*co*-glycolic acid) (PLGA) and poly(β-amino ester) (PBAE) and their surface was modified by linking the CPP termed MPG. Thanks to this system, gene correction of the ΔF508-CFTR occurred with frequencies of 10% in human CFBE cells, and 5% and 1% in the nasal epithelium and lungs, respectively, of a CF mouse model where NPs were administered intranasally [81]. A slightly different approach was recently used by Osman et al. with the purpose to restore CFTR function using a CPP-based non-viral vector for lung gene therapy [82]. In this study, authors exploited glycosaminoglycan-binding enhanced transduction (GET) peptides, which are characterized by a heparan sulfate cell-targeting sequence fused to a CPP, further improving membrane association, and thus drug internalization [83]. Osman et al. used a GET peptide consisting of a 16 amino acids portion of the fibroblast growth factor 2, coupled with CPPs and DNA *via* electrostatic interactions, to form NPs which were further PEGylated for an efficient *in vivo* delivery. These NPs were shown to pass through the mucus mesh and diffuse rapidly across CF sputum *in vitro.* Moreover, in a mouse model, they were characterized by increased biodistribution and efficient gene transfer compared to other non-viral carriers already used in pre-clinical settings, suggesting the possible exploitation of this tool for CF patients.

Most notably, as in the case of mucus penetration, rational design of NPs *per se* can ensure the overcoming of lung barriers and optimal drug interactions with target cells, by playing with adequate particle size and surface properties (e.g. charge, hydrophilicity, and shielding cloud) [84,85].

3.2. High content of proteolytic enzymes

CF lungs are characterized by chronic inflammation that drives excessive neutrophil recruitment. In response to host infection, a massive amount of proteases is released by neutrophils in the lung lumen, including for example neutrophil elastase (NE). Furthermore, elastase can be secreted by bacterial pathogens commonly affecting the respiratory tract of CF patients, such as *Pseudomonas aeruginosa* [30]. Besides NE, neutrophils can release other proteases, such as metalloproteases and cathepsins, which contribute to proinflammatory signaling and impairment of mucociliary clearance, two key hallmarks of CF pulmonary disease. Of note, cathepsins can be also released by pathogens or derive from spilling of lysosomal content from damaged cells [86]. Overall, the release of proteolytic enzymes triggers cellular damage and inflammation, which sustains chronic infection in CF lungs [51,87], but at the same time may constitute a major challenge for protein- and peptide-based drugs, given the ability of proteases to degrade peptides at preferential sites [88–91] (Table 1).

3.2.1. Strategies to counteract proteolytic cleavage

Proteolysis represents a major issue for using peptides as therapeutics for CF lung disease. As mentioned above, CF mucus is enriched in different types of proteases that, according to their specific recognition motifs, can hydrolyze peptides thereby reducing their half-life and efficacy.

A first strategy to overcome this challenge is to identify labile amino acids that are susceptible to proteolytic cut, and to modify them using natural or non-natural amino acids that may confer resistance to cleavage. To this purpose, mapping the sites that are susceptible to proteolytic cleavage, for instance by using available databases and websites (Table 2), is crucial. Once identified, labile residues can be replaced with different L-amino acids (L-AAs) that are not recognized by proteases, but still guarantee a preserved biological activity of the peptide [92]. One strategy that has been used is to change the labile amino acid with Pro or Trp that prevent recognition of the cleavage site by most proteases [93,94]. Moreover, these two residues can confer rigidity to flexible regions and therefore enhance protein stability, further conferring resistance to proteolysis [95]. This approach has been exploited for a few peptides that have been tested for CF treatment, including Splunc1, a natural peptide that inhibits the activity of the epithelial Na^+ channel (ENaC) *via* a S18 sequence in its N-terminal region, by inducing its allosteric modulation and endocytosis [96–98]. ENaC is indeed hyper-active in the absence of a functional CFTR, leading to excessive Na^+ absorption, and its inhibition is therefore an appealing strategy to restore ASL volume [99–101]. However, both the full-length sequence of the Splunc1 peptide and the minimally-active S18 motif display a canonical cleavage site for NE at amino acid 5 (GGLPVPLxxx), which makes the two sequences unsuitable for therapeutic use. To reduce the sensitivity to proteolysis, the amino acid sequence of the parental peptide was modified, leading to the generation of SPX-101© [102]. Different from the S18 region of Splunc1 that is rapidly degraded when exposed to the sputum of CF patients, or to CF-related proteases, SPX-101© is resistant to proteolysis while maintaining the biological activity of the parental peptide. More specifically, SPX-101© retains the ability to inhibit ENaC after exposure to CF sputum, and increases survival of βENaC-overexpressing mice, a well-known model of CF lung disease, when administered *in vivo* intranasally [102].

Substitution of labile amino acids has been adopted also for the

Table 1
Enzymes relevant for CF pathogenesis.

Enzymes	Preferential cleavage sites	References
Elastase	Ala, Val, Leu, Ile, Ser, Thr in P1 position	[89]
Cathepsin G	Phe, Tyr, Trp, Leu in P1 position Negatively charged amino acids in P2 position	[90]
Cathepsin B	Gly in P1 position Aromatic and aliphatic residues in P2 position	[221]
Cathepsin L	Gly, Glu in P1 position Aromatic and aliphatic residues in P2 position	[221]
Cathepsin S	Gly, Glu in P1 position Aromatic and aliphatic residues in P2 position	[221]
Proteinase 3	Val, Ile, Ala and aromatic amino acids in the P1 position	[88]
Alkaline protease (from *Pseudomonas aeruginosa)*	Lys and Arg in the P1 position	[91]
MMPs	Aliphatic and aromatic residues in P1′ position Preference for Pro in P3 position	[222]

Enzymes relevant for cystic fibrosis pathogenesis and their preferential cleavage sites. P1 position corresponds to the residue located before the cleaved peptide bond.

Table 2
Tools for *in silico* prediction of protease cleavage sites in protein sequences.

Databases and Websites	Main Feature
MEROPS	Search for proteolytic enzymes, as well as their inhibitors and substrates, and known cleavage sites in a protein of interest [223].
CutDB	Browse for individual proteolytic events and cleavage sites in proteins of interest [224].
PMAP	Search for known proteolytic events and experimentally derived protease cut-sites for a given protein target of interest [225].
PROSPER	Identification of protease substrates and their cleavage sites for twenty-four different proteases within a single substrate sequence using machine learning techniques [226].
Peptide Cutter	Identification in a given peptide sequence the possible residues that are sensitive to proteolytic cuts by a given number of proteases available [227].

LL-37 peptide, one of the several antimicrobial peptides (AMPs) that are currently under evaluation as inhaled therapeutic agents for CF [103,104]. LL-37 is effective against both *Pseudomonas aeruginosa* and *Staphylococcus aureus*, although being sensitive to proteolytic cut. Strömstedt et al. identified the biologically active sequence of the parental LL-37 peptide, called EFK17, and modified four amino acids into Trp (W) at critical protease cleavage sites [94]. Such substitutions resulted in a marked reduction in proteolytic degradation by human NE, *Staphylococcus aureus* aureolysin, and V8 protease, but failed to confer protection against *Pseudomonas aeruginosa* elastase. Intriguingly, this modified peptide has an increased bactericidal potency compared to the native sequence [94]. This study also tested a sequence where four labile L-AAs of the parental peptide were replaced by their corresponding D-amino acids (D-AAs), and found that such modification could considerably reduce peptide cleavage [94]. This is explained by the fact that peptidases and proteases are stereospecific and suggests that this approach could be generally used to confer resistance to proteolysis. D-AAs can be exploited to substitute critical residues in the amino acidic sequence, generating the so-called diastereomeric peptides, or to replace their natural counterparts *in toto* to form all-D-AAs sequences. If on the one side such modifications confer stability against proteolysis, on the other side they can negatively affect the biological activity of the peptide [105]. To avoid this issue, D-AAs can be assembled in the reverse order compared to that of the parent L-sequence. This strategy can provide structural stability, spatial orientation and resistance to proteolytic events, without affecting the biological activity of the peptide compared to the parental sequence [105].

Substitutions with D-AAs have been exploited for the optimization of not only EFK17, but also of other AMPs, including the Pin2 peptide, derived from the sequence of Pandinin-2, and the BMAP-18 peptide, known to be highly sensitive to proteases. D-Pin2 is resistant to proteases, such as trypsin and elastase, and retains a strong antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro* [106]. Similarly, the all-D-enantiomer of BMAP, called D-BMAP, is not subjected to degradation when incubated with murine bronchoalveolar lavage fluid (BAL) and retains its activity as an antimicrobial agent *in vitro*. However, D-BMAP is ineffective to treat *Pseudomonas aeruginosa* pulmonary infection in mice compared to Tobramycin, showing that there is still room for further optimization [107].

A similar approach has led to the generation of the D-enantiomer of the short α-helical AMP RR4, namely D-RR4, leading to an improvement of 32 folds in the antimicrobial activity against multidrug-resistant strains of *Pseudomonas aeruginosa,* including those colistin-resistant isolated from CF patients, indicating a potential therapeutic advantage of this peptide over other AMPs [108].

Besides protecting specific cleavage motifs within the whole peptide sequence, another method to prevent proteolytic degradation of peptides is to stabilize their extremities, which can be modified for example by N-acetylation or C-amidation [109]. This approach is highly effective, though it mostly confers protection against exopeptidases, which are abundant in human plasma but are less represented in the lungs.

Cyclization is another successful approach to reduce peptide proteolysis, since the mobile ends are fixed *via* the binding of N and C termini, resulting in conformational constraints which make difficult for proteases to access and recognize cutting sites [110,111]. Because of these constrains, cyclization also locks peptides in an active conformation, increasing their efficacy. One of the major examples of cyclic peptides used for CF therapy is colistin, a cationic polypeptide antibiotic which was abandoned in the early 1970s because of initial reports of severe toxicity. However, its use was reconsidered due to increased prevalence of multidrug resistant *Pseudomonas aeruginosa* in the lungs of CF patients [112].

Another example of cyclic peptides developed for the treatment of CF are cyclic tetrapeptides structurally related to apicidin, a natural product that acts as a histone deacetylase (HDAC) inhibitor, which correct the trafficking defect of ΔF508-CFTR [113].

An alternative strategy to circumvent the problem of proteolysis of peptide therapeutics by proteases is to directly target the activity

of proteolytic enzymes [114,115]. Since NE is the predominant protease contributing to CF lung disease, most efforts have been made to inhibit its activity [87,116]. The most promising compound is Alpha-1-Antitrypsin (AAT), also known as Alpha-1-Proteinase Inhibitor, an endogenous inhibitor of NE [117]. In CF, AAT is produced at normal levels but cannot compensate for the increased levels of NE. Supplementation of human AAT by inhalation has been tested in clinical trials, showing safety and tolerability, but its clinical efficacy is still under evaluation [5,6,118,119]. Of note, a major limitation for the usage of AAT in CF is that, besides NE, other proteases like cathepsins and metalloproteases are upregulated and contribute to lung damage, highlighting the need to develop new inhibitors with a broader spectrum of activity [2,120].

3.3. Local immunogenicity and off-target effects

A major concern in the development of novel therapeutics is represented by off-target effects, which may lead to toxic and antagonistic outcomes [121], and consequently annihilate the therapeutic potential of the medicine. Biotherapeutics present the advantage to have high specificity for their target, which limits off-target toxicities [122]. On the other hand, a major drawback of this class of drugs is their potential ability to raise an immunogenic response.

The immunogenic response can be driven by multiple factors, including on the one side the genetic background of the patient and, on the other side, the features of the therapeutic protein [123]. Despite self-tolerance, autologous peptides and proteins can elicit an immunogenic response by disturbing B- and T-cell tolerance [124]. Accordingly, several studies have shown that protein-based therapy can induce local immunogenicity, even if structurally derived from endogenous human proteins, by stimulating both adaptive and innate immune responses. Innate immune cells may recognize the drug as an antigen and, in turn, stimulate antigen presenting cells and the adaptive immune response [125]. Anti-drug antibodies (ADA) are observed after the administration of an immunogenic molecule [124] and the adaptive immune response induced by ADA is driven *via* $CD4^+$ T cell-dependent mechanisms, which mediate cell destruction and complement activation [124,126,127].

Of note, administration of endogenous proteins can lead to the production of autoantibodies [128] such as in the case of insulin. Insulin has been shown to induce an immunogenic response when administered by inhalation, but not by the subcutaneous route [129–133]. However, the level of antibodies developed after insulin inhalation is comparable with the level observed with subcutaneous administration of porcine insulin, suggesting that overall inhaled human insulin can be considered safe [134–137].

The development of antibodies against therapeutic proteins is an important safety and efficacy concern as it can lead to anaphylactic shock or interfere with the effect of the drug itself [138]. Thus, peptide-based therapeutics require strategies to limit immune responses during drug development and a detailed assessment of potential immunogenicity during preclinical safety toxicology studies [125,139].

3.3.1. Lowering undesired effects

The probability that a peptide raises an immunogenic response is determined by multiple factors. Auto-immunogenicity has been reported for proteins which are evolutionary conserved, are part of cellular structures, and display specific sequence motifs [128], like coiled-coil motifs, ELR motifs, and Zinc finger DNA-binding motifs [140,141]. In order to obtain tolerable peptides, these sequences should be rationally excluded during the design phase. Among the approaches for reducing the immunogenicity of peptides is to limit the peptide length to maximum 20 amino acids since short peptides are less immunogenic [142].

Another strategy may consist in replacing natural L-AAs with N-alkyl amino acids or D-enantiomers [143]. Nevertheless, if peptides fully or partially composed of D-AAs are less immunogenic than their corresponding L-enantiomers is still controversial. Different studies have shown that linear peptides composed of D-enantiomers can induce a unique immunogenic response [144], and lead to antibody formation at a low concentration and in a thymus-independent way [144]. Of note, D-enantiomers are specifically recognized by T-cells due to the sterical conformation of the MHC-antigen-T cell receptor complexes, which limits cross reaction between L and D sequences [144].

However, recent studies hypothesized that proteins composed of D-enantiomer are non-immunogenic [143–145]. A study has shown that a D-peptide was non-immunogenic, in contrast to its L-enantiomer which induced a strong immune response [144]. Another recent clinical study has explored the immunogenic effect of a synthetic peptide composed of D-amino acids and highlighted that, albeit development of ADA was observed, this had no impact on efficacy and safety [145]. Overall, these results support the tolerability of D-peptides as alternatives to their L-counterparts for therapeutic purposes.

Nevertheless, the contribution of specific features of a protein to immunogenicity needs case-by-case experimental confirmation [146,147], especially for biological drugs [148–150]. Accordingly, albeit *in silico* predictive methods [151] and strategic design of peptide sequence and formulation may help limiting immunogenicity, local tolerance should be carefully assessed during preclinical safety toxicology studies, especially for inhaled drugs. In this regard, it is important to underline that the immune system of humans differs from that of other mammals due to the genetic background, making difficult to predict any immunogenic response by preclinical assessment in animal models [152,153]. An intriguing possibility that is emerging to overcome this limitation is the use of cell-based assays to detect immunogenicity [154]. The immunogenic response is usually measured by the presence of immunoglobulins (IgG, IgM) in serum samples, which allow to assess the primary immune response. The predominance of IgG highlights the formation of a B cell memory since different from IgM that predominate in the early response, IgG act later to permanently eradicate pathogens through phagocytosis and opsonization [155]. However, development of an immunogenic response is hard to predict before clinical studies are performed [138]. The European guidelines encourage a systematic reporting of immunogenicity-related information of biological products [156]. Of note, immunogenicity can sometimes be detected only after a long-term follow-up, making post-marketing pharmacovigilance of utmost relevance to track immunogenicity at a large-scale and to link the appearance of adverse drug reactions (ADR) to the generation of ADA [157].

3.4. Poor aqueous solubility and aggregation

Another challenge in the use of inhaled therapeutic peptides, especially for those that are formulated as an aerosol, is poor aqueous solubility and tendency to form aggregates. The susceptibility of a peptide to aggregation depends on both extrinsic and intrinsic factors.

Extrinsic determinants include pH, ionic strength and concentration, as well as physical-chemical properties of the solvent [158,159] and likely the co-solvent [160]. Temperature is another critical factor, as it may disturb secondary, tertiary and quaternary structures of proteins, and may expose hydrophobic hot spots to the aqueous solution, leading to aggregation [159]. On the other hand,

intrinsic factors include hydrophobicity, charge and electrostatic properties, as well as size [161]. Hydrophobic and uncharged sequences are defined as hot spots due to their high susceptibility to form β-sheets, which tend to assemble into insoluble aggregates. On the contrary, electrostatic repulsion avoids contact between amino acids and thus discourages aggregation [158].

An important aspect to consider is that aggregation may affect the homogeneity of the formulation and, as a consequence, the aerosol performance of the drug. Low homogeneity may reduce the access of the drug to target cells with concomitant accumulation of peptides in localized sites of the lungs, with a negative impact on efficacy and safety. To be suitable for inhalation therapy, drugs should display an adequate aerodynamic profile, i.e. to be able to reach the alveoli in the lower tract of the respiratory system [2]. Hence, geometrical and aerodynamic size of particles should be carefully evaluated, through techniques that include Dynamic Light Scattering (DLS) and impactors [162,163]. One of the primary parameters of good performance for inhaled drugs is the aerodynamic particle size distribution (APSD) of the nebulized drug product, which is determined by the median mass aerodynamic diameter (MMAD) and the geometric standard deviation (GSD). The MMAD is the aerodynamic diameter at which half of the aerosolized drug mass lies below the stated value. Aerosol particle size is critical for inhaled therapeutics, given that only particles with an aerodynamic diameter of 1–5 μm have the highest probability to penetrate and deposit in the deep lung. On the contrary, molecules smaller than 1 μm are exhaled during expiration, whilst those sized more than 5 μm are retained in the oropharyngeal cavity or in the upper respiratory tract [164,165]. From a pharmacological perspective, two other parameters should be taken into consideration: fine particles fraction (FPF), that is the proportion of total particles with smaller than 5 μm, and respirable fraction (RF), that is the fraction of particles that are able to reach the alveoli. Usually, as long as long as MMAD decreases, FPF and RF increase, together with the chance for the drug to reach and deposit in deep lungs [166–168]. Adequate aerodynamics parameters are therefore critical to guarantee an adequate drug lung deposition, especially for peptide-based drugs that, due to the intrinsic properties of the structure, may aggregate into self-assembled particles [169,170].

Once formulated, peptides could irreversibly aggregate leading to an increase in immunogenicity and also a decrease of effectiveness secondary to the inability of the drug to reach the target organ, e.g. the lung in the treatment of CF [171]. Protein aggregation is therefore a main issue for the formulation of biotherapeutics, often requiring efforts to optimize lung targeting, homogeneous deposition, and tolerability.

3.4.1. Strategies to control solubility and aggregation

Aggregation of proteins and peptides, driven by poor solubility, shares common molecular mechanisms with the collapse of polypeptide chains into unstructured globules [172]. Among these, the contribution of both side-chains and backbone in the collapse and folding of proteins has been elucidated [173–176]. Backbone hydrogen bonding (H-bonds) interactions have long been proposed to control the collapse of proteins [177,178]. In addition to backbone H-bonds, other dipolar interactions among groups in the main-chain occur as well [179]. Of note, backbone interactions other than H-bonds (like those occurring between the dipoles of carbonyl groups, CO–CO) are common in helices and β-sheets [180], and modulate the conformation of peptides [180–182]. Moreover, CO–CO interactions are more abundant than backbone H-bonds in the collapsed or aggregated state of oligo-glycines in water [172,179,183,184]. Indeed, it is emerging that CO–CO interactions are more important than the inter-backbone H-bonds in peptide self-assembly and aggregation [179,185]. Therefore, strategical peptide design aimed at reducing CO–CO backbone interactions as well as H-bonds may result in the control of aggregation of peptide-based drugs.

Besides backbone interactions, specific residues in the side-chains play an important role in determining the solubility and folding of peptides [174,186]. While Arg generally interacts with fewer partners, Asp tends to make more side-chain to side-chain contacts [187]. Sarma et al. were also able to predict the propensity of Asn and Gln residues to aggregate [187], in agreement with their well-known role in peptide aggregation during plaque-forming diseases [188,189]. Specific substitutions in the side-chains of the therapeutic peptide would therefore significantly affect its propensity to aggregation and, ultimately, its aerodynamic properties.

Unfortunately, the solubility of an amino acid in water cannot be predicted from its hydration free energy. For example, Gln is less soluble than Val in water despite its much more favorable hydration free energy [190,191] and glycine-rich proteins display globular-like conformations even in the absence of a hydrophobic core [192]. Moreover, the idea that folding is driven by hydrophobic side-chains gravitating to the core and avoiding the interaction with the solvent has been questioned. The emerging hypothesis is that backbone CO–CO interactions, inter- and intra-solute interactions, the H-bond network, as well as solute–solvent interactions work in a cooperated manner, in synergy or opposing to each other, to drive the system to either collapse or folding [187]. Overall, this means that predicting folding and collapse of a peptide chain starting from its amino acid sequence is challenging, and only experimental validation can demonstrate the appropriate strategy of amino acid substitution for limiting aggregation.

However, strategic modelling of backbone and side chains within the peptide sequence may not always be feasible, due to the need to preserve specific amino acid residues in critical positions. Therefore, other tools have been explored to prevent aggregation and improve solubility of inhaled drugs. The most common approach for ensuring a good aerodynamic performance of inhalable dry powders is the use of excipients, like mannitol, magnesium stearate and lactose [193] or acetalated dextran [194]. Similarly, excipients can be used to improve the drug properties of aerosol formulations. As an example, cyclodextrins can be exploited to overcome some of the drawbacks that prevent the widespread clinical use of AMPs, i.e. to improve peptide solubility and pharmacokinetics [195].

A second strategy implies the use of biocompatible and/or biodegradable polymers as carriers. These include, for example, PLGA [196,197], chitosan [198,199], gelatin [200], poly(caprolactone (PCL) [201], methylcellulose (MC) [202], dextran [203] and polyacrylate [204]. The use of drug-loaded NPs proved highly effective to concomitantly enhance delivery and solubility [205]. Among others, an attractive new class of vectors is represented by poloxamine-based block copolymers [206]. Poloxamines, commercially available as Tetronic®, are x-shaped copolymers constituted of poly(ethylenoxide)/poly(propylen oxide) (PEO/PPO) blocks, bonded to a central ethylenediamine moiety. Their peculiar structure confers sensitiveness to temperature and pH, as well as ease modification of core content. More importantly for peptide delivery, thanks to their hydrophobic core poloxamines can be used to solubilize drugs in water and hydrophilic media [207]. Of note, Guan et al. demonstrated that specific synthetic peptides can self-assemble to poloxamines to form compacted NPs that are safe for lung delivery, as demonstrated in CF mice [208]. These results suggest that PEO/PPO polymers may be a valuable tool to improve solubility and delivery of peptide-based drugs, further highlighting the multiple benefits of peptide-loaded NPs for inhaled formulations. Recently, several studies reported the development of nano-

embedded microparticles (NEMs), which provide an excellent vehicle for both stabilization and delivery of drug-loaded nanoparticles and polymers to the intended site of action [209].

4. Conclusion

The data discussed in this review suggest that, despite important challenges, peptides can be successfully exploited as therapeutic compounds for the inhalation therapy of CF lung disease. A wide plethora of options, spanning from rational sequence design, use of NPs, and *ad hoc* formulation studies, can be explored for optimizing peptides and guarantee high efficacy, specificity of action, adequate target delivery and good safety profiles. Notably, many delivery systems, like engineered NPs, can be exploited to simultaneously improve drug penetration inside the CF mucus barrier and bacterial biofilms, increase intracellular uptake, counteract proteolytic cleavage, and lower undesired effects.

Evidence summarized here clearly demonstrates the possibility of exploiting peptides to target the primary cause of CF, i.e. CFTR dysfunction, but also to limit other life-threatening manifestations of the disease, such as lung inflammation and the ensuing tissue destruction. Intriguingly, peptides could be additionally leveraged as vectors for gene editing and gene transfer approaches. This is particularly relevant for those CF patients carrying stop or splicing mutations, who cannot benefit from currently available CFTR modulators [210–212], including emerging amplifiers, correctors and potentiators [213–217], and the recently approved Trikafta®/Kaftrio® triple combination [218,219]. On these grounds, gene editing strategies are under development and may take advantage of triplex-forming peptide nucleic acids and peptide-poloxamine NPs for improved DNA transfer [208,220] in the CF setting [81]. Within this scenario, peptide-based NPs display an attracting pharmacological potential yet far from being fully exploited.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandra Ghigo and Emilio Hirsch are founders of Kither Biotech S.r.l.

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Review Article

Potential therapeutic applications of AKAP disrupting peptides

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The 3′–5′-cyclic adenosine monophosphate (cAMP)/PKA pathway represents a major target for pharmacological intervention in multiple disease conditions. Although the last decade saw the concept of highly compartmentalized cAMP/PKA signaling consolidating, current means for the manipulation of this pathway still do not allow to specifically intervene on discrete cAMP/PKA microdomains. Since compartmentalization is crucial for action specificity, identifying new tools that allow local modulation of cAMP/PKA responses is an urgent need. Among key players of cAMP/PKA signaling compartmentalization, a major role is played by A-kinase anchoring proteins (AKAPs) that, by definition, anchor PKA, its substrates and its regulators within multiprotein complexes in well-confined subcellular compartments. Different tools have been conceived to interfere with AKAP-based protein–protein interactions (PPIs), and these primarily include peptides and peptidomimetics that disrupt AKAP-directed multiprotein complexes. While these molecules have been extensively used to understand the molecular mechanisms behind AKAP function in pathophysiological processes, less attention has been devoted to their potential application for therapy. In this review, we will discuss how AKAP-based PPIs can be pharmacologically targeted by synthetic peptides and peptidomimetics.

Introduction

The 3′–5′-cyclic adenosine monophosphate (cAMP) second messenger controls different biological processes and signaling pathways primarily via the activation of protein kinase A (PKA), one of the most widely researched serine/threonine kinases [1,2]. Several stimuli, such as catecholamines and neurotransmitters, bind to G protein-coupled receptors (GPCRs), and trigger the activation of heterotrimeric G proteins which, in turn, stimulate adenylate cyclase (AC) to produce cAMP from ATP. This second messenger binds the dimer of PKA regulatory subunits, promoting the release and activation of the two catalytic components of the PKA holoenzyme, which are then free to phosphorylate a multitude of intracellular substrates. Alternatively, cAMP can activate other effectors, including cyclic nucleotide-gated ion channels (CNGs) [3], Popeye domain containing proteins (POPDC) [4], and the exchange protein directly activated by cAMP (EPAC) [5].

This limited number of cAMP-dependent signal transducers appears insufficient to explain the variety of distinct cellular responses elicited by the same second messenger molecule cAMP. Therefore, the specificity of response triggered by this highly diffusible second messenger must be tightly regulated, both spatially and temporally, through further layers of complexity. The current view implies that cyclic nucleotide signals are in fact compartmentalized within the cell by localized multiprotein complexes, also known as cAMP signalosomes, that allow confined generation and destruction of cAMP as well as selective involvement of distinct signal transducers. These complexes restrain signal diffusion, assuring concentration of cAMP in subcellular domains and avoiding leakage of this secondary messenger molecule to unwanted effectors [6]. These space-restricted signalosomes generally span in their size from the nano to the micrometer scale and frequently include a selective GPCR and its preferentially activated AC isoform

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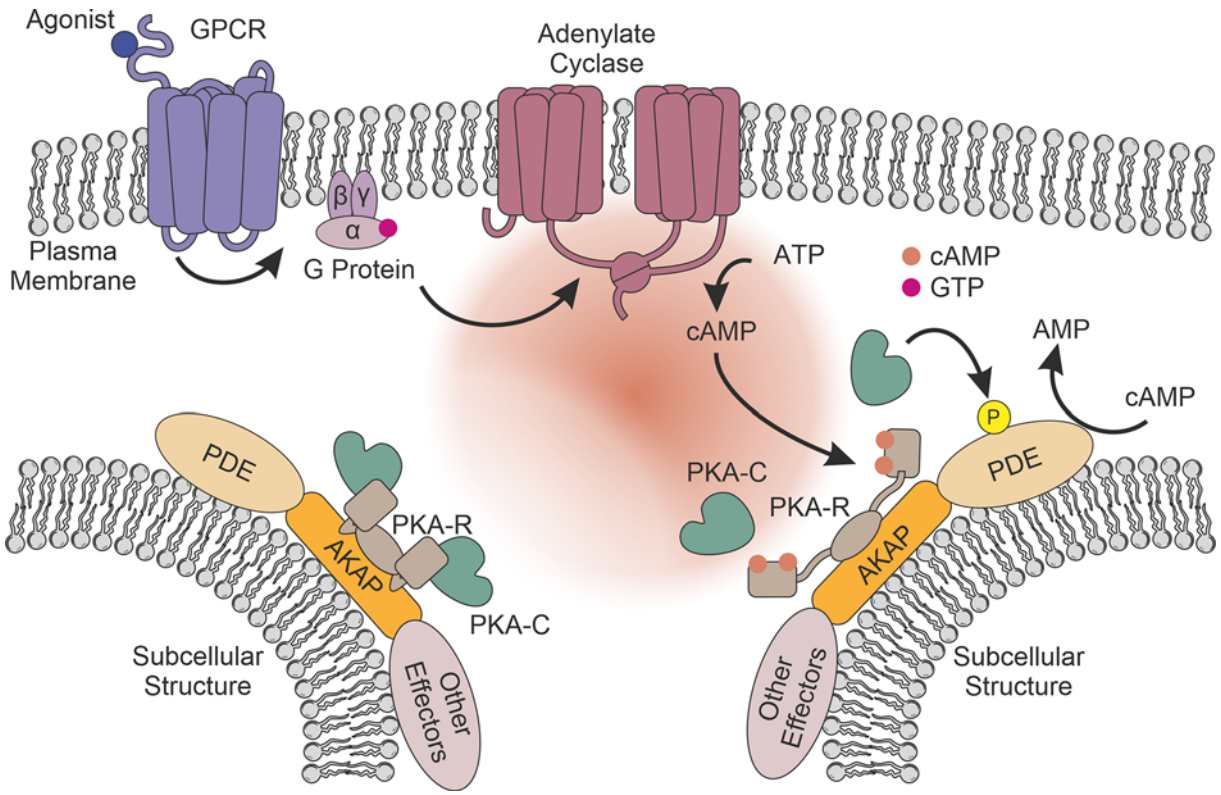

Figure 1. Schematic illustration of cAMP/PKA signaling compartmentalization

The responses elicited by cAMP are tightly regulated and organized thanks to the presence of localized protein complexes, also known as cAMP signalosomes, that allow localized generation and destruction of cAMP, eventually restraining the free diffusion of the second messenger. These multiprotein complexes generally include one GPCR that, depending on the extracellular ligand, can activate via G proteins a specific AC, another enzyme present in the complex which is responsible for the production of the second messenger. Another key player in these assemblies is the cyclic nucleotide PDE that, instead, mediates cAMP hydrolysis. Upon synthesis, cAMP activates its effectors that are also enriched in specific cell compartments. Among key effectors is PKA, which is able to phosphorylate specific substrates, such as PDEs, leading to distinct patterns of phosphorylation within the same cell in response to different external stimuli because it is anchored to specific cellular structures via AKAPs. Besides binding to PKA directly, AKAPs can associate with other enzymes, primarily members of the cAMP signaling, such as PDEs or phosphatases which dephosphorylate downstream PKA targets, but also other kinases, acting as hubs for different signaling pathways.

[7]. Typical complex components further include the cAMP effector PKA and cyclic nucleotide phosphodiesterases (PDEs), enzymes promoting cAMP hydrolysis and spatio-temporal signal containment [8,9] (Figure 1). Other signaling components can participate in these complexes and their composition can change in response to intracellular conditions.

What keeps member specificity and subcellular localization of these signalosomes are A-kinase anchoring proteins (AKAPs) [10], a group of structurally diverse scaffold proteins that directly bind the regulatory subunits of PKA as well as various signaling molecules, including key members of the cAMP signaling pathway, like PDEs and ACs (Figure 1). In addition, AKAPs can interact with other signal transduction machineries and act as hubs integrating cAMP signaling with diverse signal transduction pathways, like that of protein kinase C (PKC) or mitogen-activated protein kinase (MAPK) [10,11] (Figure 1). Precision of spatio-temporal intracellular signaling is further maintained by the ability of AKAPs to anchor specific cellular structures, such as cytoskeletal components, that allow subcellular confinement [12].

Compelling evidence suggests that cAMP compartmentalization crucially translates global external stimuli into appropriate and selective physiological responses. As such, perturbations of these finely tuned mechanisms are at the basis of a wide variety of different pathologies [13], including cardiovascular diseases, cancer, neurological disorders and inflammation.

Clinical Science (2020) **134** 3259–3282
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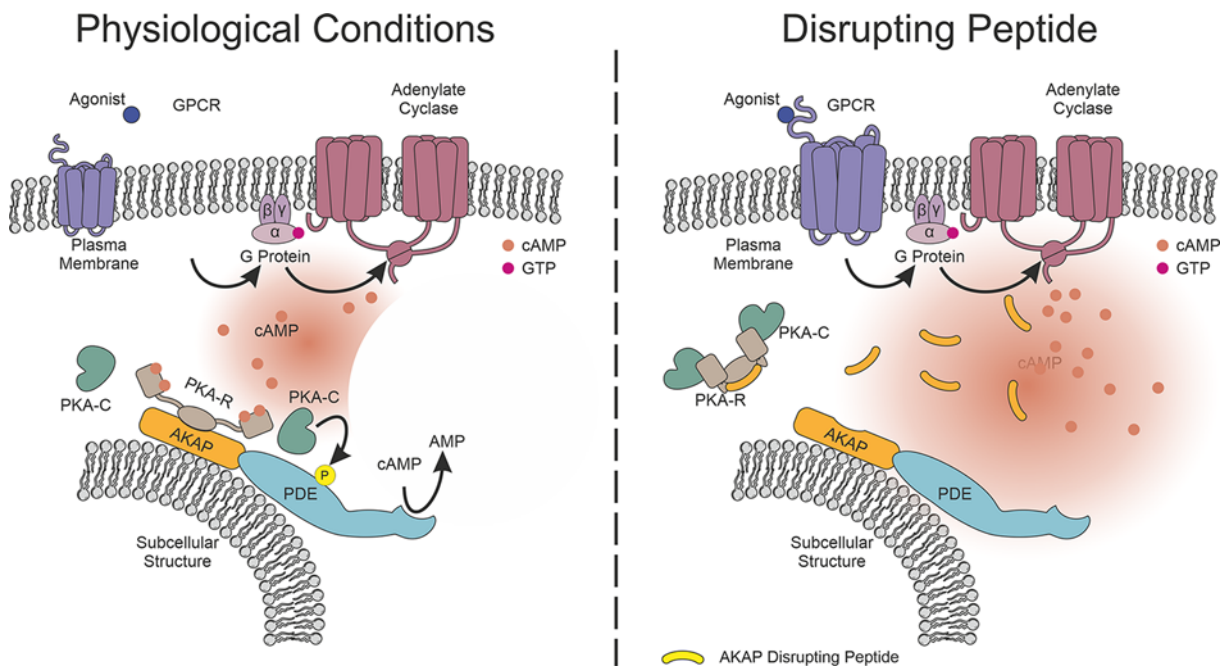

Figure 2. Schematic illustration of the mechanism of action of AKAP disrupting peptides

(Left) In physiological conditions, activation of GPCR by an extracellular ligand leads to AC-mediated production of cAMP. Two molecules of this second messenger bind to each PKA R subunit, causing a conformational change that allows the release of the two C subunits, which are then able to phosphorylate downstream targets. PKA is localized to a particular subcellular structure via binding to an AKAP, which interacts with the kinase through its amphipathic α-helical region. AKAPs also act as scaffolding proteins for other enzymes that are regulated by PKA, such as PDEs that, activated upon PKA phosphorylation, in a negative feedback loop mediate local cAMP degradation. (Right) Pharmacological modulation of specific AKAP signalosomes is achieved through peptides that compete with the AKAP for the binding with PKA. As a result, PKA is displaced from this compartment and cannot activate nearby substrates.

Several studies have explored the therapeutic possibility of targeting the protein–protein interactions (PPIs) that are key to the formation of cAMP signalosomes. This could ideally be achieved by blocking the interaction of AKAPs with either PKA or other components of the cAMP signaling pathway, such as phosphatases, PDEs and other PKA substrates [14,15]. The most widely used approach for this purpose is the use of disrupting peptides and peptidomimetics. In virtue of their high efficacy and specificity, peptides have lately gained interest as potential therapeutic [16,17] particularly in the modulation of PKA activation.

Mechanisms of PKA activation

PKA, one of the most widely researched serine/threonine kinases, is a tetramer composed of two catalytic (C) and two regulatory (R) subunits. Three different isoforms of C subunit, namely Cα, Cβ and Cγ, have similar kinetic properties and are structured as globular bilobal proteins containing an ATP binding site, a docking site for substrates binding and a domain for binding with the regulatory subunit. Four genes have been identified encoding for the R subunits, which have been subdivided into two classes: RI (RIα and RIβ) and RII (RIIα and RIIβ), respectively. Different from the C subunits, R subunits are characterized by an N-terminal dimerization/docking (D/D) domain that is connected by a flexible linker to two tandem cyclic nucleotide binding (CNB) domains. Depending on the type of R subunit involved, two different PKA holoenzymes can be formed: a type-I, which contains RI subunits (RIα or RIβ) and is primarily cytoplasmic, and a type-II holoenzyme, which contains RII subunits (RIIα or RIIβ) and is particularly associated with membranous organelles [2]. In the absence of stimuli, the PKA holoenzyme is inactive as the inhibitory sequence present in the R subunit linker binds to the active site of the C subunit, thereby preventing substrate binding [18]. PKA is activated when two cAMP molecules cooperatively bind to each R subunit, resulting in both conformational change and autophosphorylation of the inhibitory R subunits. Together, these events allow the release of the two C subunits, which are then able to phosphorylate downstream targets (Figure 2) [18].

Clinical Science (2020) **134** 3259–3282
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Remarkably, recent studies challenge this textbook view of PKA activation. For example, in rat dorsal root ganglion neurons, RII appears pre-phosphorylated in the resting state, suggesting that the autophosphorylation event can occur in the absence of cAMP and can precede the binding of this second messenger [19]. This study also shows that RII autophosphorylation as well as cAMP binding, rather than inducing the release of the C subunits, alter the topology of the RII–C interface. This notion supports the work of Scott and colleagues showing that only supraphysiological levels of cAMP mediate the dissociation of the holoenzyme, while upon physiological cAMP stimulation the PKA-II holoenzyme remains intact [20]. In agreement, R and C subunits, when fused together into a single protein, are able to restore cell viability in cells lacking free RII or C subunit proteins as a consequence of CRISPR-Cas9-mediated gene knockout [21]. On the other hand, Walker-Gray and colleagues have shown that C subunits are first released from R subunits upon cAMP binding but then rapidly recaptured [22]. Thus, despite being one of the most studied kinases, the exact mechanism of PKA holoenzyme activation and function is still incompletely understood. However, all findings converge on the view that the activity of PKA is very closely limited to the site of its activation, underlying the importance of AKAPs in anchoring the PKA holoenzyme in proximity of its substrates and in specific subcellular locations.

AKAPs and AKAP-disrupting peptides

AKAPs are a family of more than 50 structurally diverse scaffolding proteins, all binding the regulatory subunit of PKA through a shared PKA-anchoring domain [11,13,23]. The interaction of RI and RII with AKAPs is mediated by a D/D domain at the N-terminus of PKA R subunit which is known to form an X-type, antiparallel four-helix bundle [24]. AKAPs, on their side, interact with this PKA docking surface through a structurally conserved hydrophobic helical motif of their A-kinase binding (AKB) domain [25]. Although AKAPs were initially identified for their ability to bind RII subunits, a few of these scaffold proteins interact specifically with RI. In addition, 'dual specificity' AKAPs, such as D-AKAP1 and D-AKAP2, bind both holoenzymes [26]. Moreover, some AKAPs, known as 'non-canonical', show specific different ways of interacting with the R subunits. For example, pericentrin binds PKA via a 100-amino acid long motif, while other proteins such as RSK1 and α/β tubulin do not have the classical AKB domain [27–29]. Another 'non-canonical' AKAP is the phosphoinositide 3-kinase γ (PI3Kγ) which binds the RIIα subunit via its residues from 126 to 150 and a core KATHR sequence [30], an aminoacidic motif of yet unknown structure.

Studies on the canonical AKB, shared between D-AKAP1 and D-AKAP2 and able to bind with high affinity the D/D domains of both RI and RII, show that the interacting surface lays within a motif of highly conserved amphipathic helices [31]. This structural feature has allowed the design of peptides that encompass the AKB of various AKAPs and bind PKA, competitively disrupting the PKA/AKAP association (Figure 2). The first of these peptides, namely Ht31, derived from the PKA-anchoring domain of AKAP-Lbc, disrupts the interaction between canonical AKAPs and either RI or RII subunits [25,32]. Thanks to bioinformatics analysis of the minimal binding domain of several AKAPs characterized by binding to RII with the highest affinity, disruptor peptides have been designed to discriminate between RI and RII. For example, AKAP-IS is significantly more potent in disturbing interactions with RII than those with RI. Refinement of this sequence brought to the identification of superAKAP-IS, a peptide sequence that exhibits a 12000-fold preference for binding RII over RI. Conversely, the same bioinformatics-based strategy led to the design of PV38 and RI Anchoring Disruptor (RIAD), peptides specifically disrupting the interaction between AKAP and RI [14,25,33,34].

Strengths and limitations of AKAP-disrupting peptides for therapy

Despite the wealth of applications for peptides and peptidomimetics targeting PPI driven by AKAPs, most if not all the molecules described so far are still in their preclinical development. Although progress is in sight, several issues need to be optimized before a first-in-man study is attempted. Peptides are emerging as easy to design, safe and highly selective but their clinical use has not yet been fully demonstrated. Therefore, pros and cons must be carefully analyzed in face of future therapeutic applications.

General strengths

The studies summarized herein highlight the possibility of using AKAP-disrupting chemicals as useful tools to dissect physiological processes as well as promising therapeutic means in preclinical models, suggesting that these peptides/peptidomimetics might be exploited as pharmaceutical agents. Small molecule peptidomimetics have often been developed to target PPIs [35] and some of these molecules like captopril used to lower blood pressure turned into industry blockbusters. Nonetheless, targeting PPI with peptidomimetics remains a challenge, especially considering the

Table 1 Advantages and drawbacks of peptides as therapeutic agents

Advantages	Drawbacks
Small size [39]	Cost of production [39]
High specificity [17]	Short plasma half-life [41,46]
High selectivity [17]	Rapid renal clearance [38]
High efficacy [16]	Low stability [16]
Many targets [41]	Low membrane permeability [16]
Low toxicity [55]	Low oral bioavailability [46]
High activity per unit mass [39]	

intrinsic difficulty of usually flat and featureless small molecules to form a stable 3D conformation [36]. This appears as the main reason for the paucity of small molecule peptidomimetics in the current area of disrupting agents of AKAP-driven subcellular signaling. A superior performance is shown by peptides that, with a size five-times larger than small molecules and a flexible backbone, provide the ideal matching surface involved in PPI [37]. Peptides can easily target specific subcellular and signaling compartments, with consequent limited side effects. Compared with other drugs, peptides present in fact several advantages: in virtue of their small size, they penetrate the tissues better than big proteins, like antibodies. Peptides are characterized by a high activity per unit mass, meaning that small quantities are sufficient to lead to a significant effect [38,39]. Given that their degradation products are amino acids, they are characterized by low toxicity, feature that makes them potential candidates to succeed clinical trials [40]. Finally, peptides can be chemically synthesized in high quantity or purity and can be easily optimized, for example with the introduction of non-natural amino acids. Although, historically, peptides were designed as extracellular receptor agonists/antagonists as detailed in the following reviews [41,42], approaches meant to address the intrinsic difficulty of delivering peptides inside the cell have been attempted and more than 25 cell penetrating peptides are currently under clinical development. For example, AM-111, a TAT-peptide which inhibits JNK for the treatment of sudden sensorineural hearing loss, is currently under test in Phase III clinical trial (NCT02561091). P28 is a cell penetrating peptide inhibiting p53 and preferentially targeting cancer cells that, after Phase I clinical testing, results well tolerated by children affected by central nervous system malignancy [43]. Although, examples of peptides under clinical development that target AKAPs are not yet available, the number of proof-of-concept studies on such applications is steadily increasing [14].

Drawbacks of peptide-based PPI targeting and potential solutions

Although targeting AKAP-dependent PPI might benefit from the use of disrupting peptides, their advance into clinical use is hampered by the significant hurdles intrinsic to this approach. A major barrier to the therapeutic use of peptides is their relatively low stability, usually resulting from proteolytic degradation and leading to short plasma half-life [39]. In addition, due to the low membrane permeability and subsequent bioavailability, intracellular delivery is a key issue [44]. Furthermore, without extensive chemical modification, oral administration of peptides is impossible. However, peptide stability and potency can be improved by their conjugation with other molecules to form for example stapled peptides with improved pharmacodynamics [39,45]. In addition, production cost is high, albeit less expensive than that of antibodies [39] (Table 1). Finally, biochemical and functional features of AKAPs, and, above all, the conserved nature of the PKA-binding sites among most PKA-binding proteins, might reduce drug selectivity. Nonetheless, while these limitations might have restrained the clinical use of peptides, new technologies are emerging that address stability, bioavailability, delivery as well as specificity issues within the conserved binding sites in the AKAP family [46].

Stability

The short half-life of peptides may result in high frequency of administration, which can significantly impair patients' compliance to therapy. A possible strategy to improve the half-life of peptides is to increase their stability by restricting their enzymatic digestion. To this aim, specific sites which are susceptible to protease cleavage can be identified and can be substituted with other amino acids. For example, to protect peptides against exopeptidases, N-terminal and C-terminal extremities can be modified by N-acetylation or C-amidation [47], while to prevent the cleavage by endopeptidases, susceptible amino acids can be substituted by more resistant residues, such as tryptophan or proline [46,48,49]. As peptidases are stereospecific, another possibility is to create a retro-inverse peptide by replacing L-amino acids with D-amino acids. In this case, all bonds are also inverted to mimic natural amino acids. A partially or

Clinical Science (2020) **134** 3259–3282
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totally retro-inverse synthetic peptide has the same biological activity as the parental one, but the reversed sequence makes it less susceptible to proteases [46]. This method has been proven successful for several peptides, such as EFK17 [50], antimicrobial D-peptide [51], retro-inverse peptide RI-OR2 [52] and the FDA-approved Icatibant [53].

Another important issue concerning peptide stability is their low intestinal permeability as well as their rapid clearance from the organism. To improve the metabolic stability of peptides, chemical optimization can help to lock the peptide in an active conformation. This can be achieved by inducing N-methylation, stabilizing α-helices with aromatic residues, inducing a cyclization by binding N and C termini, or introducing covalent bounds to create a stapled peptide [48,54,55]. These methods latch the peptide in a bioactive conformation, maintaining chemical and structural stability, while improving its half-life. Successful examples include ATSP-7041, a cyclized α-helical stapled peptide [56], or Plecanatide, a cyclic peptide which has been recently approved by FDA [57].

Bioavailability

Biological membranes represent a barrier for peptides, which usually must enter the cytoplasm to exert their biological function. The lipophilic nature of membranes constitutes a barrier for the penetration of hydrophilic peptides by simple diffusion. To overcome this issue and generate the so-called 'cell penetrating peptides', several strategies are available. First, hydrophobic sequences or lipid moieties can be either fused or chemically bound, respectively, to function as carriers allowing diffusion through biological membranes [58–60]. For instance, the fusion of stearate residues to Ht31 peptide (St-Ht31) renders Ht31 membrane-permeable [61]. Nonetheless, to improve efficient delivery inside the cell, the most commonly used strategy is to fuse the active peptide to amino acid sequences, like Penetratin-1 and TAT (HIV transcription *trans*-activation), known to induce intracellular uptake of small proteins [46,62]. Penetratin-1 is a 16-amino acid sequence (RQIKIWFQNRRMKWKK) derived from Antennapedia homeodomain protein of *Drosophila*. Thanks to its amphiphatic nature, Penetratin-1 can adopt a random coil structure which allows the interaction between its hydrophobic components and the apolar lipid membrane and, consequently, its intracellular internalization [63,64]. An example is the cyclic peptide inhibiting Grb7, a well-known potential intracellular target in cancer therapy, which contains Penetratin-1 merged with G7-18NATE [65].

TAT is a transcription activating factor made of 86 amino acids which is internalized via endocytosis and localized at the nuclei [66]. Of note, the sequence including amino acids 48–60 of TAT (GRKKRRQRRRPPQ) penetrates inside cells more efficiently than the full-length. Being rich in arginine, the TAT sequence is positively charged, a feature that explains the electrostatic binding of this peptide to cellular membranes, composed of various anionic components, such as proteins, lipid head groups and proteoglycans [63,64]. The association of a TAT sequence to an AKAP peptide has already been exploited successfully. For example, a TAT fusion to AKAPs disrupts AKAP/PKA interaction in pancreatic B cells *in situ* [67]. Moreover, TAT-AKAD inhibits PKA localization in cardiac myocytes [68].

Delivery

Oral delivery is usually the preferred route for drug administration, due to its simplicity, high patients' compliance and low costs compared with intravenous administration. However, oral bioavailability of peptides is poor since the gastrointestinal tract represents both a chemical and a physical barrier. To be effective, peptides need to resist the low pH as well as the high amount of enzymes present in the gastrointestinal environment and finally, they have to be absorbed by the intestinal membrane [39]. As a consequence, formulations other than oral have been developed for peptides and these include aerosols, intradermal patches, transferosome, liposome, iontophoresis and sonophoresis [38]. Furthermore, different delivery systems have been developed to optimize the oral bioavailability of peptides. These include (i) lipid-based particles such as liposome [69,70], solid lipid particles [71,72], self-nanoemulsifying drug delivery system [73,74] and multiemulsion [75,76]; (ii) polysaccharide-based particles such as chitosan [77], dextran [78] or cellulose [79]; (iii) inorganic particles such as gold [80], selenium [81], silica [82–84], aluminium [85] and (iv) synthetic functional particles [86–89]. Further details about multifunctional oral delivery system can be found in a recent review of Han and colleagues [90] and Drucker [91]. Nevertheless, injection represents the main route for clinical routine administration of peptides. Although being uncomfortable for patients, it offers the advantage of high speed of action, which is not negligible in cases of emergency treatment, such as for the use of insulin [39].

Challenges for peptide-based AKAP disruptors

Several challenges must be considered in order to develop AKAP/PKA disrupting peptides as therapeutics. First, because the hydrophobic helical motif is intrinsically conserved among AKAPs, it can be challenging to target specific AKAP/PKA interactions using disruptor peptides that are based on this structure. In addition, the ubiquity of the R subunit dimer and its limited isoform diversity means that interrupting the anchoring of R subunits might result in a wide range of off-target effects. Most of the peptides that have been developed are based on the hydrophobic helical

motif conserved among AKAPs rather than on the D/D domain of PKA. This is due to the fact that the D/D-containing RII domain is not only present on PKA R subunits but also in more than 200 eukaryotic proteins that are able to bind AKAPs, reducing the possibility to target a single AKAP family member and increasing the potential off-target effects linked to the disturbing the action of many, if not all, AKAPs [92]. Nonetheless, association between AKAPs and PKA is more complex than previously expected and hydrophilic anchor points outside the amphipathic helix forming the AKB contribute to determine the affinity of the binding between an AKAP and the D/D domain [93]. Despite the relative strength of the D/D-AKAPs association, peptides encompassing these other binding sequences might in principle allow targeting of a specific AKAP family member within the large pool of different PKA-binding proteins concomitantly expressed in a cell. Furthermore, AKAPs do not only associate with PKA but also with a large variety of other signal transduction effectors (Figure 1). In general, if selective disruption of PKA binding cannot be achieved, inhibiting the PPIs between AKAPs and their other binding partners might thus represent a more specific option.

Targeting AKAP function in diseases

Studies with peptides and peptidomimetics disrupting PPI have significantly helped elucidating the mechanism of action of AKAPs. The picture emerging from these studies reveals that such peptide tools might possess distinctive functions with significant therapeutic potential. Outlined below are the most paradigmatic recent examples of proof of concept studies and preclinical applications found in elective case diseases, spanning from more common to rarer causes of death in the industrialized world.

AKAPs as targets in cardiovascular diseases

Cardiovascular diseases (CVDs) are not only the leading cause of mortality and hospitalization worldwide, but also a major cause of decline in patients' quality of life [94,95]. These vast group of pathologies including coronary artery disease, cardiac arrhythmias and hypertension, are all able to alter cardiac function, finally disrupting the vital pumping action of the heart and inducing heart failure (HF). These diseases are known to induce HF since they can cause abnormal cardiac muscle contraction and relaxation, and because they can lead to cardiomyocytes hypertrophy. Both these two processes are known to be regulated via the several AKAPs that are expressed in the heart, hinting at the possibility to target these cAMP signalosomes for therapeutic intervention. The following subparagraphs will delve into the molecular mechanisms of AKAPs-mediated regulation of Ca^{2+}-handling, excitation–contraction coupling and hypertrophic stress responses [23], and provide preclinical examples of either peptides or peptidomimetics targeting AKAPs-based PPI for the treatment of cardiac diseases.

AKAPs in Ca^{2+} cycling and cardiomyocyte contractility

cAMP has a central role in the regulation of excitation–contraction coupling, the process by which an action potential triggers myocyte contraction. AKAPs coordinate these actions and, for example, AKAP18α (also known as AKAP15), a membrane-associated AKAP, regulates cardiac contractility via anchoring PKA to voltage-gated L-type Ca^{2+} channels (LTCCs). The opening of these channels, mediated via sarcolemma depolarization, induces a localized increase in Ca^{2+}, which triggers multiple ryanodine-sensitive Ca^{2+} channels (RyRs), mediating a global release of Ca^{2+} from the sarcoplasmic reticulum (SR) and in turn the activation of several Ca^{2+}-sensitive contractile proteins, ultimately driving myocyte contraction [96–98]. A similar action is driven by AKAP5 (also known as AKAP79/150, indicating the molecular weight of the human and rodent ortholog, respectively) and this AKAP appears to be specifically connected to this machinery in the presence of catecholaminergic signaling [99]. AKAP18α localizes PKA near LTCCs through the interactions mediated by its leucine zipper (LZ) motif with the C-terminal domain of the α_1 subunit of LTCC. PKA anchored to AKAP18 mediates the phosphorylation of LTCCs, an event that is known to increase their open probability and, in turn, Ca^{2+} currents [100,101] (Figure 3). PKA inhibitors and AP2, a peptide developed to disrupt the AKAP18α–PKA interaction, strongly inhibit voltage-dependent potentiation of LTCCs in skeletal muscle cells, confirming that PKA and AKAP-mediated channel phosphorylation are required for contraction. More intriguing, though, is the design of a peptide called $AKAP15_{LZ}$ (38–54) able to disrupt the interaction between AKAP18α and LTCC [102,103]. The peptide $AKAP15_{LZ}$ (38–54) effectively binds the C-terminal domain of the α_1 subunit of LTCCs and abolishes PKA-mediated phosphorylation of the channel. This drastically reduces contractility and appears even more effective than PKA inhibitors in inhibiting voltage-dependent potentiation of LTCCs. Although this peptide has never been tested in preclinical models, its ability to prevent Ca^{2+} entry increase in response to local cAMP might suggest its use as a substitute to drugs commonly used in CVD, like β-adrenergic receptor (βAR) blockers.

Clinical Science (2020) **134** 3259–3282
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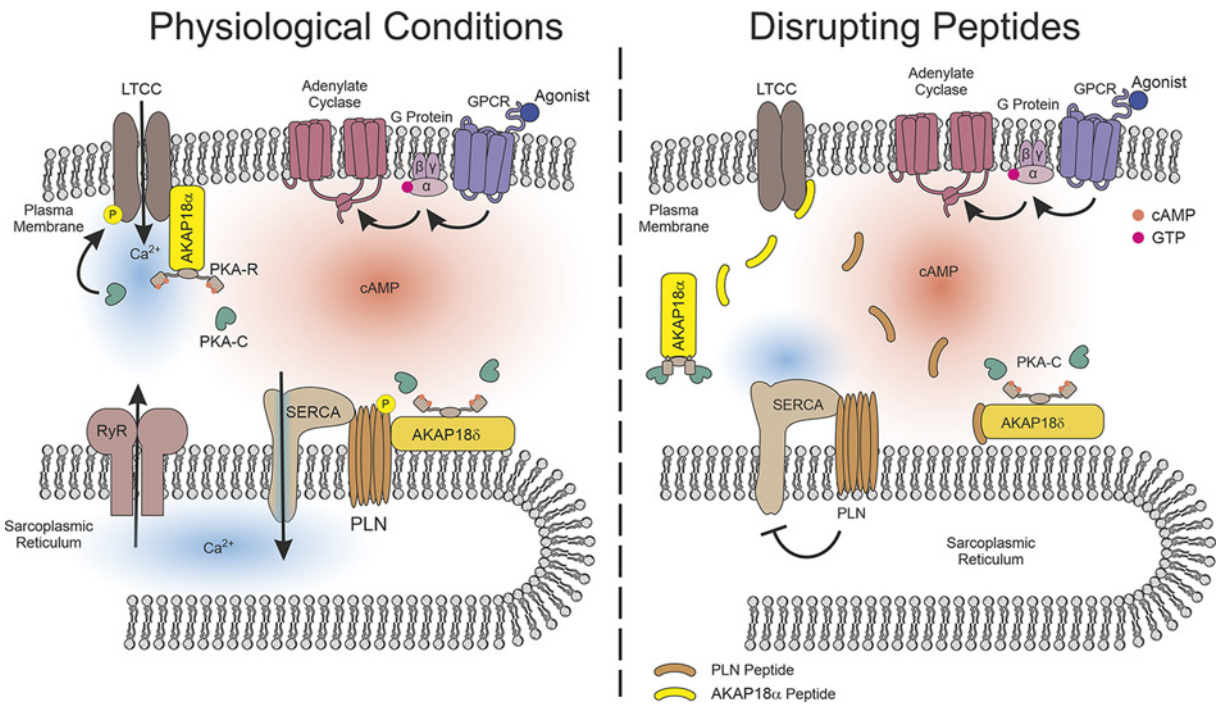

Figure 3. Major AKAPs signaling complexes involved in the control of cardiomyocyte contractility and their targeting via disruptor peptides for cardioprotection

(Left) AKAP18α is involved in cardiac contraction, since the specific pool of PKA that is anchored by this AKAP phosphorylates LTCCs, an event increasing the open probability of the channels and, in turn, Ca^{2+} currents. This increase in cytoplasmic Ca^{2+} triggers multiple RyRs, mediating a global release of Ca^{2+} from the SR and activating Ca^{2+}-dependent contractile protein. In the relaxation phase, Ca^{2+} is removed from the cytosol via SERCA2, which pumps Ca^{2+} back into the SR. When bound to PLN, these pumps are inhibited. A PKA pool anchored at the SR by AKAP18δ phosphorylates PLN, thus releasing its inhibition on SERCA, leading to relaxation. (Right) The AKAP15$_{LZ}$ (38–54) peptide, disrupting the interaction between AKAP18α and LTCC, blunts RyRs activation and the consequent cardiac contraction, suggesting its possible use as a more specific substitute of βAR blockers. Instead, the PLN peptide, designed to interfere with the interaction between PLN and the scaffold protein, leads to the inhibition of SERCA2 and it can be therapeutically useful in post-infarction to limit the chronic adrenergic signaling that represents a harmful compensatory mechanism.

Besides acting on contraction, AKAP-based complexes can control myocyte relaxation. For example, AKAP18δ, a large splice variant derived from the *AKAP18* gene, anchors the specific pool of PKA that is responsible for the phosphorylation of phospholamban (PLN) in response to βAR stimuli. This event relieves, through a still debated complex biochemical mechanism [104], the inhibition of the ATP-dependent sarcoplasmic/endoplasmic reticulum calcium pump 2 (SERCA2), the protein responsible for the entry of Ca^{2+} back into the SR, facilitating relaxation and left ventricle (LV) filling [105]. A short cell-permeable peptide, namely PLN peptide, based on the amino acidic sequence of PLN covering the AKAP18δ-binding domain, disrupts the interaction between AKAP18δ and PLN (Figure 3 and Table 2). In neonatal cardiac myocytes, this peptide selectively abolishes PKA-mediated PLN phosphorylation on Ser^{16} upon βAR stimulation and, in turn, significantly reduces Ca^{2+} re-uptake into the SR [105]. Besides representing an interesting tool for interrogating the regulation of the AKAP18δ/SERCA2/PLN/PKA complex, this peptide has limited therapeutic utility given that in most HF cases selective up-regulation, and not down-regulation, of PLN phosphorylation is desirable. Nevertheless, reducing SERCA2 activity in response to β-adrenergic stimuli by delocalizing AKAP18δ/PKA from PLN might be useful in post-infarction to limit the chronic adrenergic signaling which represents a harmful compensatory mechanism [105,106]. Furthermore, the patent US2017/0158657A1 describes a peptidomimetic disrupting the AKAP18δ–PLN interaction and eventually resulting in a promising drug for the treatment of cardiac Ca^{2+}-mediated reperfusion damage, occurring after recanalization of obstructed coronaries and leading to expansion of the infarcted area. This compound regulates SERCA2 abnormal activity via PKA RII, highlighting its potential use in the treatment and prevention of events determining chronic HF development [107].

Table 2 Major AKAP disrupting peptides and peptidomimetics and their potential therapeutic applications

Name	Type	Target	Potential therapeutic use	Current use	References
AKAP15lz	Peptide	AKAP18α/LTCC	Regulation of cardiac contraction	Research	[102]
PLN peptide	Peptide	PLN/AKAP18δ	Cardiac contractile dysfunction in HF	Research	[105]
CaNBD	Peptide	CaN/mAKAP	Regulation of cardiac hypertrophy	Research	[110]
mAKAP peptide	Peptide	Nesprin/mAKAP	Regulation of cardiac hypertrophy	Research	[112]
bs906	Peptide	Hsp20/PDE4D	Regulation of cardiac hypertrophy	Research	[115]
AKAP-Lbc peptide	Peptide	AKAP-Lbc/PKN	Hypertrophic remodeling in response to TAC	Research	[119]
EBP50 peptide	Peptide	Ezrin/EBP50	Colorectal cancer	Research	[132]
AKAP79/150-TRPV1 peptide	Peptide	TRPV1/AKAP79/150	Inflammatory heat hyperalgesia	Research	[166]
FMP-API-1/27	Peptidomimetic	AKAP-Lbc/PKA	NDI	Research	[179]
STAD-2	Peptide	AKAP/PKA	Malaria	Research	[185]
RIAD-P3	Peptidomimetic	AKAP/PKA-RI	HIV	Research	[196]

AKAPs in cardiac hypertrophy

In the heart, AKAPs not only control contractility but also the induction of pathological hypertrophy, an adaptive process triggered by different stressors, such as pressure and volume overload resulting from hypertension and myocardial infarction. Hypertrophy is characterized by an increase in cardiomyocyte size and total cardiac mass, increased myofibrillar organization, as well as up-regulation of specific genes that are only expressed during embryogenesis, namely 'fetal' genes [108,109]. Despite being beneficial at early stages, this defense mechanism in the long run can turn maladaptive, and lead, in association with abnormal Ca^{2+} handling, to cardiomyocyte death, HF or arrhythmia [108].

A large body of evidence indicates a crucial role in cardiac hypertrophy of cAMP signaling compartmentalization, and thus of AKAPs [109]. Among these, a key player is mAKAP that, localized at the perinuclear membrane via its binding with Nesprin, recruits the phosphatase calcineurin (CaN) controlling the activity of transcription factors, like NFAT and MEF2, critically involved in the activation of the hypertrophic transcriptional program. The Ca^{2+} pool released after RyR phosphorylation by PKA activates CaN, which de-phosphorylates NFAT or MEF2, triggering their nuclear translocation and the subsequent transcription of the hypertrophic gene program [110,111] (Figure 4, left panel). In the attempt to modulate these PPIs for therapeutic purpose, Li and colleagues report a peptide based on the CaN-binding site of mAKAP named CaNBD [110] (Table 2). Expression of this peptide in HEK293 cells significantly inhibits the mAKAP/CaN association, reducing NFAT phosphorylation and consequent nuclear localization (Figure 4, right panel). More importantly, primary neonatal rat cardiac myocytes expressing CaNDB do not show any increase in cross-section area after a 2 days long adrenergic stimulation of hypertrophy, thus demonstrating the ability of the peptide to counteract the hypertrophic remodeling (Figure 4, right panel) [110]. mAKAP can also induce cardiac hypertrophy via ERK5-mediated inhibition of PDE4D3, which increases the local concentration of cAMP, and as a consequence, enhances the PKA/RyR/cytoplasmic Ca^{2+}/CaN/nuclear NFATc3 pathway [112,113]. mAKAP localized at the perinuclear membrane by its binding with Nesprin anchors PDE4D3 which acts as a scaffold protein for ERK5, MEK5 and the cAMP-activated Epac1. Activated ERK5 phosphorylates PDE4D3 on Ser^{579} inhibiting its activity, while activated Epac1 halts ERK5 activity, thus preventing PDE inhibition [112]. Cytokines known to activate ERK5 are indeed able to induce eccentric cardiac hypertrophy, characterized by an increase in length of cultured rat neonatal ventriculocytes. On the contrary, ERK5 inhibitors block hypertrophy. Notably, this protective effect can be achieved by displacing mAKAP from the perinuclear membrane via disrupting its binding with Nesprin with an mAKAP competing peptide (residues 582–1286), pointing to the possible development of therapeutic tools targeting mAKAP-organized PPI [45,112] (Figure 4, right panel and Table 2).

Like mAKAP, AKAP-Lbc is also involved in the development of cardiac hypertrophy. AKAP-Lbc anchors PKA to its substrate, the heat-shock protein of 20 kDa (Hsp20). Remarkably, when phosphorylated by PKA at Ser^{16}, Hsp20 protects against cardiac hypertrophy [114]. Hsp20, in turn, binds to PDE4D that, through a negative feedback loop, hydrolyzes cAMP, halting PKA activation as well as Hsp20 phosphorylation (Figure 4, left panel) [114]. With the idea of targeting this Hsp20/PDE4D association and enhancing PKA-mediated phosphorylation of Hsp20, Martin and colleagues report the mapping of the protein–protein interface by a peptide array and the development of a disrupting peptide, named bs906 (Figure 4, right panel and Table 1). Treatment with bs906 is able to attenuate cardiac myocyte hypertrophy in cell culture but, more importantly, to protect mice from the decline in cardiac contractility

Clinical Science (2020) **134** 3259–3282
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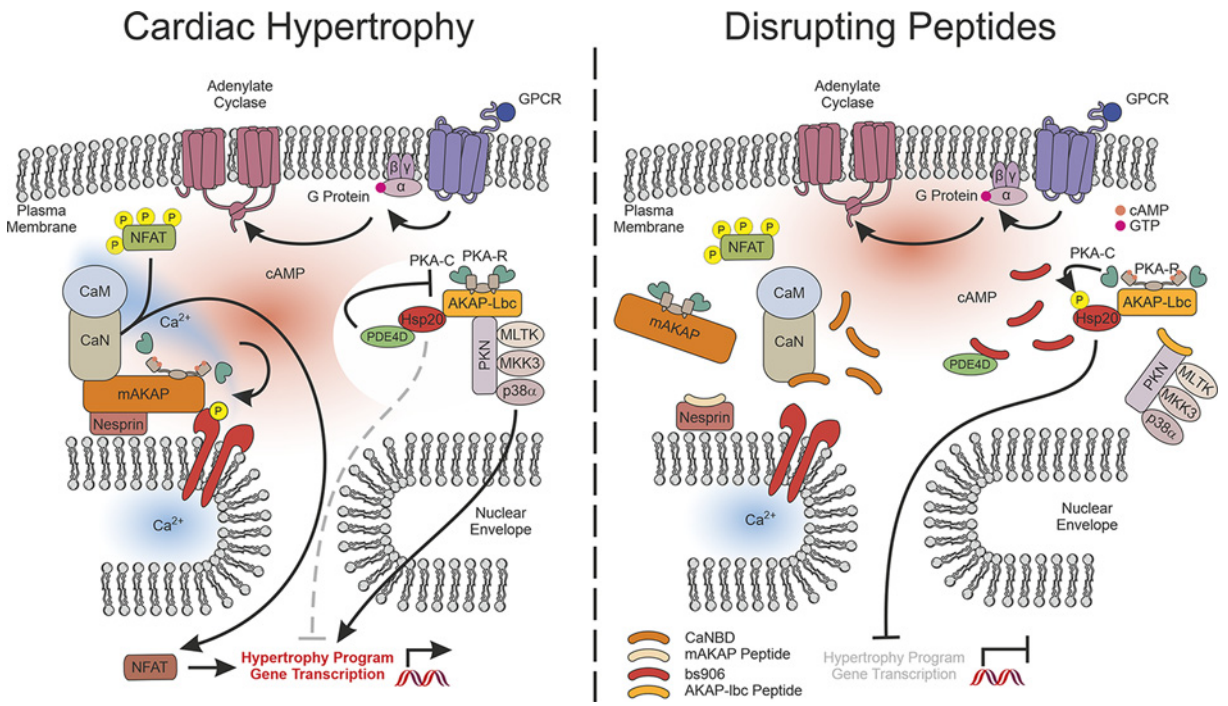

Figure 4. Major AKAPs signaling complexes involved in cardiac hypertrophy and their targeting via disruptor peptides for cardioprotection

(Left) several AKAPs are known to modulate hypertrophy-inducing enzymes. In physiological conditions mAKAP, localized at the perinuclear membrane via Nesprin, anchors PDE4D3 which acts as a scaffold protein for its negative regulator ERK5 that, when activated, phosphorylates PDE4D3 inhibiting its activity and leading to cardiac hypertrophy. The local increase in cAMP due to PDE inhibition also enhances the PKA/RyR/cytoplasmic Ca^{2+}/CaN/nuclear NFATc3 pathway, leading to the activation of the hypertrophic transcriptional program. Another AKAP involved in cardiac hypertrophy regulation is AKAP-Lbc, which anchors PKA to its substrate Hsp20, mediating its phosphorylation that is known to protect against this pathological process. Hsp20, in turn, binds to PDE4D, that through a negative feedback loop hydrolyses cAMP, halting PKA activation as well as Hsp20 phosphorylation. AKAP-Lbc can also interact with several RhoA effectors like PKNα, MLTK, MKK3, leading to the activation of p38 MAPK and consequent hypertrophy. (Right) The mAKAP peptide, disrupting the interaction between Nesprin and mAKAP, displaces the scaffolding protein from the perinuclear membrane, inhibiting NFAT activity. The CaNBD peptide, which disrupts the interaction between mAKAP and CaN, blunts NFAT translocation into the nucleus, inhibiting the hypertrophic transcriptional program. Hsp20 inhibition can be induced via the usage of a disruptor peptide called bs906, that blocks the binding between this protein and AKAP-Lbc. The AKAP-Lbc peptide, disrupting the interaction between this scaffold and PKN, inhibits p38α activity. All these disrupting peptides exert a cardioprotective effect against stress-induced hypertrophy, and can be possibly used as therapeutics in the future.

induced by transverse aortic constriction (TAC). Mice treated with 10 mg/kg bs906 twice a week for 4 weeks show better LV contractility than mice injected with a scrambled control peptide [115]. AKAP-Lbc also acts as a GEF for the small GTPase Rho, another trigger of pathological hypertrophy. While, on the one hand, the Rho-GEF activity of AKAP-Lbc is enhanced by α1 adrenergic receptors (α1-ARs), on the other hand Rho-GEF function is inhibited by the association with 14-3-3 triggered by PKA-mediated phosphorylation of AKAP–Lbc itself [116,117]. Finally, the AKAP–Lbc complex includes RhoA effectors, like PKNα, MLTK, MKK3, which activate the p38 MAPK and its consequent hypertrophic response (Figure 4, left panel) [118]. Perez Lopez and colleagues report a transgenic-mouse model overexpressing an AKAP–Lbc disruptor peptide inhibiting the interaction between the AKAP and p38 signaling module. Overexpression of this inhibitory peptide protects mice from hypertrophic remodeling in response to TAC (Figure 4, right panel) [119], thus showing that the PPI between AKAP–Lbc and p38 can represent a valuable target for therapeutic intervention (Table 2).

Cancer

Cancer is the second commonest cause of death globally, accounting for an estimated 9.6 million deaths in 2018, with more than 3 million people diagnosed with cancer in Europe only [120]. Considering the number of patients that have to be diagnosed with the pathology every year worldwide, and also the fact that several current therapies can lose their effectiveness due to resistance, it is crucial not only to find new mechanisms that can be targeted therapeutically, but also new biomarkers that can allow a prompt clinical intervention. Among the several signaling hubs known to play a role in cancer development and progression, a growing body of evidence indicates the involvement of AKAPs in several cancer-related cellular processes such as cell proliferation, survival and migration [13]. Mutations in multiple AKAPs have been observed in cancer patients and this has prompted to investigate AKAPs as potential cancer biomarkers. For example, AKAP3, also called AKAP110, is expressed in several types of cancer, such as hepatocellular carcinoma (HCC), ovarian, breast, lung and colon cancer [121], while in physiological conditions it is expressed only in the testis, where it is involved in sperm motility regulation [122]. Several studies have shown a correlation between AKAP3 expression, histological grade and clinical stage of ovarian cancer [121,123,124]. Similarly, AKAP4, another AKAP expressed in testis and known to control sperm motility in physiological condition, is up-regulated in cancer cells and could represent an interesting biomarker [125]. AKAP–Lbc, also known as AKAP13, is overexpressed in several cancers, such as HCC, breast cancer, esophageal cancer and acute myeloid leukemia and controls cancer cell migration through PKA and RhoA activation [125]. Furthermore, AKAP–Lbc is implicated in the onset of resistance to tamoxifen in patients suffering from breast cancer, through a mechanism dependent on Src1 [126], and could represent an interesting target for cancer therapy. Similar to AKAP–Lbc, overexpression of the AKAP Ezrin promotes cell survival in colon cancer cells, through a PKA-dependent up-regulation of the pro-survival factors XIAP and survivin [127]. This appears to be regulated by an increase in Ezrin phosphorylation triggered by either IGF1R or TGF-β receptor. Therefore, interfering with Ezrin-mediated PPI is suggested as a new treatment for colon carcinoma [127]. In agreement with the strategy of targeting PPIs organized by AKAPs, a proof of principle is provided by the finding that disruption of AKAP/PKA-RII binding by the St-Ht31, the cell permeable version of the Ht31 peptide, decreases proliferation of breast cancer cells [128].

Cancer cell growth is usually accompanied by metabolic adaptations that involve mitochondria and that are in part regulated by AKAPs, such as AKAP1, which is overexpressed in a wide variety of high-grade cancer tissues. This protein, which resides in the outer mitochondrial membrane, is a transcriptional target of Myc and supports cancer cell growth via the activation of the mTOR signaling pathway. AKAP1 can recruit Sestrin2, and consequently remove the inhibitory constraint of Sestrin2 on leucine-mediated mTOR activation. Silencing or inhibition of this scaffold protein disturbs leucin-mediated activation of mTOR and thus slows down tumor growth, suggesting AKAP1 as a promising target for cancer therapy [129,130].

Given the role of AKAPs in immunomodulation, targeting these proteins can also be exploited to boost the immune system in the fight against cancer. cAMP is a potent negative regulator of T cells and is responsible for the dysregulation of T-cell function in cancer, lowering anti-tumoral immunity [131–133]. Stokka and colleagues report that the AKAP Ezrin is involved in the regulation of T-cell function and is found in lipid rafts of T cells. This localization occurs through the binding with Ezrin-radixin-moesin-Binding Phosphoprotein 50 (EBP50), which promotes the formation a macromolecular complex with C-Terminal Src Kinase (Csk) and Phosphoprotein Associated with Glycosphingolipid-enriched membrane microdomains (PAG) [132]. Once activated, PKA phosphorylates Csk, which negatively regulates T-cell function by phosphorylating and inhibiting the T-cell receptor signal transducer Lck (Figure 5, left panel). A peptide deriving from the EBP50 sequence (EBP50 peptide) and disrupting the interaction between Ezrin and EBP50, reverses the inhibitory effect of cAMP on T-cell function (Figure 5 right panel, Table 2) [132,133] but whether this peptide is of clinical use awaits further studies.

Neuronal diseases

Beside their function in CVDs and cancer, by playing a crucial role in the regulation of neuronal signaling pathways, AKAPs can represent interesting targets for the treatment of neuronal damage/dysfunction with disruptive peptides. Brain injuries and neurological disorders represent another critical disease area with growing yearly incidence rates and greatly unmet medical need. Neurodegenerative diseases severely affect industrial countries, as the prevalence increases with the age of the population [134,135]. Up to now, no effective treatment exists to cure, slow down or prevent neurological disorders [136].

Recent evidences indicate that targeting AKAPs can be useful in better understanding as well as potentially treating these conditions. On the one hand, AKAPs are emerging as orchestrators of protective mechanisms in some neurological disorders, such as Parkinson's disease (PD) and axonal degeneration in the retina [15]. In this context, peptides

Clinical Science (2020) **134** 3259–3282
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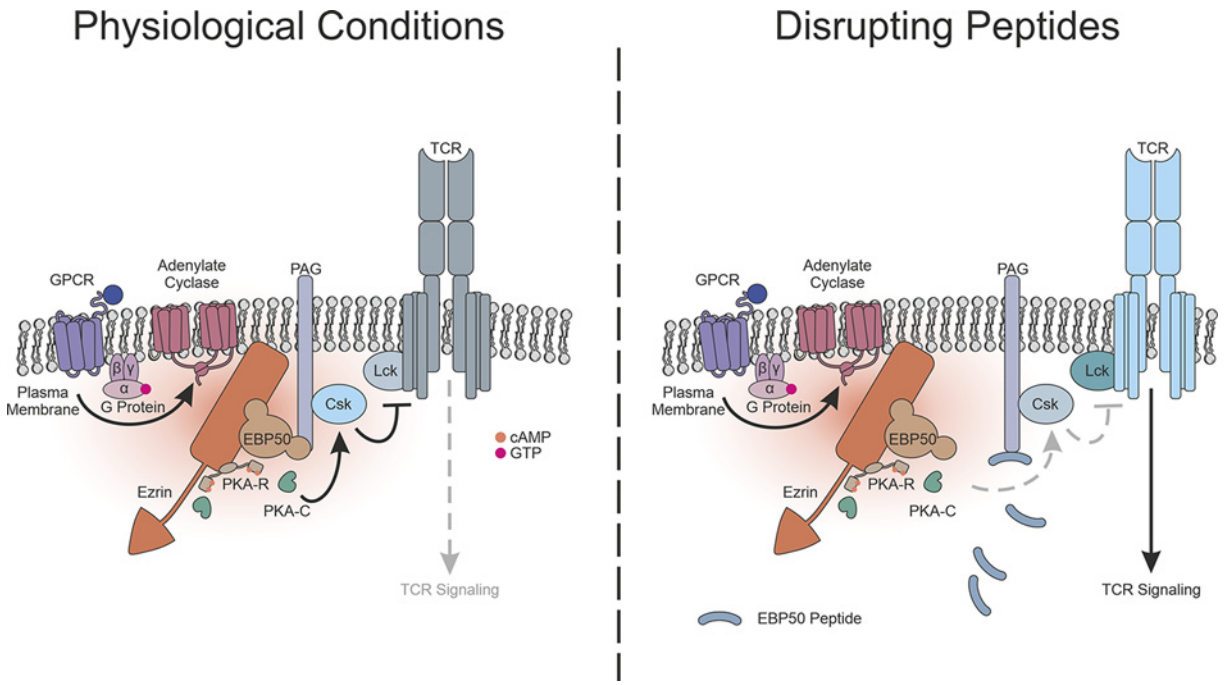

Figure 5. Schematic illustration of Ezrin function in T cells and the effect of AKAP disrupting peptide on immunomodulation

(Left) Ezrin is located in lipid rafts of T cells through the binding with EBP50 and promotes the formation of a macromolecular complex with Csk and PAG. Following the activation of GPCR, cAMP activates PKA that phosphorylates Csk which, in turn, inhibits Lck and the downstream T cell receptor (TCR) signaling. (Right) The use of EBP50 peptide disrupts the interaction between Ezrin and EBP50, reversing the inhibitory effect of cAMP on TCR.

disrupting PPI organized by specific AKAPs do not represent therapeutic targets but rather new valuable tools to interrogate disease mechanisms. On the other hand, specific PPI organized by selected AKAPs are turning out as key pathological determinants. This is evident not only in the development of some neuronal and neurodegenerative disorders, such as Alzheimer's Disease (AD), where no therapies can cure or slow disease progression [137], but also in other invalidating and hard to treat conditions, like seizure, chronic pain and addiction [15]. As such, the frequently opposing roles of AKAPs in these conditions highlight the importance to develop disrupting peptides with high degree of specificity, in order to specifically disrupt detrimental but not neuroprotective AKAPs.

Parkinson's disease

PD is an example where targeting AKAPs cannot be exploited for therapeutic strategies but can still be useful for a better understanding of the disease mechanism. PD is the second most common age-related neurodegenerative disease after AD, with an estimation of 10 million affected people worldwide [138]. PD is caused by the progressive loss of dopaminergic neurons in the *substantia nigra* [139], resulting in different symptoms, such as bradykinesia (slowed movement), resting tremors and rigidity [140].

Most of the currently known proteins involved in PD, like for example PTEN-induced kinase 1 (PINK1), are linked to mitochondrial quality control processes. Therefore, mitochondrial damage in dopaminergic neurons appears as a major contributor of the selective neuronal vulnerability observed in PD [139,141,142]. Through an unclear mechanism, PINK1 modulates a PKA pool associated with D-AKAP1 that regulates mitochondrial trafficking in dendrites [143] The D-AKAP1–PKA interaction induces the phosphorylation of the mitochondrial Rho GTPase Miro2, which in turn promotes mitochondrial transport and energy supply to dendrites, thus maintaining neuronal survival [137,139]. Oxidative stress is responsible of the disruption of PINK1-PKA signaling, inducing mitochondrial damage and mitophagy. Whether disruptors of D-AKAP1/PKA interaction can cause PD is yet unclear but future studies are envisaged to address this issue.

Neuronal survival in the retina

Survival of retinal ganglion cells (RGCs) relies on cAMP signaling and requires muscle A-kinase anchoring protein α (mAKAPα) [144]. A leading cause of blindness is retinopathy triggered by the death of RGCs, that are responsible for the visual information transmission via the optic nerve, from the retina to the lateral geniculate, pretectal and superchiasmatic nucleus [144]. mAKAPα is expressed at the outer nuclear membrane of RGCs [144,145] and induces a cAMP/PKA-dependent signaling responsible of a positive effect on neuronal survival and axon growth [146]. Besides associating with enzymes involved in cAMP signaling, mAKAPα binds to mitogen-activated protein kinases MEK5 and ERK5, known to be essential for neuronal survival [112,147–149]. In line with this finding, genetic deletion of mAKAPα in mice does not disturb prenatal RGC development but predisposes to increased death of RGCs in a model of axonal degeneration of the optic nerve. This supports the notion that mAKAPα/ERK5 axis is not implicated in physiological processes but rather in RGCs survival in stress conditions [144] and, although proof-of-concept studies are missing, suggests that disturbing this interaction can contribute to blindness.

Alzheimer's Disease

While AKAPs seem protective in PD and axonal degeneration in the retina, they appear to play an opposite role at least in some of the processes occurring in AD. Affecting 50 million people worldwide, AD is the commonest neurodegenerative cause of memory loss, cognitive impairment, dementia and death [150]. Two key pathological hallmarks are present in brains of AD patients: neurofibrillary tangles and amyloid plaques. Amyloid plaques are generated by the accumulation of the highly toxic Aβ oligomers, which result from the excessive cleavage of the amyloid precursor protein (APP). Aβ formation suppresses synapses functions and may represent a causative trigger to the typical AD symptoms [151]. AKAP-regulated proteins can participate in this toxic effect of β oligomers [15]. For example, CaN is recruited to AKAP79/150–LTCC complex [35] to dephosphorylate cytosolic NFAT and to allow nuclear translocation of this transcription factor. Nuclear NFAT then unleashes a neurotoxic gene program, promoting dendritic spine morphological changes and AD progression [15]. The use of CaN antagonists prevents this morphological change in a transgenic mouse model, increasing memory function [99]. Sadly, CaN antagonists have severe systemic side effects that do not allow their application in this specific disease condition. An alternative may reside in the usage of a peptide disrupting the interaction between NFAT and CaN called VIVIT, which is able to cross the blood–brain barrier (BBB) via its conjugation with the dNP2 sequence. This molecule, based on the common CaN-NFAT binding motif, can effectively inhibit NFAT activity regulating T-cells function in a model of multiple sclerosis [104]. Whether this effect can be translated to AD is yet to be defined and future experiments are expected to test this hypothesis.

Seizure

Another area of potential use of peptides disrupting AKAP-orchestrated signaling hubs is the treatment of seizures. With an average of 5 million people diagnosed each year, seizures are one of the most common neurological disorder affecting people of all ages. [152]. Seizures are caused by an excessive and hypersynchronous electrical discharge of cortical neurons and can be related to epilepsy, if recurrent and without any provoked factor [153,154]. A cause of seizures is the dysfunction of voltage-gated potassium (K_v) channels, which are crucial to limit neuronal excitability by contributing to membrane repolarization and hyperpolarization [155]. AKAP79/150 is involved in the suppression of the K_v current, enhancing neuronal excitability. Therefore, disruption of AKAP signaling might supress the pathologically elevated neuronal excitability responsible of seizures. In support to this hypothesis, Tunquist and colleagues showed that mice lacking AKAP79/150 are more resistant to seizures due to a PKC-dependent increase in the open probability of K_{v7} channels [156]. Along the same line, valproic acid inhibits palmitoylation of AKAP79/150, which is increased during seizure [157], and thus reduces AKAP79/150-dependent K_{v7} channel suppression [158]. Therefore, stronger insight into AKAP79/150-driven interactions might pave the way to a new peptide-based treatment for seizure.

Chronic pain

While in most neurologic conditions the proof-of-concept that peptides disrupting AKAP-mediated PPI might carry therapeutic potential is still missing, clearer examples of their use come from the area of chronic pain treatment. Chronic pain is a major societal problem, as pain-related disease and pain are the leading cause of disability worldwide. In U.K., a systematic review highlights that 13–50% of the adult population is affected by chronic pain [159]. In contrast with acute pain, chronic pain is due to an excessive stimulation of nociceptive receptors, which leads to alterations in the pathways responsible for physiological pain sensation, such as overexpression of pain-stimulated receptors. Among these proteins, N-methyl-D-aspartate (NMDA) receptors (NMDARs) and transient receptor potential vanilloid receptor subtype 1 (TRPV1) represent key players in pain-related pathways [160]. NMDAR, a tetrameric

Clinical Science (2020) **134** 3259–3282
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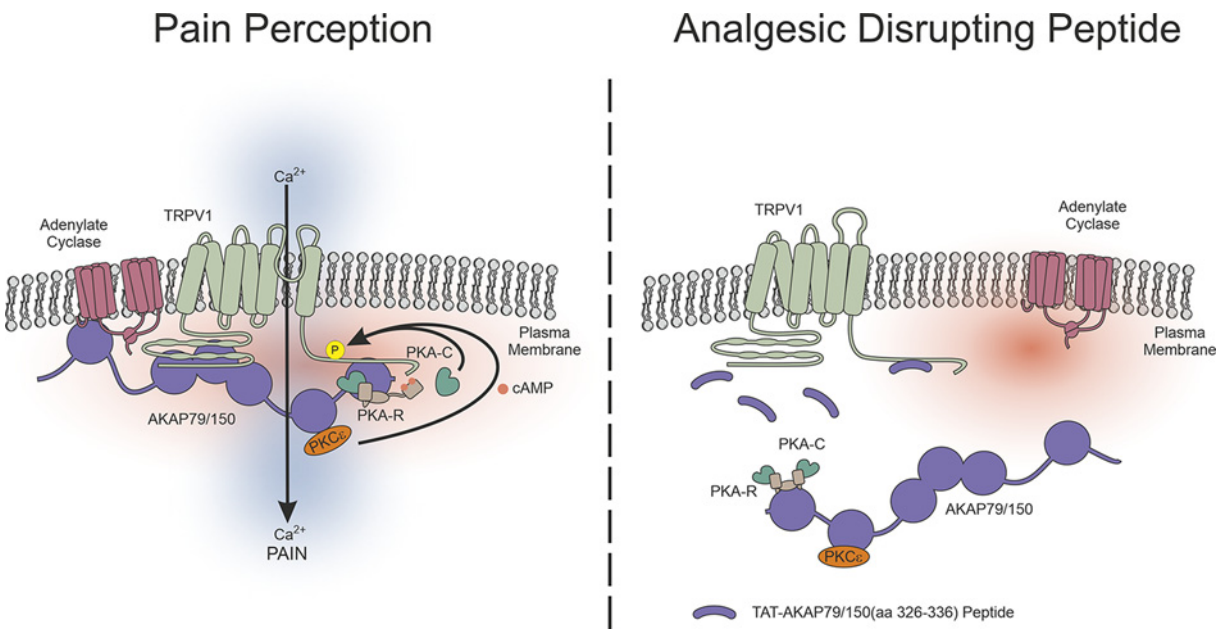

Figure 6. Schematic illustration of pain perception mediated by AKAP79/150 and the effect of a specific disrupting peptide

(Left) AKAP79/150 interacts with TRPV1 and regulates the localization of PKA and PKC nearby the receptor. Both kinases can phosphorylate TRPV1, inducing a calcium-dependent thermal-induced pain stimulus. (Right) AKAP79 (TAT-326-336) peptide disrupts AKAP79–TRPV1 interaction, preventing TRPV1 activation and the consequent pain stimulus.

protein constituted by different subunits, namely GluN1, GluN2 (isoform A-B-C-D) and GluN3 (isoform A-B) [161], is involved in nociceptive transmission in spinal dorsal horn, where the prevalent subtypes are GluN1 and GluN2 [162]. Peripheral inflammation induces spinal GluN2B accumulation and consequently NMDAR hyperfunction, which therefore leads to the initiation and development of chronic pain. Despite extensive efforts in targeting this receptor to treat chronic pain, clinical outcomes with several of these antagonists have been disappointing [161], leaving this medical need unmet. The inhibition of PKA activity reduces NMDAR-mediated synaptic transmission [163,164], suggesting inhibition of selective subsets of PKA as a strategy to promote analgesia [165]. Wang and colleagues report that delivery of the cell-permeable peptide St-Ht31 into mice dorsal horn neurons influences the glutamatergic transmission of nociceptive signals, without affecting motor function. The analgesic action of St-Ht31 correlates with the inhibition of synaptic GluN2B receptor, meaning that NMDAR-mediated nociceptive transmission is prevented by AKAP/PKA disruption allowing the reduction in inflammatory pain *in vivo* [61].

In another study, Fisher and colleagues highlighted the potential analgesic effect of disturbing AKAP79/150–TRPV1 interaction with a cell-permeable peptide [166]. TRPV1, an ion channel expressed in nociceptive primary afferent nerve fibers, is a mediator of thermal-induced pain stimulus and AKAP79/150 has a critical role in TRPV1 sensitization in primary nociceptive neurons (Figure 6, left panel). TRPV1-deficient mice display a defective response to noxious thermal stimuli, demonstrating that this channel is essential for thermal nociception [167] and suggesting TRPV1 as a target for analgesic drugs. In agreement, Btesh and colleagues report the design of a cell-permeable AKAP79/150–TRPV1 peptide derived from AKAP79/150 (TAT-326-336) that disrupts AKAP79/150–TRPV1 interaction, which is crucial for the TRPV1 sensitization mediated by PKA or PKC in dorsal root ganglion (DRG) neurons (Figure 6, right panel and Table 2). This peptide does not affect the interaction of AKAP79 with other targets and successfully reduces inflammatory hyperalgesia in a mouse model of inflammatory pain, while having no effect on hyperalgesia without inflammation, demonstrating a high specificity of action [168]. Contrary to usual TRPV1 antagonists, this peptide has no effect on acute pain threshold, and thus holds great promise for chronic pain treatment.

Addiction

Other examples of peptides with therapeutic potential that disturb PPI orchestrated by AKAPs come from the search of ways to treat addiction to drugs and alcohol. Addiction is affecting 31 million people worldwide and is a chronic relapsing disorder, characterized by the compulsive use of addictive substances despite negative personal and societal

impact [169]. The mesocorticolimbic circuit, also known as brain reward circuit, has a key role in the addiction process. Reward stimuli induce an increase in dopamine (DA) neuron activation in the ventral tegmental area (VTA), releasing DA which modulates various targets, primarily in the nucleus accumbens (NAc) [170,171]. During drug abuse, DA release is altered leading to a compulsive drug-seeking behavior, characteristic of the addiction. The VTA also sends γ-aminobutyric acid (GABA)-ergic projections to the NAc and prefrontal cortex, which inhibits DA neurons [172–174]. GABA neurons are therefore directly linked to acute and chronic effects of drugs [101], such as tolerance, dependence and withdrawal. AKAP–PKA interaction underlie drug-mediated plasticity involving GABA synapse in the VTA, and is known to contribute in particular to cocaine-induced neuronal adaptations [175]. AKAP79/150 is expressed in VTA dopamine neurons and may be localized in GABAergic synapses. The AKAP79/150–PKA interaction is important in the maintenance of GABA receptor basal activity, and its inhibition by the Ht31 peptide leads to the blockade of this process [173].

AKAP79/150 is also expressed at excitatory synapses on GABAergic medium spiny projection neurons (MSNs) of the NAc, where it is implicated in the relapse of cocaine-seeking behavior, important feature of addictive pathology following withdrawal [176]. A proteomic study highlights the increase in AKAP79/150 expression after 2 weeks of cocaine administration followed by 2 weeks of extinction. By using the inhibitor peptide St-Ht31, reinstatement of cocaine-seeking is impaired [177]. Disruption of AKAP79/150–PKA interaction may thus provide a way to inhibit this behavior and reduce the risk of relapse.

Metabolic diseases: interference with AKAP signaling in nephrogenic diabetes insipidus

Metabolic diseases are another emerging interesting area of application of interference with AKAPs. This is evidenced, for example, by the finding that, among the various forms of diabetes, nephrogenic diabetes insipidus (NDI) is triggered by abnormal AKAP-dependent cAMP signaling. Patients suffering from this disease produce an excess of hypo-osmotic urine and consequently suffer from polydipsia. The consequent dehydration might lead to hypernatremia, causing neurologic symptoms in severe forms such as neuromuscular excitability, confusion, seizure or coma [178]. NDI is caused by mutations in genes encoding for vasopressin receptor 2 (V2R) and aquaporin 2 (AQP2) [179], key players in the maintenance of water balance, expressed in collecting ducts' cells. The dysregulation of AVP–V2R–AQP2 system results in an abnormal response of the collecting ducts to vasopressin (AVP), the hormone responsible of water conservation, in a context where AVP production and secretion are normally regulated by the hyperosmotic stimuli [178]. In physiologic conditions, V2R is activated by AVP, eliciting a cAMP rise responsible of PKA-mediated phosphorylation of AQP2 on Ser^{256} [180,181]. This results in membrane translocation of AQP2 and subsequent increase in water permeability of renal collecting ducts. Compartmentalized AQP2 phosphorylation is regulated by the binding with AKAP220 in the medullary collecting ducts [182]. AKAP18 is also involved as it localizes in the same vesicles containing AQP2 and PKA [180] but a conclusive proof of its involvement in NDI is still missing.

Although current therapies for NDI aim at elevating cAMP levels independently of V2R, they often result unsuccessful, especially due to a failure in increasing urine osmolarity [179]. In this scenario, AKAPs appear promising targets for NDI treatment in virtue of their ability to regulate AQP2 phosphorylation. In a recent study, Ando and colleagues highlight the potential role of AKAP-PKA disruptors to increase AQP2 trafficking [179]. While Ht31 usually inhibits PKA, in cortical collecting duct (CCD) cells this peptide induces PKA activation and the consequent AQP2 phosphorylation at Ser^{256}, leading to an increase in apical AQP2 expression, thus rising water reabsorption. Nonetheless, the *in vivo* uses of Ht31 are limited by its short half-life and its low cell permeability. Conversely, the peptidomimetic small molecule FMP-API-1 (3,3′-diamino-4,4′-dihydroxydiphenylmethane) carries functional properties comparable with Ht31 with strongly improved cell permeability and stability (Table 2). Like Ht31, FMP-API-1 activates PKA in CCD cells, increasing apical AQP2 expression and eventually rising osmotic water permeability. *In vivo* experiments performed in mouse models of NDI confirm the therapeutic effect of FMP-API-1 and its ability to increase urine concentration at a level equal to that induced by the endogenous activator vasopressin. Whereas the pharmacological properties of this molecule are not yet optimal, further lead optimization looks even more promising. Derivatives of FMP-API-1 like, for example, FMP-API-1/27, enhance Ser^{256} AQP2 phosphorylation in the kidney only, thus providing improved safety, especially considering that it does not target cardiac PKA [179]. Nonetheless, while this molecule is designed to act as a tissue-specific drug, its effect is not AKAP-specific and consequently might disturb various AKAP–PKA interaction in the kidney. Results of preclinical testing are not yet available and whether its lack of selectivity might lead to unwanted side effects is currently unclear.

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Plasmodium falciparum infection

Modulation of cAMP signaling through the interference with AKAP-mediated PPIs has some potential even in unexpected fields such as treatment of parasitic and infectious diseases. One of these areas of intervention is malaria. Only in 2017, malaria caused the death of 435000 people worldwide, with 93% of deaths reported only in the WHO African Region [183]. *Plasmodium falciparum* is the parasite responsible for this endemic disease in which red blood cells are infected and shelter the various stages of the parasite's lifecycle, leading to typical symptoms which might end in life-threatening complications. Despite constant efforts to eradicate this deadly disease, no current treatments have succeeded to overcome this parasite [184]. Remarkably, once infected, red blood cells release ATP that, in turn, increases intracellular cAMP concentration via extracellular receptors, leading to PKA activation, a critical event regulating parasite life cycle and infection [131]. Flaherty and colleagues report that a constrained hydrocarbon-stapled peptide, STAD-2, designed to disrupt the interaction between AKAPs and human PKA-R, selectively targets only red blood cells that are infected by *P. falciparum in vitro* (Table 2). In addition, this peptide localizes inside the *Plasmodium* almost immediately after treatment and rapidly kills the parasite, suggesting this compound as a promising anti-malarial therapy. The specific mechanism of action of the STAD-2 peptide is still unclear, but evidence shows that it does not associate directly with PKA, suggesting a PKA-independent mechanism [185]. Hence, a better understanding of the mechanism of action and a better optimized lead are awaited before further clinical testing.

Human immunodeficiency virus

Several studies show that cAMP elevation and PKA activation are involved in different steps of the disease triggered by the Human Immunodeficiency Virus (HIV), such as cell cycle arrest and enhancement of infectivity in resting T cells [133,186–189]. According to UNAIDS, 38 million of people were living with HIV in 2019, and 690000 patients died from Acquired Immune Deficiency Syndrome (AIDS)-related illness worldwide [190]. After its entrance into T-helper lymphocytes via the interaction with CD4 and CCR5 chemokine co-receptor, HIV integrates into the human genome, where it is transcribed to promote replication and disease spreading [191,192]. In the last phase of the disease, the virus cytopathic action induces a massive loss of T cells, responsible for the immunodeficiency and the consequent decreased lifespan [190].

HIV patients show a drastic increase in intracellular cAMP level in T cells, which acts as a negative regulator of immune function by decreasing T-cell responses and, consequently, acquired immunity [186,193]. PKA inhibits T-cell proliferation of HIV patients and this inhibition is reversed by the use of PKA antagonists [133,194]. RIAD is a highly specific peptide targeting the interaction between AKAPs and PKA-RI, which is the most abundant subunit in T cells [195,196] (Table 2). Singh and colleagues show that this peptide is effective in blocking HIV replication *in vivo* and in stabilizing T-cells levels. RIAD or its peptidomimetic RIAD-P3 decrease cAMP, leading to inhibition of viral replication and stabilization of $CD4^+$ cells *in vivo* in a murine model of AIDS [196]. Although, in this context, the mechanism of action of RIAD is still incompletely understood and its specificity is still disappointing, disrupting PKA/AKAP in T cells appears as a promising new approach for HIV treatment.

Conclusions

In the last decades, researchers have uncovered the crucial role of AKAPs and of compartmentalized cAMP signaling in several physiological processes as well as in disease. These scaffolding proteins are indeed responsible for the correct and specific propagation of the cAMP signal in space and in time through the orchestration of supramolecular complexes, involving PKA, its substrates and other signaling effectors. The number of such effectors that has been discovered is steadily increasing and might include proteins, lipids and even RNAs [7]. The assembly of these complex cAMP signaling hubs is based on PPI that have emerged as key regulators of multiple cellular functions as well as pathogenetic responses. The large body of proof of concept studies outlined here indicate that AKAPs, by orchestrating these PPIs, represent targets for pharmacological intervention. Given that protein surfaces involved in these PPI are usually large and endowed with high chemical complexity, design and identification of small molecule peptidomimetics meant to reproduce these spatial features is a difficult task. On the other hand, synthetic peptides are now emerging as useful tools to effectively, safely and selectively disrupt PPI. Pharmacology of such peptides is still in its infancy, but new approaches are addressing and solving most of the drawbacks associated with stability, delivery and bioavailability. Future studies are awaited to confirm the clinical relevance of manipulating AKAP-associated signaling pathways with PPI disrupting peptides.

Competing Interests

E.H. and A.G. are founders and stakeholders of Kither Biotech, a pharmaceutical product company focused on respiratory medicine not in conflict with statements in this review. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.
The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AC, adenylate cyclase; AD, Alzheimer's disease; AIDS, acquired immune deficiency syndrome; AKAP, A-kinase anchoring protein; AKB, A-kinase binding; AQP2, aquaporin 2; cAMP, 3′–5′-cyclic adenosine monophosphate; CaN, calcineurin; CDD, Cortical collecting duct; Csk, C-Terminal Src Kinase; DA, dopamine; D/D, dimerization/docking; EBP50, Ezrin-radixin-moesin-Binding Phosphoprotein 50; EPAC, exchange protein directly activated by cAMP; FDA, Food and Drug Administration; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; HCC, hepatocellular carcinoma; HF, heart failure; HIV, human immunodeficiency virus; Hsp20, heat-shock protein of 20 kDa; K_V, voltage-gated potassium channnel; LTCC, L-type Ca^{2+} channel; LV, left ventricle; LZ, leucine zipper; mAKAPα, muscle A-kinase anchoring protein α; MAPK, mitogen-activated protein kinase; MEF, Myocyte Enhanced Factor; NAc, nucleus accumbens; NDI, nephrogenic diabetes insipidus; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; NFAT , Nuclear Factor of Activated T cells; PD, Parkinson's disease; PDE, phosphodiesterase; PINK1, PTEN-induced kinase 1; PKA, protein kinase A; PKC, protein kinase C; PLN, phospholamban; PPI, protein–protein interaction; RGC, retinal ganglion cell; RIAD, RI anchoring disruptor; RyR, ryanodine-sensitive Ca^{2+} channel; SERCA2, sarcoplasmic/endoplasmic reticulum calcium pump 2; SR, sarcoplasmic reticulum; St-Ht31, stearate residues to Ht31 peptide; TAC, transverse aortic constriction; TRPV1, transient receptor potential vanilloid receptor subtype 1; VTA, ventral tegmental area; V2R, vasopressin receptor 2; βAR, β-adrenergic receptor.

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Clinical Science (2020) **134** 3259–3282
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MINI REVIEW
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Mechanisms of Anthracycline-Induced Cardiotoxicity: Is Mitochondrial Dysfunction the Answer?

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Cardiac side effects are a major drawback of anticancer therapies, often requiring the use of low and less effective doses or even discontinuation of the drug. Among all the drugs known to cause severe cardiotoxicity are anthracyclines that, though being the oldest chemotherapeutic drugs, are still a mainstay in the treatment of solid and hematological tumors. The recent expansion of the field of Cardio-Oncology, a branch of cardiology dealing with prevention or treatment of heart complications due to cancer treatment, has greatly improved our knowledge of the molecular mechanisms behind anthracycline-induced cardiotoxicity (AIC). Despite excessive generation of reactive oxygen species was originally believed to be the main cause of AIC, recent evidence points to the involvement of a plethora of different mechanisms that, interestingly, mainly converge on deregulation of mitochondrial function. In this review, we will describe how anthracyclines affect cardiac mitochondria and how these organelles contribute to AIC. Furthermore, we will discuss how drugs specifically targeting mitochondrial dysfunction and/or mitochondria-targeted drugs could be therapeutically exploited to treat AIC.

Keywords: mitochondria, anthracycline, reactive oxygen species, mitochondria-targeted drug, cardiotoxicity after chemotherapy

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INTRODUCTION

Advances in cancer therapy resulted in marked improvements in patient survival, with anthracyclines (ANTs) probably being the most potent antineoplastic therapeutics available for the clinical practice, and still representing one of the pillars in the treatment of different tumors. In 2018 more than 3 million people were diagnosed with cancer in Europe only, and it has been estimated that currently 14.5 million people are living with a history of cancer in USA, with this number rising up to 19 million over the next 10 years (1, 2). Notably, 50% of people diagnosed with cancer today will survive at least 10 years after diagnosis, and this proportion is even higher for childhood cancer survivors. However, this improvement in survival of cancer patients has led to a greater recognition of the long-term adverse effects of antineoplastic therapies like ANTs, mostly involving the cardiovascular system. In a cohort of almost 2,000 cancer survivors monitored over 7 years, 33% of deaths were related to cardiovascular conditions while cancer-related mortality accounted for 51% of deceases. Given the concrete possibility of incurring in ANT-induced cardiotoxicity (AIC), and that the number of cancer survivors is constantly increasing, in the upcoming years there will probably be a Cardio-Oncology "epidemic." For this reason, cardiologists, oncologists, and basic scientists are combining their efforts in order to better characterize the molecular mechanisms

behind this pathology (3). In this regard, in recent years the role of mitochondria has strongly emerged, since several compounds exert their cardiotoxic effects targeting these organelles (4, 5). This is due to the fact that mitochondria are particularly important for the heart because of its high demand in energy. Since mitochondria are the organelles dedicated to ATP production, dysfunctional mitochondria are repeatedly replaced by newly synthesized ones with the purpose of sustaining the constant need for ATP, underlying the importance of mitochondria dynamics and mitophagy. Drugs that impair the proper activity of mitochondria likely cause a substantial decrease in ATP levels that, eventually, leads to myocardial dysfunction (6). For this reason, drugs preserving mitochondrial function and metabolism are receiving increasing attention in order to treat or prevent cardiotoxicity induced by several drugs, including ANTs. In this review, we will describe the crucial role in AIC of mitochondria, organelles of fundamental importance for the heart, and we will discuss about specific treatments targeting their function and metabolism.

AIC: FROM DEFINITION TO CURRENT TREATMENT

ANTs, such as doxorubicin (DOX), daunorubicin and epirubicin, are antibiotic agents highly effective as anticancer therapeutics, and for this reason they have been registered by the World Health Organization as essential medicines (7). However, it was noticed early on that their use is associated to the development of heart failure (HF) (8, 9). Already in the seventies, Von Hoff et al. analyzed retrospectively more than 4,000 DOX-treated subjects and found that the overall incidence of congestive HF caused by the treatment was 2.2%. Notably, the number of patients affected by AIC in this study is probably underestimated since it was based only on clinician-identified signs and symptoms of congestive HF. Moreover, it was already clear that the probability of incurring in AIC is strictly dependent on the total dose administered and that the use of smaller, divided doses of DOX decreases the likelihood of developing cardiotoxicity, while there is a sharp increase in the prevalence of HF occurring at increasing doses of the drug (10). Importantly, anthracyclines are rarely administered as single agents and are more often combined with radiotherapy or modern targeted therapies, like monoclonal antibodies, which importantly exacerbate toxicity (11).

AIC can manifest acutely, early after infusion, strongly compromising cancer treatment since it may require dose modification or even cessation of anticancer therapies (12). Almost 30% of patients are affected by this type of cardiotoxicity, that is characterized by electrocardiogram abnormalities, including atypical ST changes, reduced QRS voltage, tachycardia, and supraventricular premature beats. Yet, acute AIC is a rare complication and the most prevailing and significant form of AIC is the chronic one. It is characterized by left ventricular systolic dysfunction, with a reduction in left ventricular ejection fraction (LVEF), which can be very insidious since it is asymptomatic in the early stages. It can eventually progress to dilated cardiomyopathy and congestive heart failure (CHF), which is nowadays one of the main co-morbidity in childhood cancer survivors (11, 13, 14). These patients have a 12-fold increased chance of developing congestive heart failure (CHF) up to 30 years after treatment, with an occurrence of AIC up to 30% (15–17). Of notice, some cancer patients already have pre-existing cardiovascular diseases or at least cardiovascular risk factors that strongly increase the likelihood of developing cardiac issues, and specifically AIC, in these individuals.

The assessment of AIC primarily relies on evaluation of clinical symptoms and/or detection of systolic function (LVEF) by echocardiography, acquisition scans, and magnetic resonance imaging (18). In particular, cardiotoxicity is currently diagnosed when a decline of 5–55% in LVEF with HF symptoms, or an asymptomatic decline of 10 to below 55%, is observed. Nevertheless, recent studies highlight the limitations of these ejection fraction-based screenings, proposing new diagnostic strategies. In particular, strain rate imaging and troponin (Tn) leakage in the peripheral blood could be used to identify patients with early clinical signs of cardiotoxicity (19–21). From a therapeutic point of view, unfortunately there is no specific treatment targeting AIC. Efforts are being made to develop strategies to prevent AIC that, depending on their mechanism of action, are classified as primary, when focused on preventing the disease concomitantly with ANT treatment, and secondary, when prompted to prevent symptomatic progression (22). For now though all the secondary preventive strategies have limited follow up, also because of the difficulties related to monitoring cardiotoxicity in both adults and children (22). Some clinical trials have shown modest success with the usage of the standard pharmacological regimen for HF. Notably, it has been reported that the non-selective β adrenergic receptor (βAR) blocker, carvedilol, can prevent DOX-induced left ventricular dysfunction through its antioxidant properties, and can ameliorate cardiac function and survival in cancer patients under ANT therapy (23–25). More recently, it was demonstrated that early treatments with the angiotensin converting enzyme I (ACE-I) enalapril, either alone or in combination with carvedilol, are able to fully or partially recover LVEF in 82% of patients manifesting signs of cardiotoxicity within the first year after the end of ANT treatment (13). Unfortunately, these regimens are far from optimal for AIC treatment, and this is probably due to the fact that the mechanisms involved in this specific type of cardiomyopathy are different to those underlying other types of cardiac disease, like ischemic, post-infectious, and idiopathic dilated cardiomyopathies (22). This underlies the need for more specific therapeutics, and so, of a better understanding of the molecular mechanisms behind this condition.

MITOCHONDRIA: KEY PLAYERS IN AIC

If the molecular processes behind the anticancer effects of ANTs are well-known and studied, the mechanisms underlying their cardiotoxic effects are still poorly understood and controversial. It is well-established that ANTs exert their anticancer action by directly targeting and inhibiting topoisomerase 2 (Top2) in cancer cells, more specifically the 2α isoform, halting DNA transcription, and replication (26). However, the same mechanism can hardly explain the toxic effect of ANTs

on the heart, since cardiomyocytes are for definition non-dividing cells, thus leaving an open question for cardio-oncology researchers (27, 28). Recent evidence suggests that DOX cardiotoxicity is causally linked to inhibition of a Top2 isoform which is preferentially expressed by differentiated cells, like cardiomyocytes, namely Top2β, the only Top2 expressed in mitochondria (27, 29). Moreover, a number of other mechanisms of AIC, which are not necessarily linked to Top2β inhibition, have started to emerge. Interestingly, both pathways have been reported to impact on the activity of mitochondria. In the next paragraphs, we will describe Top2β-dependent (or direct) and Top2β-independent (indirect) mechanisms of DOX cardiotoxicity and how these signaling pathways converge on the dysregulation of mitochondrial activity and metabolism in cardiomyocytes.

"Direct" Mechanisms of AIC Involving Mitochondria

As mentioned above, the cellular targets of DOX are topoisomerases, more specifically of the Top2 class (30). DOX can bind both DNA and Top2 in order to form the ternary Top2-DOX-DNA cleavage complex which triggers cell death. As mentioned before, besides inhibiting Top2α in proliferating cells, ANTs can target Top2β, which is also the only known type 2 topoisomerase present in cardiac mitochondria [**Figure 1**; (27)]. In their study, Zhang et al. demonstrated that DOX treatment induces significant changes in the expression of genes controlling both mitochondrial structure and metabolism (oxidative phosphorylation pathways) in cardiomyocytes expressing Top2β ($Top2\beta^{+/+}$), but not in Top2β knockout mice ($Top2\beta^{\Delta/\Delta}$) (29). More specifically, among the genes downregulated after DOX treatment in $Top2\beta^{+/+}$, and not significantly affected in $Top2\beta^{\Delta/\Delta}$ cardiomyocytes, are Ndufa3 (encoding the NADH dehydrogenase 1-α subcomplex 3), Sdha (encoding succinate dehydrogenase complex II, subunit A), and Atp5a1 (encoding the ATP synthase subunit α). In agreement, mitochondria fail to maintain their membrane potential in DOX-treated $Top2\beta^{+/+}$ but not in $Top2\beta^{\Delta/\Delta}$ cardiomyocytes (29). In addition to modulation of genes involved in mitochondrial function and metabolism, DOX was also shown to decrease the transcription of *Ppargc1a* and *Ppargc1b*. These two genes encode for PGC-1α and PGC-1β, respectively, that by interacting with crucial transcription factors, namely NRF-1, NRF-2, and ERRα, push the expression of genes implicated in mitochondrial biogenesis (29). In keeping with their preserved mitochondrial function, cardiomyocyte-specific Top2β knockout mice are protected from DOX-induced progressive HF. Indeed, after 5 weeks of DOX treatment, $Top2\beta^{+/+}$ mice show a decrease in ejection fraction up to 50%, whereas this parameter is not altered in $Top2\beta^{\Delta/\Delta}$ mice. Zhang et al. also demonstrated that reactive oxygen species (ROS) production is reduced by 70% in the hearts of $Top2\beta^{\Delta/\Delta}$ as compared to $Top2\beta^{+/+}$ mice (29). Of note, the finding that Top2β silencing only partially reduces ROS production in cardiomyocytes treated with ANTs suggests that ROS may be generated in response to DOX by additional Top2β-independent mechanisms that will be discussed in the next paragraph.

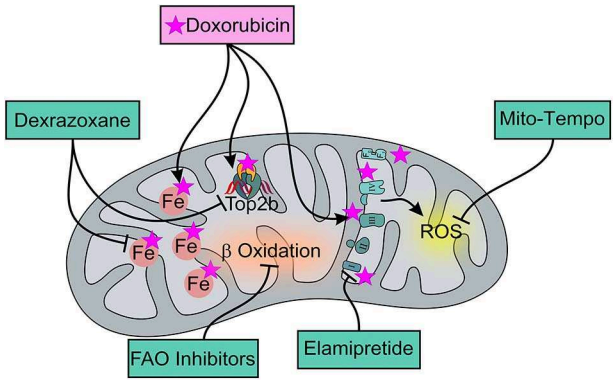

FIGURE 1 | Effects of DOX and of mitochondria-targeted drugs on mitochondrial function and metabolism. DOX preferentially accumulates within mitochondria thanks to its ability to specifically bind to the phospholipid cardiolipin, causing membrane perturbation and ETC disruption that can be limited by Elamipretide, a tetrapeptide that improves the efficiency of electron transport and restores cellular bioenergetics. ETC dysfunction mainly induces ROS production that can be though limited by the usage of the mitochondria-targeted antioxidant, Mito-Tempo, a specific scavenger of mitochondrial superoxide. Moreover, DOX can directly interact with iron to form reactive ANT-iron complexes resulting in an iron cycling between Fe^{3+} and Fe^{2+} which is associated with ROS production and altered iron homeostasis. Dexrazoxane, as an iron-chelator, can inhibit the production of ROS ensuing from the interaction between ANT and non-heme iron, ultimately alleviating DOX-induced mitochondrial oxidative stress. Moreover, Dexrazoxane can prevent DOX from binding to the Top 2β-DNA complex. For AIC treatment, FAO inhibitors can also be used for their ability to enhance glucose oxidation and prevent a decrease in intracellular ATP levels, thereby ensuring the proper maintenance of cellular homeostasis.

"Indirect" Mechanisms of AIC Involving Mitochondria

Since the initial discovery of ANT cardiotoxicity, the generation of excessive ROS has represented the most widely accepted mechanistic explanation. Even if in cardiomyocytes ROS can be produced, at least in part, as a consequence of ANT-mediated Top2β inhibition (see previous paragraph for further detail), several "indirect" or Top2β-independent mechanisms significantly contribute to ROS production and mitochondrial dysfunction. In the next paragraph, we will describe mechanisms of AIC which are unrelated to Top2β inhibition and that culminate in alterations of mitochondrial function and metabolism.

Mitochondrial ROS Production and Metabolism Dysregulation

Recent evidence suggests that ANTs, in particular DOX, preferentially accumulate in the mitochondria of cardiomyocytes, strongly impacting on both the structure and the activity of these organelles. Indeed, DOX can directly bind to the abundant phospholipid cardiolipin, located in the inner mitochondrial membrane (31, 32). This interaction hampers the electron transport chain (ETC), since it inhibits complex I and II, leading to ROS production (**Figure 1**). More specifically, a quinone moiety in the C ring of DOX can accept electrons for NADH or NADPH and is thus reduced by the respiratory

chain complex I, generating a reactive semiquinone free radical (33, 34). On one hand, this mechanism decreases the electron flow through the ETC, removing electrons normally used for ATP production; on the other hand, the reduced semiquinone can transfer the electron to O_2, generating the superoxide anion O_2^-. DOX can be generated back by this process, in a reaction known as the "redox cycling," and can be reduced again if NADH is present, producing O_2^- continuously. O_2^- can be transformed into the low-toxic hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) or into other ROS (35, 36). ANT-mediated production of these reactive species in turn can activate different pathways leading to cardiomyocytes death, including apoptosis and necrosis. Intriguingly, DOX-induced cardiomyopathy has been recently linked to another form of regulated cell death, the less characterized iron-dependent cell death, also named ferroptosis, which is driven by iron-dependent lipid peroxidation. Indeed, ANTs produce ROS also because they can chelate free iron, leading to the formation of reactive iron-DOX complexes that can interact with O_2 [**Figure 1**; (37)]. Moreover, it has been shown that DOX can upregulate heme oxygenase 1, the enzyme responsible for heme degradation, and releases free iron in cardiomyocytes, leading to oxidation of lipids of the mitochondrial membrane and to a further release of free iron in cardiomyocytes, thus feeding this vicious cycle of ROS production (37). In addition, Ichikawa et al. showed that DOX specifically triggers iron accumulation in the mitochondria of isolated cardiomyocytes, without altering total cellular iron levels. Intriguingly, this preferential accumulation is also found in the hearts of DOX-treated patients. Mechanistically, the increase in mitochondrial iron levels upon ANT administration is mediated by the downregulation of the ATP-binding cassette subfamily B member 8 (ABCB8), a transporter protein mediating mitochondrial iron export. ABCB8 overexpression protects mice from DOX-induced oxidative stress and cardiomyopathy and preserves mitochondrial structure and cardiomyocyte viability. Conversely, in the absence of ABCB8, DOX-induced ROS production and mitochondrial damage are increased compared to controls, underlying the cardio-protective role of this transporter (37, 38). Notably, other aspects of mitochondrial metabolism and energy production can be disrupted by ANTs. It has been demonstrated that β-oxidation, the main process used by the healthy heart to generate energy, is inhibited upon DOX treatment through the down-modulation of carnitine palmitoyltransferase 1 (CPT-1), while glycolysis is increased by 50% within few hours as a compensatory response. However, this metabolic adaptation is reversed with time, with a strong decrease in glucose oxidation that has been demonstrated both *in vitro* and *in vivo*. This may be due to the reduction of glucose supply after the induction phase or because of the poor availability of one of the key enzymes of the process, namely phosphofructokinase (PFK) (39).

Calcium Homeostasis Dysregulation

The metabolic changes induced by DOX, and the consequent reduction in ATP levels, are known to negatively impact myocardial contractility, which may be exacerbated by an impairment of myocardial Ca^{2+} signaling. It is known that DOX affects Ca^{2+} homeostasis and signaling via several mechanisms, also involving ROS. On one hand, the lipid peroxidation elicited by DOX-mediated ROS production can alter the activity of membrane-residing proteins, such as mitochondrial calcium channels (40, 41). In addition, ANTs can impair the expression and activity of key players of myocardial contraction, namely the cardiac ryanodine receptor (RyR2) and the sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA2) (42). In physiological conditions, the action potential mediating contraction is detected by L-type Ca^{2+} channels that activate RyR2, which are responsible for Ca^{2+} release from the sarcoplasmic reticulum (SR). This latter increase in cytoplasmic Ca^{2+} level triggers muscle contraction. Ca^{2+} levels are eventually restored to basal via the activation of SERCA2, mediating the reuptake of Ca^{2+} into the SR (42). DOX and its main metabolite, doxorubicinol (doxOL), are known to activate and increase the open probability of RyR2, though this effect is acute and detectable only right after administration of the drug and at low concentrations (42). Instead, doxOL was found to oxidize RyR2 thiols and this irreversible modification causes a significant inhibition of the channel. Interestingly, it has been shown that SERCA2 can be inhibited via the same oxidation process, which leads to a dramatic increase in cytoplasmic Ca^{2+} levels (42). In addition, this process is exacerbated by the fact that ANTs can negatively affect the transcription of the channel (42). More importantly, DOX is able to activate Calcium/Calmodulin-dependent protein kinase-II (CaMKII), which alters mitochondrial Ca^{2+} homeostasis and promotes apoptosis. CaMKII increases Ca^{2+} influx in mitochondria through mitochondrial calcium Ca^{2+} uniporter (MCU) via activation of the nuclear factor-kappa B (NF-kB) and p53. This, in turn, leads to the opening of the permeability transition pore (MTP) at lower levels of Ca^{2+} compared to normal conditions, resulting in dissipation of the mitochondrial membrane potential and in increased permeability to apoptotic factors (43, 44). Moreover, ANT-mediated ATP depletion (as described in the previous paragraph) also reduces the mitochondrial membrane potential and causes MTP opening, further dysregulating Ca^{2+} homeostasis (45).

Autophagy and Mitochondrial Dynamism Impairment

Among all mammalian cells, cardiomyocytes emerge for having the highest mitochondrial density and also the greatest respiratory capacity. This might be the reason why preserving the homeostasis of these organelles is a physiological imperative for the heart. In agreement, mitochondria damaged by DOX have to be promptly removed to maintain a healthy heart. Unfortunately, ANTs are known to disrupt the major degradative/recycling process of mitochondria, namely autophagy (46, 47). Several studies found that acute administration of high-dose ANTs can induce the accumulation of both LC3 and p62, the major autophagy markers, with a reduction in ATP levels in mouse hearts, and a significant suppression of oxygen consumption rate (OCR) in their mitochondria (46). Further analysis from Li et al. demonstrated that DOX blocks cardiomyocytes autophagic flux mediating a strong accumulation of undegraded autolysosomes. This is due to defects in lysosomal acidification caused by

DOX-mediated suppression of the activity of V-ATPase, the proton pump that generates and maintains pH gradients in this organelle (48). Furthermore, ANTs inhibit the phosphorylation of one of the positive regulators of autophagy initiation, AMPK, suggesting that ANTs dampen autophagy not only by impairing the autophagic flux but also by inhibiting its initiation. Starvation prior to ANT treatment restores AMPK signaling and autophagy, ultimately protecting the heart against cardiac dysfunction (49). Another mechanism by which DOX impairs autophagy involves the PI3Kγ pathway. Li et al. recently showed that DOX activates a PI3Kγ/Akt/mTOR cascade which ultimately converges on autophagy inhibition, while genetic or pharmacological inhibition of PI3Kγ restores the autophagic flux and protects mice against AIC (50).

Along with impaired autophagy, AIC is characterized by defective mitochondrial dynamics, which refers to organelle fusion, fission, and mitophagy, a specific autophagic mechanism targeting mitochondria. The mitochondrial fusion proteins, mitofusin1 and 2 (Mfn1 and Mfn2), and optic atrophy 1 (Opa1), as well as the mitochondrial fission protein, dynamin related protein (Drp)1, are highly expressed in the mammalian heart, wherein their genetic ablation causes dramatic cardiac dysfunction. Mfn2 levels are decreased in cardiomyocytes after treatment with DOX and this event is associated with increased mitochondrial fission, leading to mitochondrial fragmentation, mitophagy, decreased antioxidative capacity, and ultimately cell death. Accordingly, increased expression of Mfn2 in cardiomyocytes, or the use of the mitochondria-targeted antioxidant Mito-Tempo, a specific scavenger of mitochondrial superoxide, attenuate DOX-induced mitochondrial fission and prevent cardiomyocyte mitochondrial ROS production and apoptosis (51). Mito-Tempo though is not the only known compound to counteract AIC. Several others are now being investigated and will be extensively described in the following paragraphs.

TARGETING MITOCHONDRIA AND THEIR METABOLISM FOR THE TREATMENT OF AIC

In-depth study of the intertwined molecular mechanisms underlying ANT-induced mitochondrial toxicity has recently paved the way to the development of approaches potentially useful to treat AIC. However, targeting AIC in the clinical setting is still challenging, since a major requirement for these medications is that they do not interfere with the antitumor activity of ANTs. Below we will describe the most promising therapeutics for AIC, with a major focus on those targeting either ROS and their production, or mitochondrial metabolism.

Dexrazoxane

Dexrazoxane is not only one of the most studied cardioprotective adjuvant for DOX chemotherapy, but it is also the only Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved drug for AIC prevention (12, 52). Thanks to its ability to act as an iron-chelator, dexrazoxane inhibits the production of ROS ensuing from the interaction between ANTs and non-heme iron, ultimately alleviating DOX-induced mitochondrial oxidative stress [**Figure 1**; (53, 54)]. However, the concept that dexrazoxane promotes cardioprotection only by virtue of its antioxidant properties is debated, especially in view of the finding that other antioxidant drugs, such as vitamin A, vitamin E, and N-acetylcysteine, failed to provide benefits in the treatment of AIC (55–57). An additional mechanism that may account for the cardioprotective action of dexrazoxane is its ability to prevent DOX from binding to the Top2β-DNA complex. X-ray crystal structure analyses revealed that dexrazoxane can bind to the two ATP binding sites at the N terminus of Top2 and bridges two Top2 monomers in the closed-clamp configuration [**Figure 1**; (58)]. Moreover, it has also been demonstrated that dexrazoxane forms a tight complex with the ATPase domain of human Top2α and Top2β, suggesting that this compound prevents ANT from binding to Top2 (59). In addition, dexrazoxane has been shown to interact with Poly(ADP-ribose) (PAR) monomers, acting as a PAR Poly(ADP-ribose) polymerase (PARP) inhibitor (60). In agreement, inhibition of this enzyme improves cardiac function and decreases mortality without altering the anticancer activity of DOX in several animal models of DOX-induced cardiomyopathy (61). Consistent with its mechanisms of action, dexrazoxane is exploited to prevent rather than treat AIC and its use appears to be most appropriate in patients with stage A of HF, i.e., at high risk of developing the pathology. However, Ganatra et al. demonstrated that dexrazoxane exerts its cardioprotective function also in stage B HF (62). In a small cohort of patients showing pre-existing asymptomatic, systolic left ventricular (LV) dysfunction, the administration of dexrazoxane 30 min before each ANT dose was enough to allow patients to complete their planned chemotherapy, with a minimal decrease in LVEF and no elevation in HF biomarkers. On the contrary, the three patients that did not receive dexrazoxane had a marked reduction in heart function and developed HF. Of note, two of them died from cardiogenic shock and multi-organ failure (62).

Concerning the clinical efficacy of dexrazoxane, it has been shown in multiple trials that it can reduce the incidence of CHF and LVEF decline in patients treated with ANTs (63–65). These findings were also corroborated by a more recent study in which Marty et al. found that, based on both LVEF and CHF results, 164 relapsed breast cancer patients treated with dexrazoxane have significantly lower overall cardiac events in comparison with the control group treated with DOX or epirubicin only (66). Similarly, dexrazoxane has been shown to abrogate DOX-mediated mitochondrial dysfunction in childhood cancer survivors. Lipshultz et al. found that, in peripheral blood mononuclear cells (PBMCs), DOX-damaged mitochondria expand their mtDNA, which encodes for 13 polypeptides involved in oxidative phosphorylation, as an attempt to compensate for the injury and improve mitochondrial metabolism (67). Treatment with dexrazoxane, together with DOX, reduces the number of mtDNA copies per cell compared to the group treated with DOX only, suggesting preserved mitochondrial function in patients receiving the combination

therapy (67). Intriguingly, besides proving the efficacy of dexrazoxane in counteracting AIC-related mitochondrial dysfunction, this study also suggests that mitochondrial injury, and the ensuing increase of mtDNA in peripheral blood, might represent a biomarker for early detection of cardiotoxicity, which still represents an unmet clinical need.

Despite evident clinical benefits, in 2011 EMA contraindicated the usage of dexrazoxane in children since its efficacy in this subpopulation was not assessed. In addition, it was proposed that dexrazoxane could not only attenuate the anticancer effects of ANTs and increase the risk of secondary malignancies, but could also cause myelotoxicity (64–66). Nevertheless, this view has been recently refuted by a number of studies (68). A phase-III clinical trial, involving more than 500 children and adolescents affected by T-cell acute lymphoblastic leukemia (ALL) or lymphoblastic non-Hodgkin lymphoma, was conducted to investigate not only the cardio-protective effects of dexrazoxane but also its safety as well as its potential impact on the antineoplastic efficacy of ANTs (69). In addition, Lipshultz et al. found that dexrazoxane attenuates DOX-induced cardiac injury in children with acute lymphoblastic leukemia, without compromising its antileukemic efficacy (70). It was also reported that dexrazoxane alone does not increase the risk of second primary malignancies (SPMs), which are instead related to the usage of three Top2 inhibitors used in combination (doxorubicin, etoposide, and dexrazoxane) and mostly etoposide (71). For these reasons, EMA has approved the administration of dexrazoxane to children supposed to be given more than 300 mg/m^2 of ANTs (12, 52, 68).

Mito-Tempo

The novel drug named mitochondrial-targeted Tempo l (Mito-Tempo) is a well-known superoxide dismutase (SOD) mimetic. Mitochondria are the only organelles having a unique type of superoxide dismutase, the manganese-containing SOD2, which is crucial for protecting against excessive production of O_2^-, a key feature of AIC (**Figure 1**). Mice that do not express this protein develop a severe cardiomyopathy already at 10 days after birth, while mice missing one allele of SOD2 (SOD2$^{+/-}$ mice) develop hypertension with time and if challenged with an high-salt diet, suggesting a role for this enzyme in cardiac protection (72). Mito-Tempo consists of the tempol moiety bound to a triphenylphosphonium cation that allows the molecule to enter mitochondria, and this is the reason why this molecule may be highly effective in organs, such as the heart, which are rich in these organelles. Mimicking the activity of SOD, Mito-Tempo acts as an antioxidant drug in rats, and in mice it has also been shown to alleviate oxidative stress and cardiac toxicity induced by DOX (73, 74). Indeed, already in the 90's, it was demonstrated that Mito-Tempo significantly reduces the contractile impairment as well as the lipid peroxidation observed in rat heart treated acutely with DOX (75). In all these *in vivo* studies, Mito-Tempo was used in combination with ANTs in patients with no pre-existing heart disease, suggesting that it might be exploited to prevent AIC likely in patients in stage A HF. In addition, in a guinea pig model of non-ischemic HF, Mito-Tempo reversed the pathological phenotype, suggesting that this compound can also have a therapeutic effect in patients in later stages of ANT-induced HF (76). More recently, Mito-Tempo was used in combination with dexrazoxane and this combinatorial treatment ameliorates DOX-induced cardiomyopathy without altering the antitumor activity of DOX (77).

Elamipretide

Elamipretide is one of the first drugs developed to target selectively the mitochondrial ETC in order to improve the efficiency of electron transport and restore cellular bioenergetics [**Figure 1**; (78)]. More than one mechanism of action has been proposed for this tetrapeptide. It penetrates cell membranes, localizing to the inner mitochondrial membrane where it can interact with the phospholipid cardiolipin. Cardiolipin has a crucial role in maintaining the functional positioning of the ETC complexes and supercomplexes within the inner mitochondrial membrane, allowing for efficient electron transfer down the redox chain, minimizing reactive oxygen species production. This binding between cardiolipin and the tetrapeptide prevents peroxidation of the phospholipid, thereby maintaining membrane fluidity and supercomplex formation and enhancing electron transport chain function, ultimately increasing ATP synthesis and reducing mitochondrial ROS (79–82). Several studies conducted in rats showed that elamipretide can significantly improve myocardial mitochondrial ATP content, reduce myocardial infarct size and improve cardiac function (83–85). Moreover, treatment with elamipretide improves left ventricular function in animals with HF (84). Saba et al. also demonstrated a significant improvement in ejection fraction in dogs with HF treated with elamipretide for 3 months (86). In addition, this compound can ameliorate left ventricular relaxation via restoration of cardiac myosin binding protein-C (84, 86, 87). A clinical trial of elamipretide in patients with heart failure with reduced ejection fraction (HFrEF) has also been conducted to evaluate safety, efficacy, and tolerability of the compound. Daubert et al. reported that no subjects suffered any serious adverse events, and only one stopped the treatment after a single administration. Moreover, all patients had stable hemodynamic parameters of blood pressure and heart function, suggesting that elamipretide is well-tolerated also together with current standard HF medications. Most notably, patients treated with elamipretide showed a significant reduction in left ventricular volumes in comparison with placebo, despite the small sample size of the trial (88). Of course, larger studies are required to determine its safety as well as its efficacy in patients with HF, but up to now elamipretide seems to be an optimal therapeutic option for targeting mitochondrial dysfunction in the future. On note, elamipretide has not yet been tested in a specific model of AIC but all these studies suggest that this molecule can both ameliorate and prevent different aspects of mitochondrial dysfunction, leading to envisage its use in patients at different stages of the disease. Unfortunately, there is still no evidence that this drug does not alter the antineoplastic activity of ANTs, which might be a possibility because of its known ability to inhibit apoptosis (84). Further studies are needed to prove the possibility of using this molecule in Cardio-Oncology.

Autophagy-Targeting Drugs

Until now, no compounds targeting autophagy have been used in clinical trials to prevent AIC or any cardiac disease. Targeting autophagy in AIC, as well as in any disease context, is still controversial, since this process is critical to the maintenance of cellular homeostasis and it has to be finely tuned, with any perturbation being either beneficial or detrimental (47, 89). Some attempts to modulate this process have been reported in animal models and have shown promising starting results, suggesting that inhibiting this process can be protective and that can be used in the future in patients in stage A of AIC. Sciarretta et al. also demonstrated that the autophagy activator trehalose can protect from myocardial infarction-induced cardiac remodeling, suggesting the possible use of this molecule as a therapeutic agent for HF (90). Sishi et al. showed that rapamycin, a known potent activator of autophagy, is able to improve the negative effects mediated by DOX treatment when administered in combination with the anticancer therapy, leading to a decrease in ROS production, and enhanced mitochondrial function (91). Pharmacological inhibition of PI3Kγ phenocopies mTOR blockade and restores the autophagic flux, ultimately preventing AIC (50). However, boosting the autophagic process can negatively impact on the efficacy of cancer treatments since it may make the tumor resistant to chemotherapy. In agreement, autophagy inhibitors, instead of activators, have been tested in oncology so far. Several trials have been carried out inhibiting autophagy with hydroxychloroquine (HCQ), the only clinically-approved autophagy inhibitor (92), raising some concerns about the possible future usage of autophagy-activators for curing AIC.

Inhibitors of Mitochondrial Fatty Acid Beta Oxidation

Members of this category are Trimetazidine, Ranolazine, and Perhexiline and their use results in the reduction of myocardial fatty acid (FA) uptake and oxidation (**Figure 1**). In pathological conditions, such as HF, cardiac fatty acid and glucose metabolism are altered and contribute to impaired heart efficiency and function. More specifically, there is an increase in the amount of fatty acids that are oxidized by cardiac mitochondria (93–95). Since FA oxidation (FAO) consumes more energy in comparison with glucose oxidation, requiring 10% more oxygen for a given amount of ATP that is produced, an increase in the amount of FA oxidized by the mitochondria can potentially reduce cardiac efficiency and impair heart function (96). Therefore, FAO inhibitors might represent promising drugs for treating AIC in patients at more advanced stages of the disease, such as B and C, since they lead to an enhanced glucose oxidation and prevent a decrease in intracellular ATP levels, thereby ensuring the proper functioning of ionic pumps and maintenance of cellular homeostasis (97–99). Nevertheless, early and sustained inhibition of CPT-1, the crucial and limiting enzyme of FAO, was shown to prevent LV dysfunction and remodeling, as well as efficiently slowing down the development and progression of the disease, in a dog model of HF, suggesting the possible usage of FAO inhibitors also in stage A HF (100). Of note, these compounds could also provide the opportunity to target cancerous cells as well, since they depend on FAO for several aspects such as proliferation, survival and drug resistance (101).

Trimetazidine is an antiischemic agent able to specifically inhibit the long-chain mitochondrial 3-ketoacyl coenzyme A thiolase enzyme that can help cardiomyocytes to maintain proper energy metabolism. No clinical trial has been conducted using this drug for the treatment of AIC, or more generally HF, but its safety and tolerability have been proven through its use in acute coronary syndrome (102). Several studies demonstrated that trimetazidine is effective in improving LVEF, decreasing the rate of hospitalization and reducing brain natriuretic peptide (BNP) levels in subjects with HF (103–106). Moreover, it can also improve cardiac function and reduce HF symptoms when administered together with metoprolol, a βAR blocker.

Ranolazine, if used at high concentrations, is a partial inhibitor of fatty acid beta-oxidation (107). Its main mechanism of action is indeed related to its capability to inhibit late inward sodium channels. In failing myocytes, these channels are hyperactivated, leading to calcium overload and in turn contractile dysfunction and increased oxygen consumption (108). Ranolazine is approved for the treatment of chronic angina, but there is evidence suggesting its clinical effect also for HF treatment (109). Up to now, it has been demonstrated that ranolazine mediates diastolic benefits, by restoring myocyte relaxation, reducing resting tension as well as left ventricular end diastolic pressure in animal studies conducted in dogs (110, 111). Further improvements have also been reported when this drug is used in combination with βAR blockers (112). Concerning clinical trials, a small sample size study has been conducted in HF patients with preserved ejection fraction, revealing that ranolazine can provide improvement in hemodynamics, but no evidence was provided of improvement in relaxation parameters (113).

Perhexiline is another drug acting on metabolism that was originally thought as an antianginal medication and its usage was declined for several side effects, including hepatotoxicity and neurotoxicity (114). More recently, its toxicity has been found to be preventable with individualized dosing, but its clinical use remains difficult. Its activity as a fatty acid beta-oxidation inhibitor was demonstrated on rat hearts that showed a reduction of fatty acid utilization of 35%, with a concurrent increase in cardiac output of 80 mL/min/g. More specifically, it was demonstrated that perhexiline can inhibit CPT-1, known to control access of long chain fatty acids to the mitochondrial site of beta-oxidation (115). Concerning its clinical use for HF treatment, a small sample size clinical trial has been performed, particularly focused on studying its effect on oxygen consumption. A clear improvement in peak oxygen consumption was found following perhexiline treatment compared to no change in patients treated with a placebo, and improved ejection fraction was also observed, suggesting its possible and effective future employment also for AIC (116).

BEYOND CARDIOMYOCYTES

An important aspect to consider from a therapeutic perspective is that, although the majority of the studies in the field of Cardio-Oncology have focused their attention on the effects of ANTs on cardiomyocytes, these are not the unique cellular population found in the heart. The emerging view is that anticancer compounds also target cardiac fibroblasts and endothelial cells. It has been shown that, both *in vitro* and *in vivo,* DOX affects the differentiation of fibroblasts into myofibroblasts which in turn produce a huge amount of extracellular matrix components, leading to cardiac fibrosis. This process is driven by DOX-dependent ROS that activate TGF-β, the main responsible for fibroblast differentiation (117, 118). Moreover, DOX also modulates the activity of ATM, a kinase which is activated in response to DNA damage induced by oxidative stress. Interestingly, this activation occurs only in cardiac fibroblasts and not in cardiomyocytes, suggesting that this may be a cell-type specific mechanism contributing to AIC (119). How ANTs affect mitochondria in fibroblasts is still unexplored and requires additional work. Instead, more information is available on the role of these organelles in cardiac endothelial cells. Apart from increasing cell permeability and leading to edema formation, DOX can also reduce ATP levels and, in turn, mitochondrial function in these cells (120). Moreover, by means of its interaction with the nitric oxide (NO) synthase, DOX can also interfere with NO production that is essential for endothelial homeostasis (121). However, further studies are needed to further explore the role of these other cardiac cell populations in AIC, hopefully paving the way to the development of new therapeutic options.

FUTURE PERSPECTIVES

Besides the urgent need for new effective therapeutic approaches, another still unresolved issue in the field of Cardio-Oncology is how to predict who is likely to develop cardiotoxicity. Anthracycline dose, patient's age and pre-existent cardiovascular disease only partially explain the interindividual susceptibility to AIC and the prevailing hypothesis is that the sensitivity to anthracyclines has a genetic basis (122). Unveiling the genetic variants that contribute to AIC is of upmost importance since it may give the clinicians the opportunity to identify patients at risk prior the treatment, and potentially modify the therapeutic regimens by using alternative drugs or cardioprotective agents. Early candidate gene association studies (CGAS) and genome-wide association studies (GWAS) have started to reveal the first genes, that are primarily related to drug metabolism and transport, iron metabolism, DNA repair, oxidative stress, and calcium homeostasis, with no genes being directly linked to mitochondrial function regulation (123, 124). However, given the small sample sizes of these studies, additional work is warranted to conclusively validate these variants and to discover new genes implicated in AIC susceptibility. In this scenario, human-induced pluripotent stem cells (hiPSCs) represent an emerging powerful tool since they can be obtained non-invasively from blood samples, can be renewed *in vitro* and are genetically identical to the patients from whom they are derived making them the ideal experimental model for pharmacogenomics research. By exploiting hiPSCs, Knowles et al. recently discovered a number of new genetic variants which also include some genes involved in mitochondrial function regulation (125). In addition, being able to faithfully recapitulate *in vitro* the inter-individual susceptibility to AIC (126), hiPSCs offer the unique opportunity to verify *in vitro*, before the drug is administered to the patient, that the treatment does not cause toxicity, paving the way toward a personalized medicine approach in the field of Cardio-Oncology (123).

CONCLUSIONS

It is now well accepted that mitochondrial dysfunction underlies a broad spectrum of pathologies, ranging from cancer to neurodegenerative and cardiovascular disease. It is not surprising that mitochondria play a key role also in the pathogenesis of AIC, considering the ability of ANTs to bind a phospholipid of the inner mitochondrial membrane, cardiolipin, and thus to accumulate within mitochondria. A number of drugs specifically targeting mitochondrial pathways which are deregulated in pathology as well as a new class of mitochondria-targeted compounds have been developed. While most of them have already been tested in preclinical models of HF, little is still known about their therapeutic potential in the treatment of AIC. Further studies in the appropriate preclinical murine and human models of AIC are awaited to fill this gap.

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Conflict of Interest: AG and EH are co-founders and board members of Kither Biotech, a startup biotech focused on the development of PI3K inhibitors.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW ARTICLE

Inhaled Biologicals for the Treatment of Cystic Fibrosis

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Abstract: ***Background***: Cystic Fibrosis (CF), one of the most frequent genetic diseases, is characterized by the production of viscous mucus in several organs. In the lungs, mucus clogs the airways and traps bacteria, leading to recurrent/resistant infections and lung damage. For cystic fibrosis patients, respiratory failure is still lethal in early adulthood since available treatments display incomplete efficacy.

Objective: The objective of this review is to extend the current knowledge in the field of available treatments for cystic fibrosis. A special focus has been given to inhaled peptide-based drugs.

Methods: The current review is based on recent and/or relevant literature and patents already available in various scientific databases, which include PubMed, PubMed Central, Patentscope and Science Direct. The information obtained through these diverse databases is compiled, critically interpreted and presented in the current study. An in-depth but not systematic approach to the specific research question has been adopted.

Results: Recently, peptides have been proposed as possible pharmacologic agents for the treatment of respiratory diseases. Of note, peptides are suitable to be administered by inhalation to maximize efficacy and reduce systemic side effects. Moreover, innovative delivery carriers have been developed for drug administration through inhalation, allowing not only protection against proteolysis, but also a prolonged and controlled release.

Conclusion: Here, we summarize newly patented peptides that have been developed in the last few years and advanced technologies for inhaled drug delivery to treat cystic fibrosis.

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1. INTRODUCTION

Cystic Fibrosis (CF) is one of the most common among rare diseases, affecting over 75000 patients worldwide. CF is caused by congenital mutations disrupting the function of the CF Transmembrane Conductance Regulator (CFTR) channel [1, 2]. More than 2000 mutations in the *CFTR* gene have been identified to date. Among these, the loss of the amino acid phenylalanine (F) at the 508th position (F508-del mutation) accounts for approximately 70% of disease-related mutations in CF patients. The mutated F508-del CFTR channel is not properly folded, displaying minimal membrane levels and gating in CF epithelial cells. This defect, in turn, results in the production of abnormally viscous mucus, sweat and digestive fluids, which obstruct airways, intestine, pancreas and liver.

Improvements in the available therapies contributed to such an extension in life expectancy that the current view is to no more consider CF a pediatric disease, but rather a pathology of the young adult (some patients reaching 50 years of age). However, respiratory failure is still lethal in early adulthood, due to limited availability and incomplete efficacy of current treatments [3]. Indeed, although most CF patients have disease manifestations in multiple organs, respiratory failure remains the leading cause of morbidity and mortality, due to irreversible airways obstruction, recurrent and chronic infections, and progressive loss in pulmonary function.

In this review, we summarize the most up to date and relevant peptide-based approaches for CF therapy via the inhalation route. Unbiased search for "peptide" and "Cystic Fibrosis" keywords in Patentscope database retrieved patented inventions spanning from those improving mucus clearance, to those targeting the primary defect in CFTR function (Fig. **1**). Some remarkable insights on carriers and available medical devices for inhaled delivery, as retrieved by PubMed investigation, are also provided. An in-depth

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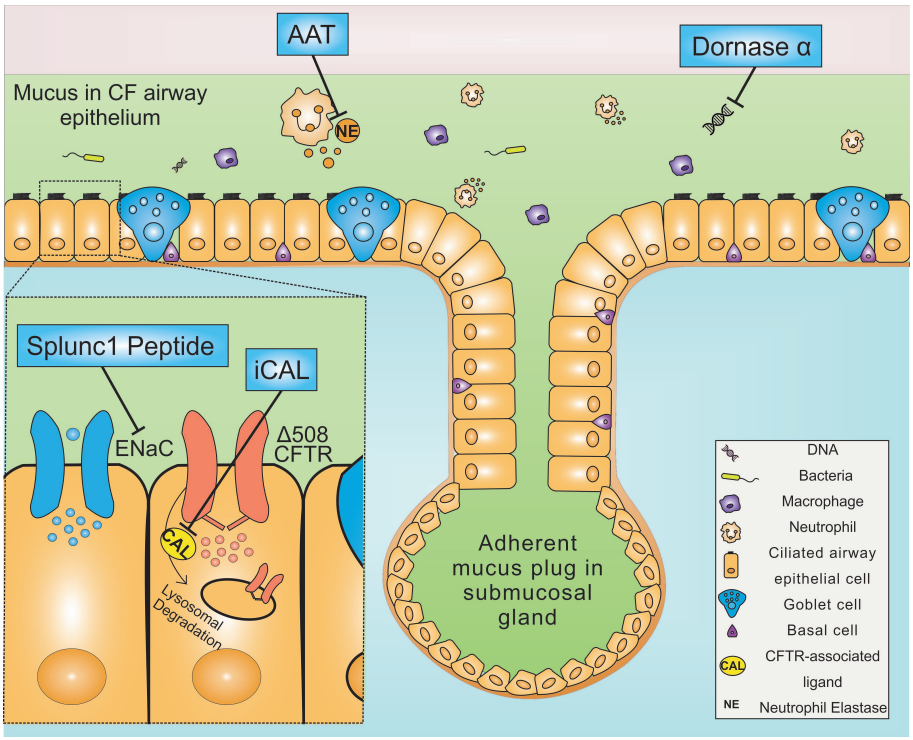

Fig. (1). In Cystic fibrosis, depletion of the airway surface liquid is due to the absence of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-mediated fluid secretion, accompanied by increased absorption via ENaC epithelial sodium channel. The dehydration of the liquid layer associated with a mutant CFTR contributes to mucus stasis, and eventually to plugging in the submucosal gland or in proximity of the originating goblet cells. These events lead to the onset of a proinflammatory airway environment that promotes proliferation of bacteria and attracts inflammatory cells, including neutrophils and macrophages. Neutrophils, in turn, release proteases, which further enhance mucus viscosity and ultimately lead to lung damage.

albeit not systematic approach has been used to retrieve salient information that is considered useful for the reader. A critical perspective has been used to comment on available data and provide suggestions for future research.

2. CURRENT TREATMENTS OF CYSTIC FIBROSIS

Current treatments for CF combine several agents which delay either pulmonary (mucolytics, bronchodilators, antibiotics, corticosteroids, chest physiotherapy, airway clearance, and exercise) or gastroenteric dysfunctions (pancreatic enzyme replacement, fat-soluble vitamin replacement and high-caloric density diets). As a consequence, adherence to such complex and time-consuming therapies is often incomplete [4, 5].

Moreover, most of the available therapies achieve only a symptomatic relief and lack a mechanistic rationale, possibly due to the gaps in the comprehension of CFTR complex biology. Accordingly, only three medicinal products targeting the molecular defects were developed, so far, by Vertex Pharma, by means of high throughput screening and massive money investments. These molecules, which aim at restoring normal salt/water transport across epithelia, have been approved in the EU, and are marketed as Kalydeco® (which is effective in a minor subset of patients), Orkambi®, and the recently developed Tezacaftor [6, 7].

Orkambi® has been recently approved for the treatment of CF patients carrying the genetic F508del mutation in homozygosity, occurring in around 40% of the cases. Notably, Orkambi® is the combination of a CFTR corrector and a potentiator. However, its clinical efficacy is unsatisfactory: treatment shows only limited improvement in lung function, not all the patients respond to therapy, and drug costs are elevated. Despite the recent approval of these new compounds restoring CFTR function, the quality of life and life expectancy of CF patients remain poor, and the current mean age of death is around 30 years [3].

However, clinical results with Orkambi® indicate that the abnormal CFTR is *per se* a druggable target. This prompted the development of new drugs for oral CF treatment, including the Tezacaftor/Ivacaftor combined therapy, which is now in Phase III (NCT03150719), for patients carrying the homozygous F508-del *CFTR* gene who discontinued treatment with Orkambi®. Unfortunately, the cost of such regimens is elevated, possibly leading to failure of reimbursement agreements. Thus, effective and affordable drugs for CF therapy are still an unmet need.

3. RECENT DEVELOPMENTS ON PEPTIDE-BASED INHALED DRUGS

Given their attractive properties, such as high specificity, excellent safety and tolerability, several peptides have been proposed as therapeutic agents for respiratory diseases. Some of these are currently under development for CF systemic therapy, including the Vasoactive Intestinal Peptide (VIP) and an amino acid fragment of the CB subunit of crotoxin from Crotalus durrissus terrificus venom. VIP is indeed able to rescue F508-del CFTR trafficking to the apical cell mem-

brane and restore protein function [8] while CB directly interacts with F508-del CFTR NBD1 domain and behaves as both potentiator and corrector [9]. Moreover, antimicrobial peptides have been considered for the treatment of CF-related infections, including the tetra-branched M33 peptide (and its derivatives) [10-12] and Esculentin-1a-Derived Peptides, currently under evaluation for their activity against *Pseudomonas aeruginosa* lung infections [13].

Interestingly, the antibiotic Tobramycin, which was initially delivered via the parenteral route, was lately administered as an inhaled medication that was recently optimized by the development of Tobramycin Inhalation Powder (TOBI Podhaler®) [14]. Therefore, we cannot exclude that peptides that have been initially tested for systemic and/or topical delivery might be implemented for inhaled delivery.

3.1. Peptides Reducing Mucus Viscosity

3.1.1. ENaC Inhibition

In CF, accumulation of de-hydrated mucus in the airways is a major pathogenic process. The balance of Cl^- secretion and Na^+ absorption across the airway is critical for the regulation of Airway Surface Liquid (ASL) volume [15, 16]. Indeed, airway epithelia can both secrete chloride (Cl^-) through the CFTR and absorb sodium (Na^+) through the Epithelial Sodium Channel (ENaC), the apical channel responsible for Na^+ absorption across a wide range of epithelia, including renal, gastrointestinal, and airway [17]. Therefore, modulation of ENaC by peptide-based drugs emerged as a promising therapeutic approach to maintain ASL in respiratory diseases [18].

In CF, the absence of CFTR results in ENaC hyperactivity, leading to uncontrolled absorption of Na^+ and depletion of ASL volume [19], further resulting in lack of chloride and bicarbonate transport. The consequent defect in mucus clearance promotes the formation of biofilms colonized by pathogens, like *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenza*. Notably, when submerged in biofilms, bacteria are less targetable by drugs, thus promoting the development of antibiotic resistance.

Short palate lung and nasal epithelium clone 1 (Splunc1) is a ~25kDa protein which acts as a volume sensor of ASL, and contains an ENaC inhibitory domain, the so-called S18 region [20-24]. Unlike conventional ion channel antagonists blocking the pore, Splunc1 inhibits ENaC by inducing allosteric modulation and endocytosis [25-28]. Since Splunc1 failed to regulate ENaC in CF lungs due to protease degradation, Spyryx Biosciences is currently developing SPX-10®, a Splunc1-derived peptide**,** which functions in CF airways as an ENaC inhibitor [29-33]. The restored ASL hydration is predicted to improve mucociliary clearance and decrease infection/inflammation in CF lungs [34, 35]. Inhaled SPX-101® already concluded a Phase I study in CF patients (NCT03056989), and the HOPE-1 randomized, double-blind, placebo-controlled Phase II study for efficacy and safety is ongoing (NCT03229252).

Interestingly, S18-derived peptides are intrinsically disordered thanks to their peculiar amino acidic sequence and display a high degree of flexibility in their polypeptide chain, which lacks a stable 3D structure. As a consequence, they are heat-stable and can achieve a high contact area and binding efficiency with their target proteins [36]. In addition, S18-derived peptides are protease resistant, do not freely cross the respiratory epithelium barrier, and do not reach kidneys, thus avoiding the undesired induction of hyperkalemia caused by small molecule ENaC antagonists like amiloride [37, 38]. For many peptide-based drugs, the inability to cross the respiratory epithelium and reach the bloodstream after inhaled delivery may be an advantage, as it would result in low, if not absent, systemic bioavailability, thus reducing both on-target and off-target effects in other organs. For example, in the case of CF, inhaled antibiotics achieve higher concentrations with fewer side effects than orally delivered antibiotics [39]. Chronic inhalation of peptides could eventually induce a local immunogenic response, but, given that Splunc1-derived peptides are naturally occurring, at least in normal lungs, immunogenicity is unlikely for SPX-101® [22].

3.1.2. Neutrophil Elastase (NE) Inhibition

Airway recruitment of neutrophils is another major hallmark of CF lung disease and is responsible for tissue remodeling and, ultimately, destruction as a consequence of the excessive release of Neutrophil Elastases (NEs). The activity and level of NEs are closely related to the ongoing inflammation, and NE levels are elevated in the Bronchoalveolar Lavage (BAL) fluid of CF patients [40]. Besides inducing lung damage, NEs cleave and activate ENaC, further reducing mucus hydration and clearance [41-44]. A number of NE inhibitors have been described [45]; among these, hypersulfated disaccharides were disclosed for the treatment of CF.

Alpha-1-Antitrypsin (AAT), also known as Alpha-1-Proteinase Inhibitor (API), is an endogenous NE inhibitor, which is predicted to improve pulmonary function [46, 47]. In CF, AAT levels are normal, but the burden of NEs is so large that it overwhelms AAT protective effect. To date, clinical grade AAT is obtained by extraction and purification from human plasma, a procedure that has been patented about three decades ago [48]. Five patents recently addressed the production of AAT (years 2013 to 2015) [49-53], as summarized in [54].

Intravenous infusion of AAT purified from human plasma is indicated for the chronic therapy of adults affected by severe hereditary API deficiency [55]. However, the need for prolonged and repeated intravenous access, as recommended for AAT deficiency-associated lung diseases, as well as for chronic diseases like CF, implicates an increased risk of thrombosis and infections [41]. To overcome these major issues, an inhaled biological therapy based on human AAT has been developed, and completed a phase I trial in CF patients (NCT01347190). Three phase II trials were also completed (NCT01684410 and NCT00486837 sponsored by Grifols Therapeutics; NCT00499837 sponsored by Kamada) and one was terminated (NCT02010411). Overall, inhaled AAT has been shown to be safe and well tolerated, albeit its clinical efficacy is still debated [41, 56-59].

However, a potential limitation of AAT is that several other proteases, including cathepsins and metalloproteases, are upregulated in CF and may contribute to lung damage, without being blocked by AAT [60].

3.1.3. DNA Clearance

CF airways are characterized by high levels of DNA and actin in the lung lumen, which are released by necrotic neutrophils [61-63]. Extracellular DNA accumulation, in turn, adversely alters mucus rheology, leading to decreased mucociliary clearance.

The first patented invention concerning the ability of DNase1 to cleave extracellular DNA, and thus decrease mucus viscosity, in CF lungs has been described in the nineties [64, 65]. Dornase alfa, or rhDNAse, is a recombinant version of human DNase1 protein used as a therapeutic for CF [66]. Pulmozyme® is a FDA/EMA approved recombinant version of human DNase1 marketed by Genentech for the treatment of CF (Phase IV trial completed in 2013, NCT01712334), where it has been shown to reduce the incidence of infections [67]. Pulmozyme® is administered via nebulization by means of different nebulizers, including Hudson T Up-draft II® and Marquest Acorn II® with Pulmo-Aide®, PARI LC® Plus or PARI BABY™ with PARI PRONEB®, Durable Sidestream® with Mobilaire™ or Porta-Neb®, and the eRapid™ Nebulizer System.

rhDNAse is nowadays considered a gold-standard drug. Notably, it was the first peptide-based drug showing the safety and efficacy requirements needed to be successfully developed and marketed as an inhaled therapeutic for CF.

3.2. Peptides Targeting the Primary CFTR Defect

3.2.1. NEG2

The pioneer of peptide-based drugs for CF is a short peptide derived from the R domain of CFTR (amino acids 817-838), named NEG2 peptide, which was shown to play a critical role in channel activity regulation. Exogenous NEG2 peptide derivatives were shown to interact with CFTR, exerting both stimulatory and inhibitory effects on the function of the channel. Unfortunately, despite leading to a remarkable progress in the knowledge of CFTR biology, NEG2-derived peptides were not developed further for CF therapy [68, 69].

3.2.2. Thymosin Alpha 1

Thymosin Alpha 1 (Tα1) is a naturally occurring polypeptide of 28 amino acids. Tα1 is well known for its immunoregulatory properties [70], and has been suggested as an anti-inflammatory agent and a CFTR modulator in CF [71].

Tα1 composition may be adapted for oral, parenteral, topical and inhaled administration [72]. Of note, the ability of Tα1 to correct/potentiate the F508-del CFTR mutant has been reported [71]. However, this finding has been questioned by a recent publication showing that Tα1 does not correct F508-del CFTR in CF airway epithelia, raising concerns on the future development of Tα1 as a CFTR modulator [73], but rather as an anti-inflammatory agent.

3.2.3. iCAL

In CF patients, F508-del CFTR is improperly folded, resulting in increased Endoplasmic Reticulum (ER) degradation and decreased Golgi trafficking. Moreover, the residual CFTR is removed from the apical membrane and degraded in lysosomes due to pathological interactions with CFTR-Associated Ligand (CAL), a CFTR-interacting PDZ domain protein that associates predominantly with Golgi [74]. Recently, CAL Competitive Inhibitor (iCAL) peptides have been shown to selectively bind to CAL PDZ domain, preventing the interaction with CFTR [75]. iCALs selectively inhibit CAL, but not NHERF, increasing F508-del CFTR at the membrane, resulting in increased Cl^- conductance in the bronchial epithelium of CF patients [75, 76].

Unfortunately, these isolated sequences are not suitable as drugs, due to their inability to penetrate cell membranes and reach their intracellular CAL target. CT007 is an optimized iCAL drug compound that represents a breakthrough for the selective stabilization of F508-del CFTR in CF and that acts by maintaining functional CFTR at the cell surface. CT007 shows additivity or even synergy in combination therapy with VX-809. The compound displays cell permeability, solubility, targeted intracellular release, enhanced *in vivo* stability, low immunogenicity and physical-chemical properties suitable for the formulation into an inhaled dry powder or solution form that allows topical dosing and immediate uptake in airway epithelial cells [77, 78].

3.3. Nanocarriers

Albeit promising in terms of efficacy, peptides might be difficult to handle, because of their instability in body fluids due to the action of proteases, and during storage or delivery due to interactions with surfaces, air/water interface, presence of proteolytic enzymes, and sensitivity to ultrasounds and shear stress. Accordingly, some protein-based therapies failed during clinical translation, being labile and prone to proteolysis in the blood, compared to conventional small molecules [79]. Combining peptides with nanocarriers was therefore explored as a strategy to provide both protection against proteolysis and prolonged/controlled release [80, 81].

Several types of nanocarriers have been developed, including liposomes, which are able to carry active substances both inside and in/on their surface, as well as chitosan-modified liposomes [82, 83]. Thanks to this successful strategy, a number of nebulizable liposomal formulations have reached clinical trials, including Arikace® (liposomal amikacin) and Pulmaquin® (liposomal ciprofloxacin) antibacterial formulations, which are currently in advanced stages of development.

Alternative to liposomes, PLGA, alginate-chitosan hybrid, and hyaluronan particles have been designed to prolong residence in the lung milieu [79, 80]. PLGA nanoparticles can be used, for example, as carriers of triplex molecules that bind or hybridize to a target sequence in the human *CFTR* gene [84]. However, peptides that either target pathogens or epithelial cells need to penetrate the biofilm, a matrix composed of polysaccharides and DNA that contribute to increased mucus viscosity. Interestingly, when tested, the efficiency of compounds conjugated to liposomes or PLGA particles was enhanced in most cases, compared with the corresponding free drugs [81, 85].

3.4. Inhaled Drug Delivery

The preferred route of administration of drugs targeting respiratory dysfunctions in CF is ideally inhalatory. Notably,

inhalation is not only preferable for the diminished side effects, compared to oral and/or systemic delivery, but is also convenient considering the large surface area and high vascularization of the lungs [82, 83].

On the other side, to be suitable for inhalation therapy, drug particles should meet specific requirements, including adequate ADME features (pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion) and an appropriate respirable size, namely 1-5μm, which depends also on the performance of the device used for delivery [4].

To this aim, new formulations were engineered to improve lung targeting and dose consistency, so that could be adapted to the high number of delivery systems that are now available, including portable inhalers and nebulizers [86].

In 1956, Riker Laboratories developed the first pressurized Metered Dose Inhaler (MDI) [87]. The introduction of the pMDI denoted the beginning of modern aerosol industry, which now provides also Dry-Powder (DPI) and Soft-Mist Inhalers (SMI), ultrasonic and jet nebulizers, vibrating-mesh as well as smart devices [88].

pMDIs, SMIs and DPIs can deliver relatively small quantities of medication to the lungs, when compared to medical nebulizers. As an example, pMDI and DPI usually deliver small drug amounts to the airways, so they require repetitive delivery to provide effective doses [89]. An exception might be the preparation in the form of pulmospheres [86], which allows loading of drug particles up to 95% in weight [90] and guarantees low interpatient variability, enabling treatment of patients of any age, even in the presence of severe airflow obstruction [91]. Moreover, use of pMDI and DPI inhalers can be challenging, potentially leading to improper handling that can significantly reduce drug delivery to the lungs and therapeutic effectiveness [92].

Ultrasonic nebulizers are not suitable for nebulization of proteins such as AAT, because the molecule is destroyed by frictional forces. On the contrary, jet nebulizers, such as the Pari Boy® S with the LC Plus® nebulizer (Pari), are suitable, although able to deliver only up to 40% of the aerosol to the lungs [93]. Vibrating-mesh devices, such as the MicroAir NE-U22V® nebulizer (Omrom Healthcare), the AeroNeb® OnQ aerosol generator (Inspiration Medical), and the eFlow® (Pari), conveniently generate aerosols by perforated meshes actuated by piezo-electric vibrational crystal or ceramic elements.

Even at high outputs, the delivery rates to the lungs can be further improved by timing drug delivery to the inspiratory phase of the respiration cycle [94, 95]. To this aim, a smart card has been designed to optimize aerosol pulse to the patient's pulmonary function. Currently, two smart devices equipped with such technology are available: The I-neb, a small stand-alone nebulizer, and the Akita®, hooked to a Pari jet or eFlow nebulizer [94, 96]. However, devices equipped with such technology are still very expensive.

Unfortunately, the shearing provided during nebulization of aerosol droplets may induce physical stress on liposome bilayers and peptides themselves, causing a loss in therapeutic efficacy. Hence, formulation composition, nebulizer design and an adequate training program of patients should be optimized, with the aim of i) reducing the detrimental effect of shearing on the drug, and ii) maximizing deposition in the lower airways [97, 98].

CONCLUSION

Peptides are versatile therapeutic candidates for the systemic and inhaled treatment of several diseases. Accordingly, during the past few years, peptide-based therapeutics have been developed for the treatment of CF.

Evidence discussed above highlights the emerging peptide-based drugs as promising, albeit challenging, therapeutic agents for the inhalatory treatment of CF patients.

CURRENT & FUTURE DEVELOPMENTS

An emblematic case recapitulating the challenges in the development of peptide-drugs for inhaled delivery is that of insulin [99]. Massive efforts in developing inhaled insulin formulations were pushed on one side by the global burden of diabetes worldwide, and, on the other side, by the need to overcome the distress of multiple daily injections. This new formulation was expected to be successful, especially when Exubera® was approved by the FDA/EMA and released to the market in 2006/2007.

A number of other inhaled insulin formulations were in late stage of clinical development and expected to reach the market. However, when Pfizer withdrew Exubera® from the market due to limited commercial success, inhaled insulin was removed from several pipelines, despite positive clinical results and high acceptance from patients. MannKind was the only one to progress with Afrezza® inhaled insulin. Sanofi brought this product to the market in 2014, but returned it to MannKind shortly afterward, because of poor sales. As learned from the insulin story, a successful development of peptide-based drugs for inhaled delivery might be challenging even in the case of a non-rare disease like diabetes.

Of note, CF-related diabetes is a major co-morbidity of CF, affecting over half of middle-aged CF patients [100]. However, inhaled delivery for CF therapy, *per se*, has an additional, intrinsic challenge: given the thick mucus layer and the chronic inflammation in the lungs, it is likely that peptide-based drugs, like insulin and others, do not efficiently reach the target cells. Nevertheless, peptides can survive sufficiently long in the mucus layer to diffuse and reach their targets. In addition, reduction of the mucus burden with different physiotherapeutic techniques prior to the administration of the compound can further help to overcome the potential problem of delivery through the mucus.

Future trials in CF should be therefore grounded on such concepts and take in serious consideration the need of optimization in the delivery of peptidic drugs in a potentially adverse environment [85].

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REVIEW ARTICLE

PI3K Signaling in Chronic Obstructive Pulmonary Disease: Mechanisms, Targets, and Therapy

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Abstract: Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disorder characterized by irreversible chronic inflammation and airflow obstruction. It affects more than 64 million patients worldwide and it is predicted to become the third cause of death in the industrialized world by 2030. Currently available therapies are not able to block disease progression and to reduce mortality, underlying the need for a better understanding of COPD pathophysiological mechanisms to identify new molecular therapeutic targets. Recent studies demonstrated that phosphoinositide 3-kinase (PI3K) signaling is prominently activated in COPD and correlates with an increased susceptibility of patients to lung infections. PI3Ks have thus emerged as promising alternative drug targets for COPD and a wide array of pan-isoform and isoform-selective inhibitors have been tested in preclinical models and are currently being evaluated in clinical studies. Here, we summarize the recent knowledge on the involvement of PI3K enzymes in the pathophysiology of COPD, and we will discuss the most recent results arising from the preclinical as well as the clinical testing of PI3K inhibitors as novel therapeutics for COPD.

Keywords: COPD, PI3K, inflammation, targeted therapy.

1. INTRODUCTION

The rise in lifespan worldwide is accompanied by an increase in the prevalence of chronic diseases. Among these, Chronic Obstructive Pulmonary Disease (COPD) is one of the most prevalent and dangerous conditions for the health status of elderly patients, being one of the main causes of comorbidity and death [1]. The prevalence of COPD in individuals older than 65 years of age was recently estimated to be 14.2%, twice the number evidenced in individuals between 40 and 60 years of age. In general, COPD is predicted to become the third cause of death in the industrialized world by 2030. Bronchodilators, alone or in combination with corticosteroids, are milestones in the treatment of COPD, but novel treatments are urgently needed, as the current therapy does not block disease progression, nor reduces mortality [2].

Therefore, a better understanding of the underlying pathophysiological mechanisms is necessary to identify new molecular targets and develop new drugs.

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2. COPD: FROM DEFINITION TO CURRENT TREATMENT

COPD is a chronic respiratory disease characterized by irreversible chronic inflammation in the lung, leading to airflow obstruction, acute recurrences, exacerbations and progressive lung destruction and ultimately, the decline in lung function. The development of COPD is closely related to smoke, but it is also induced by air pollution, occupational exposure, indoor air contamination and infections, all conditions that produce an exaggerated inflammatory response in people with genetic susceptibility. Based on clinical and radiological characteristics, COPD can be classified into different subtypes which, however, share the same pathophysiology [3]. Clinically, patients may have similar characteristics (such as age, body mass index and smoke exposure), but they may display a different number of exacerbations and/or grade of dyspnea, and this phenomenon is likely explained by a difference in the genetic background [4].

Environmental stimuli trigger leukocyte infiltration of mucosa, submucosa and glandular tissue, and ultimately results in mucus hypersecretion, remodeling and narrowing of the small airways, with obstruction or

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destruction of the lung parenchyma, and consequent loss of the alveolar attachment, known as emphysema [5, 6]. Radiological tests, such as chest X-ray and high resolution computed tomography, allow to differentially diagnose bronchitis, characterized by small airway involvement, and emphysema, characterized mainly by alveolar breakdown.

COPD patients present respiratory symptoms, such as dyspnea, cough, and sputum production, which can all be considered a consequence of inflammation and permanent damage of the airways, leading to air trapping and mucus hypersecretion [7]. The first instrumental examination to confirm a suspected diagnosis of COPD is spirometry, which evaluates air trapping as a reduction in forced expiratory volume in 1 second (FEV1). This parameter is also used to classify four different stages of the pathology on the basis of the obstruction grade, according to the Global Initiative on Obstruction Lung Disease (GOLD) [5].

Irrespective of disease severity, smoking cessation and no exposure to risk factors are recommended, while the prescribed pharmacological therapy varies depending on the GOLD stage. Current pharmacological treatment of COPD is based on bronchodilators and anti-inflammatory agents [8]. Bronchodilators include short- and long-acting agonists of β2-adrenergic receptors (SABAs, LABAs), long-acting muscarinic antagonists (LAMAs), and methylxanthines. SABAs produce prominent airway smooth muscle relaxation but could precipitate cardiac rhythm disturbance in susceptible patients. Methylxanthines are used in combination therapy when symptoms are not controlled yet. Anti-inflammatory agents include inhaled corticosteroids (ICS), oral glucocorticoids and phosphodiesterase-4 (PDE-4) inhibitors. ICS improve lung function and reduce exacerbations, ameliorating the health status of COPD patients and are relatively well-tolerated [8]. However, in COPD ICS are rarely used alone, rather they are employed in association with LABA to obtain a better control of the disease. A recent meta-analysis [9]reported a greater efficacy of LABA/LAMA combination compared to LABA alone, or LABA in association with ICS, in term of improvement in FEV1, dyspnea and exacerbation rate. However, all these therapies are merely symptomatic and fail to modify the natural course of the disease and to improve the quality of life of patients.

3. THE ROLE OF INFLAMMATION IN COPD PATHOPHYSIOLOGY

COPD is characterized by chronic inflammation of lower airways and lung parenchyma, which increases further during acute exacerbations [10]. Several stimuli, primarily cigarette smoke and environmental factors, combined with genetic susceptibility, trigger the production of cytokines and activate cells of both innate and adaptive immunity [3].

The central event of COPD pathophysiology is infiltration of neutrophils and macrophages to the site of inflammation, with mast cells also being implicated. Macrophages and neutrophils are importantly augmented in the bronchoalveolar lavage (BAL) of smoking COPD patients, and mast cells primarily accumulate in the lung parenchyma [11]. Of note, macrophages from COPD patients exhibit peculiar features that may account for the existence of different COPD phenotypes. In the lung of smoking COPD patients, there is a 5- to 10-fold higher number of macrophages than in smoking non-COPD individuals, because COPD macrophages are more responsive to inflammatory mediators. Moreover, macrophages from COPD patients release reactive oxygen species (ROS) and elastolytic enzymes with high activity, although they have a defective phagocytic activity. These macrophages not only have different characteristics in terms of immune response, but they are also more resistant to corticosteroids. This is probably due to downregulation of histone deacetylase (HDAC) 2 activity, which critically controls pro-inflammatory gene expression [3, 12]. In addition, smoking may stimulate the release of hematopoietic growth factors, such as GM-CSF, by lung macrophages, eventually leading to neutrophil release from bone marrow and migration to the airways. Neutrophilic infiltration, another key feature of COPD, is driven by upregulation of E-selectin on endothelial cells. This process eventually results in a massive release of neutrophil proteases which, ultimately, are implicated in alveolar destruction and mucus hypersecretion. Of note, also mast cells, which play a critical role in the pathogenesis of allergic disorders, may be involved in COPD development and progression [13, 14]. Mast cell numbers are increased in inflammatory infiltrates of COPD patients and correlate with reduced lung function, airway remodeling and emphysema15. Exposure to environmental challenges results in mast cell activation, degranulation and cytokine release. The ensuing coordinated release of proinflammatory mediators eventually leads to pathologic chronic inflammation [16]. For instance, TNF-α and IL-8 contribute to neutrophilic inflammation, together with mouse mast cell protease-6 (mMCP-6), which is known to promote neutrophil and macrophage chemotaxis as well as chemokine expression [17, 18]. Furthermore, it has been shown that high levels of mast cell–derived

hTryptase are associated with more severe airflow obstruction [19, 20]. Another study demonstrates that macrophages and tryptase-expressing mast cells are required for the development of COPD, with mMCP-6 contributing to macrophage accumulation in the airways and with hTryptase-β inducing macrophages to increase their expression of pro-inflammatory cytokines and chemokines [21].

In addition to innate immunity, COPD patients display a predominant infiltration of CD8+ T lymphocytes, which are typically activated during bacterial infections, and which produce enzymes, like perforins and granzyme B, that are implicated in cytolysis and alveolar destruction. In addition, some studies demonstrate that, in COPD lungs, B cell counts are increased. Similarly, there is an elevation of the number and the size of B cell-rich lymphoid follicles, which is known to be linked to the development of emphysema [22, 23]. In agreement, mediators promoting B cell maturation, activation and survival are upregulated in the lungs of COPD patients [24]. Similarly, B cell products, such as autoantibodies directed against lung cells, cells components or extracellular matrix proteins, are found in COPD lungs [25]. These autoantibodies, which likely originate from increased oxidative stress, may further contribute to lung inflammation and injury in COPD patients. By forming immune complexes, these antibodies may, in turn, activate complement components and contribute to the development of emphysema [22, 26]. Smoke and oxidative stress may also induce a kind of autoimmune response, through the production of damaged proteins. In particular, it has been demonstrated that oxidative stress induces the formation of carbonylated proteins which activate the immune system and the production of autoantibodies [3].

Finally, airway inflammation may involve not only leukocytes, but also structural cells, such as epithelial cells, endothelial cells and fibroblasts. Besides activating inflammatory cells, smoke and other noxious agents activate epithelial cells, which release inflammatory mediators such as cytokines (TNF-α, IL-1β, IL-6), chemokines (CXCL1 - 8), growth factors, lipids and free radicals. While in physiological conditions epithelial cells produce antioxidant agents, antiproteases and defensins against noxious agents, in COPD patients this defense system is impaired, probably in irreversible manner. In addition, in these patients, the levels of some growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), which are normally implicated in the maintenance of lung integrity, are reduced [3].

4. PHOSPHOINOSITIDE 3-KINASES (PI3KS)

Among the key signaling cascades involved in the control of inflammation, and of other key pathogenic events of COPD, is the phosphoinositide 3-kinase (PI3K) pathway. In the last decade, the availability of transgenic mouse models and of small molecule inhibitors allowed the validation of these enzymes as new and promising drug targets for the treatment of chronic inflammatory disorders, including airway diseases [27, 28].

PI3Ks are lipid and protein kinases involved in intracellular signal transduction, mainly functioning by phosphorylating phosphoinositides on the D3 position of the inositol ring. Historically, PI3Ks have been divided into three classes (class I, II and III) based on the structure of the catalytic subunits, the type of the adaptor subunit bound, and the preferred lipid substrate [29]. Among them, class I PI3Ks are the most studied and phosphorylate phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) to produce a key intracellular second messenger, namely phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3 or PIP3). Less characterized are instead class II and III, which are responsible for the generation of other two major signals: phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2) and phosphatidylinositol 3-phosphate (PtdIns3P or PI3P).

Class I PI3Ks can be further subdivided into class IA and class IB, depending on the adaptor associated to the catalytic subunit, and the type of upstream, membrane activating receptor. Class IA PI3Ks, including PI3Kα, β, and δ, are heterodimers composed by a p110 catalytic subunit (p110α, p110β and p110δ) and an adaptive subunit of the p85 family. This adaptor stabilizes and localizes the heterodimer at the membrane, where phosphatidylinositol substrates are accessible, upon activation by receptor tyrosine kinases (RTKs), such as cytokine and growth factor receptors. In response to ligand stimulation, the SH2 domain of the p85 adaptor binds to phosphorylated tyrosine residues, in turn recruiting and activating the p110 catalytic subunit. On the other hand, PI3Kγ is the only member of class IB PI3K. It is composed by a p110γ catalytic subunit and either a p101 or p84/87 adaptor, and it has long been considered to be triggered by G protein-coupled receptor (GPCR) agonists exclusively [30]. However, recent studies show that PI3Kβ can also be activated by GPCRs [31, 32,]whereas PI3Kγ can be engaged by RTKs in a Ras/p87-dependent way, thus introducing a new model of class I PI3K activation [33].

Following engagement by RTKs or GPCRs, class I PI3Ks produce PIP3 which accumulates at the plasma membrane. In turn, PIP3 serves as a docking site for proteins carrying lipid-binding domains, primarily the pleckstrin homology (PH) domain, such as phosphoinositide-dependent kinase-1 (PDK1), mechanistic target of rapamycin complex 2 (mTORC2) subunit mSin1 and protein kinase B (Akt/PKB). Besides their well-known lipid kinase activities, PI3Ks can also function as protein kinases that directly phosphorylate protein substrates [34,] although studies exploring this aspect of PI3K biology are limited.

Class II PI3Ks, including PI3K-C2α, PI3K-C2β and PI3K-C2γ, and the only member of class III PI3K, Vps34, are all less characterized monomers. Based on their ability to produce PtdIns(3,4)P2 and PI3P, they emerge as key regulators of vesicular trafficking, but no evidence suggests an implication of these isoforms in airway physiology and pathology. Thus, in the next paragraphs, we will focus on the role of class I PI3Ks in chronic airway diseases.

5. PI3K SIGNALING IN COPD

Class I PI3K isoforms display different expression patterns, implying distinct and non-redundant roles. While PI3Kα and PI3Kβ are ubiquitously expressed, PI3Kδ and PI3Kγ have a more restricted, although not exclusive, expression in leukocytes. Accordingly, murine models with PI3Kα or PI3Kβ deficiency show embryonic lethality, whereas PI3Kγ or PI3Kδ knockout mice are viable and fertile [35].

The development of isoform-selective small molecule inhibitors, and the generation of transgenic mice depleted of either PI3Kγ or PI3Kδ, allowed characterizing these two isoforms as key regulators of migration, activation and differentiation of immune cells *in vivo*27. Mice deficient for PI3Kγ (PI3Kγ-/-) exhibit defects in T-cell development and activation. Similarly, animals expressing a kinase-inactive PI3Kδ (PI3KδD910/A910 mutants) show impaired B and T cell antigen receptor signaling, as well as defective B cell development and differentiation, accompanied by an impairment in T cell differentiation into Th1, Th2 and Treg lineages [36].

PI3K signaling pathway is also a key regulator of neutrophil and macrophage migration. In inflammatory models, PI3Kγ-/- animals show a reduced number of infiltrating cells, with macrophages and neutrophils displaying impaired migration in response to different GPCR stimuli [37]. Indeed, PI3Kγ catalytic activity crucially controls morphological changes associated with cell polarization through PIP3 generation and regulation of Rac activity as well as cytoskeleton rearrangement. Also PI3Kδ-dependent production of PIP3 contributes to cell polarization and asymmetric F-actin synthesis during neutrophil chemotaxis, which are fundamental for cell motility and contraction [38]. The use of the pan-PI3K inhibitor LY294002, and of the PI3Kδ-selective IC87114 further confirmed the role of this specific isoform in regulating monocytic integrin activation, and the general role of PI3K signaling during diapedesis [39]. The usage of LY294002, together with wortmannin, also demonstrated that PI3K signaling pathway strongly impairs FcεRI-mediated mast cells degranulation [40]. However, in mice only PI3Kδ seems to be required for an optimal IgE/Ag dependent hypersensitivity response and its genetic or pharmacological inactivation in mast cells leads to an impaired allergen-IgE-induced degranulation and cytokine release [41, 42]. Interestingly, PI3Kγ inhibition reduces mast cell degranulation only at later stages. In agreement, animals lacking PI3Kγ are resistant to passive systemic anaphylaxis and this is due to an autocrine/paracrine effect of mast cells-released GPCR agonists upon FcεR stimulation43. All these results indicate that PI3Kδ and PI3Kγ are involved in different stages of mast cells degranulation, with PI3Kδ acting earlier in response to IgE and PI3Kγ later to maximize degranulation [42, 43]

Intriguingly, most of the processes orchestrated by PI3Ks and described above are critically involved in the development and the progression of chronic inflammatory airway disease. Of note, the PI3K pathway is prominently activated in COPD. The negative regulator of the PI3K pathway, PTEN, is significantly decreased in peripheral lung tissue of COPD patients compared to healthy subjects, and positively correlates with the severity of airflow obstruction. Conversely, phosphorylated Akt, a marker of PI3K activation, shows a negative correlation with PTEN protein levels. Exposure to cigarette smoke extract (CSE), and the consequent ROS production decreases PTEN protein content in primary bronchial epithelial cells, and the knockdown of the protein potentiates Akt phosphorylation and enhances the production of proinflammatory cytokines, such as IL-6, CXCL8, CCL2, and CCL5. This evidence suggests a model where ROS production triggers a reduction in PTEN protein levels, resulting in increased PI3K signaling and amplification of inflammation in COPD [44].

Furthermore, CSE can induce cellular senescence in human bronchial epithelial cells, and the ROS/

PI3K/AKT/mTOR signaling pathway may play an important role in this process, since its inhibition can attenuate this phenotype [45]. This increase in ROS production has been found crucial to induce PI3Kα activation and the consequent elevation of microRNA-34a. This induction, in turn, correlates with a reduction of Sirtuin-1 (SIRT1) and SIRT6, two NAD-dependent deacetylases mediating resistance to oxidative stress, in bronchial epithelial cells (BEAS2B), and this process is also observed in peripheral lung samples of patients affected by COPD [46].

Interestingly, enhanced activation of the PI3K signaling pathway is associated with an increased susceptibility of COPD patients to infections, in particular to influenza virus, which exacerbates their condition and increases morbidity and mortality. Accordingly, the usage of a PI3Kα inhibitor restores protective antiviral responses, suppresses infection, and improves lung function in these individuals [47]. Activated PI3Kα is also found in cytologically normal proximal airway epithelial cells of smokers at risk for lung cancer. This suggests that PI3Kα is activated in the airways before tumorigenesis and that patients carrying a hyperactive PI3Kα are more susceptible to lung injury and cancer development [48].

The view that hyperactivation of the PI3K signaling pathway correlates with enhanced sensitivity to lung disease is further sustained by the work of Angulo *et al*. reporting PI3K activation in patients affected by a primary immunodeficiency (PID) called Activated PI3k-δ Syndrome (APDS). This disease is associated with a dominant gain-of-function mutation (E1021K) in the p110δ protein, which has been found present in 17 patients from seven unrelated families. Notably, APDS is characterized by recurrent respiratory infections, progressive airway damage, lymphopenia, increased circulating transitional B cells, increased IgM and reduced IgG2 levels in serum, and impaired vaccine responses. From a molecular point of view, this immunodeficiency is caused by enhanced membrane association and kinase activity of PI3Kδ. Patient-derived lymphocytes display increased levels of PI(3,4,5)P3 and of phosphorylated Akt, and are prone to activation-induced cell death phenotype. This correlation between PI3Kδ hyperactivation and immunodeficiency in APDS is suggestive of a specific role of this isoform in the development of respiratory infections and airway damage. This work also suggests new possible therapeutic strategies for these conditions, since selective p110δ inhibitors, such as IC87114 and GS-1101, reduce the activity of the mutant enzyme at least *in vitro [*49].

In the next paragraph, we will describe recent advances in the development as well as preclinical and clinical testing of PI3K inhibitors for COPD therapy.

6. PI3K INHIBITORS IN COPD ANIMAL MODELS

In the last decade, a number of studies proved the therapeutic effects of inhibiting PI3Kγ and/or PI3Kδ in different respiratory diseases [50-54]. Aerosolized TG100-115, the dual-PI3Kγ/δinhibitor, significantly reduces neutrophil accumulation in two widely used preclinical models of COPD, i.e. lipopolysaccharide (LPS)- and cigarette smoke (CS)-induced COPD55. In mice, CS can induce COPD-like pathological features, such as infiltration of macrophages and neutrophils in the pulmonary parenchyma, airway fibrosis and emphysema [56, 57]. Another preclinical model is represented by *β*ENaC overexpressing mice (*β*ENaC-Tg), which is characterized by airway mucus obstruction and chronic airway inflammation, two key features of both COPD and cystic fibrosis-like lung disease. Galluzzo *et al.* report that the PI3Kγ specific inhibitor, AS-605240, significantly reduces neutrophil infiltration in BAL, and genetic deletion of PI3Kγ remarkably inhibits emphysematous changes in the lung of *β*ENaC-Tg mice [58.]Recently, a new dual-PI3Kγ/δinhibitor, RV-1729, was tested in murine models of asthma and COPD and shown to significantly inhibit allergen-induced inflammation [59].

Steroid resistance is another major reason of inflammation-induced chronic airway obstruction in COPD patients. It results from reduced expression of histone deacetylase 2 (HDAC2) and the subsequent increased transcription of several pro-inflammatory genes [12]. Compelling studies demonstrate that PI3Kδis involved in the regulation of inflammatory gene expression as well as in oxidative stress-induced corticosteroid insensitivity [60]. Of note, PI3Kδ but not PI3Kγ is a major determinant of oxidative stress-induced corticoid resistance in the CS-induced mouse model. Accordingly, PI3Kδinhibition prevents HDAC2 activity reduction in the presence of oxidative stress. Importantly, this action restores the anti-inflammatory function of glucocorticoids, including suppression of neutrophil infiltration and proinflam-matory cytokine release, in CS–induced COPD61. Interestingly, low doses of theophylline effectively inhibit PI3Kδ activity *in vitro,* and reverse corticoid resistance in CS-exposed

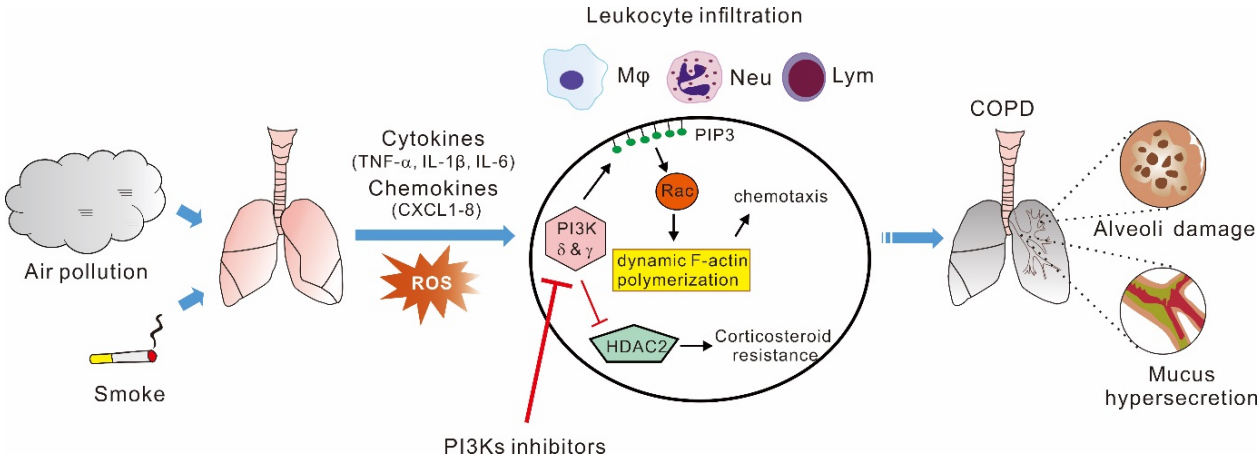

Fig. (1). PI3K signaling and targeting in COPD pathogenesis. Cigarette smoke and air pollution initiate an inflammatory response in the respiratory tract by promoting the release of pro-inflammatory mediators, like cytokines, chemokines, growth factors and free radicals (reactive oxygen species, ROS) from epithelial cells and leukocytes. These signals in turn trigger the infiltration of macrophages, neutrophils and lymphocytes in the lung, ultimately causing irreversible alveoli damage and airflow obstruction. PI3Kγ & δ critically control leukocyte recruitment by increasing PIP3 productions and activating the downstream signaling components, including Rac GTPase, which leads to polymerization of dynamic F-actin and leukocyte chemotaxis. Furthermore, PI3Kγ & δ promote ROS-induced corticosteroids resistance, which is elicited by down-regulation of HDAC2 expression and the subsequent increased transcription of pro-inflammatory genes. Accordingly, inhibitors of PI3Kγ & δ significantly reduce the inflammatory response, restore corticosteroids sensitivity and concomitantly stimulate alveoli regeneration.

Table 1. Preclinical and clinical effects of PI3K inhibitors in COPD

Inhibitor	Target	Effects on COPD	Model	Ref
IC87114	PI3Kδ	Restores glucocorticoid responsiveness; Inhibits GM-CSF and CXCL-8 release	LPS- and CS-induced COPD mouse model	[35, 37]
TG100-115	PI3Kδ and PI3Kγ	Reduces neutrophils and TNFα accumulation	Preclinical study	[31]
GSK2269557	PI3Kγ	Inhibits pro-inflammatory cytokines release	Phase 2 clinical trial	[36]
RV1729	PI3Kδ and PI3Kγ	Inhibits CXCL-8 release and neutrophils accumulation	Phase 1 clinical trial	[34]
AS-506240	PI3Kγ	Reduces neutrophil infiltration	βENaC-Tg mice	[33]
Wortmannin	Pan-PI3K	Induces alveolar regeneration	Elastase-induced COPD mouse model	[39]

mice, in synergy with the PI3Kδ-selective inhibitor IC87114 [62].

Finally, Horiguchi M. *et al* confirm that the pan-PI3K inhibitor Wortmannin significantly stimulates alveolar repair and recovers lung function in elastase-induced murine COPD model, suggesting that inhibition of PI3Ks is also able to induce differentiation of human alveolar epithelial stem cells [63].

7. PI3K INHIBITORS IN COPD CLINICAL TRIALS

In addition to animal experiments, many clinical studies have focused on the efficacy of PI3K inhibitors in COPD patients or samples. The selective PI3Kδ inhibitor, IC87114, restores glucocorticoid responsiveness through suppression of LPS-induced GM-CSF and CXCL-8 release in monocytes of COPD patients treated with dexamethasone [60]. More recently, an inhaled PI3Kδ inhibitor, GSK2269557, was tested for safety, tolerability, pharmacokinetics and dose-response effects in COPD patients. After 14 days of daily inhalation with GSK2269557, IL-8 and IL-6 levels are markedly decreased in the sputum. However, a significant improvement of lung function was not detected in GSK2269557-treated patients [64]. Nowadays, two selective PI3K inhibitors, RV1792 and

GSK2269557, are being tested into phase 1 and 2 clinical studies for COPD. According to process filing of preclinical studies, GSK2269557 is considerably more selective and significantly more potent than RV1792 [59]. As reported in the clinical study by Cahn A. *et al*, GSK2269557 induces only a few adverse events, such as nausea, cough, headache and arthralgia compared to placebo. Furthermore, in COPD patients enrolled in the clinical trial, there is no report of abnormal laboratory results, vital signs or ECG recordings during the treatment. Similarly, there is no evidence for paradoxical bronchospasm following treatment with GSK2269557 [64]. Thus, considering the acceptable safety profile of inhaled GSK2269557, this compound may potentially be developed into a promising drug for COPD patients.

In summary, these works suggest that targeting PI3Ks holds promise as a new effective molecular strategy for treating COPD (summarized in Table **1**).

CONCLUSION

Nowadays, the major translational goals in the treatment of chronic respiratory disease are to modify the natural history of the disease and to improve the quality of life as well as the survival of patients. Despite the escalation of new combinations of current therapies, and the introduction of innovative inhalator devices that allow to more efficiently reach the affected lung structure, a prolongation of life expectancy seems to be even now not achievable.

The development of other therapeutic strategies implies a more detailed knowledge of the mechanisms underlying the onset and the progression of the pathology. On these grounds, recent research efforts identified the PI3K pathway, and in particular PI3Kγ and δ isoforms, as key actors in COPD-related inflammation. Accordingly, PI3K inhibition has started to be evaluated as a novel therapeutic strategy for chronic inflammatory airway diseases: non-selective (dual γ/δ) and selective (δ) inhibitors have been developed and extensively tested in preclinical models, and clinical trials are ongoing. The hope is that the promising results of these clinical tests will soon translate into a novel therapeutic chance for such as a debilitating disease, like COPD.

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CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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PI3Ks in Diabetic Cardiomyopathy

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Abstract: Diabetic cardiomyopathy is a heart disease in diabetic patients, identified as ventricular dysfunction in the absence of coronary artery disease and hypertension. The molecular mechanisms underlying diabetic cardiomyopathy are still poorly understood. The protein and lipid kinase phosphoinositide 3-kinases (PI3Ks) have been suggested to regulate cardiac injury during diabetes. In this review, we will summarize the role of different PI3K isoforms and of their downstream signaling in the pathogenesis of diabetic cardiomyopathy, including the regulation of cardiac metabolism, contractility, hypertrophy, myocardial cell death, and inflammation.

Key Words: phosphoinositide 3-kinases, PI3Ks, diabetic cardiomyopathy

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INTRODUCTION

Diabetes is a common and chronic metabolic disorder characterized by insulin resistance and/or deficiency, leading to elevated glucose levels in plasma. All diseased subjects (5%–10%) are affected by type 1 diabetes mellitus exhibiting no insulin production, whereas type 2 diabetes mellitus is characterized by insulin resistance and accounts for the remaining ~90% of all cases of diabetes.[1–3] Diabetes mellitus is considered as one of the major threats to human health of the 21st century, as it is considered the sixth leading cause of death worldwide.[4] Over the past decades, an increase in sedentary life, a "westernized-diet" high in fat and sugar and environmental changes have been responsible for a steep rise in the incidence of the disease.[5] In 2015, an estimated 415 million adults are living with diabetes and this number is expected to reach 642 million by 2040, leading diabetes to become a striking economic burden, particularly in developed countries, requiring an estimated 12% of the worldwide health care expenditure for the treatment and prevention.[6]

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In diabetic patients, the complications associated with the pathology remain the main cause of morbidity and mortality. The primary cause of death resides in cardiovascular disease, with an increased risk of developing hypertension and coronary artery disease (CAD), in turn leading to heart failure (HF). Nevertheless, for these patients the risk of developing HF remains increased despite adjustment for CAD and hypertension. Therefore, diabetes-associated functional and structural myocardial changes, in the absence of CAD and hypertension, describe a condition termed "diabetic cardiomyopathy," referring to a combination of molecular abnormalities that predispose to the development of heart dysfunction.[7] It is characterized by ventricular hypertrophy, interstitial and perivascular fibrosis, diastolic, and often also systolic dysfunction, increased apoptosis, mitochondrial impairment, and exaggerated oxidative stress.[8] Several factors have been proposed to explain the pathogenesis of diabetic cardiomyopathy, with cardiac metabolic disturbances emerging as one of the most relevant. Under physiological conditions, insulin stimulates the uptake of glucose in different metabolic tissues, including the cardiac muscle, to maintain glycemic homeostasis.[9] However, a reduction in insulin signaling or insulin resistance, both hallmarks of diabetes mellitus, together with a decrease in glucose transport causes the loss of metabolic flexibility leading to lipotoxicity and impaired calcium signaling, which eventually manifest as myocardial stiffness, hypertrophy, and diastolic dysfunction.[10] Other symptoms, including contractile dysfunction, cardiac hypertrophy, and inflammation, can be either consequent complications of metabolic disturbances or independent factors, rendering diabetic cardiomyopathy a complicate pathology. Many molecular signal pathways are involved in the pathogenesis of diabetic cardiomyopathy.[11] Among them, PI3K pathway is a key player due to its well-known roles in regulating not only insulin signaling but also cell growth, survival, and inflammation.[12] In fact, perturbation of this signaling pathway has been demonstrated to be implicated in metabolic imbalance, reduced contractility, hypertrophy, and inflammation. In this review, we will discuss the role of different PI3Ks isoforms and of their downstream signal in different aspects of diabetic cardiomyopathy (Fig. 1).

PHOSPHOINOSITIDE 3-KINASES

Phosphoinositide 3-kinases (PI3Ks) are a group of lipid and protein kinases that catalyze the phosphorylation of phosphatidylinositols (PtdIns) on the D3 position of the inositol ring. PI3Ks include 8 different isoenzymes that are grouped into 3 classes, based on the structure of the catalytic subunits, the type of adaptor subunits that associate with them, and the preferred lipid substrate.[13] Among them, class I

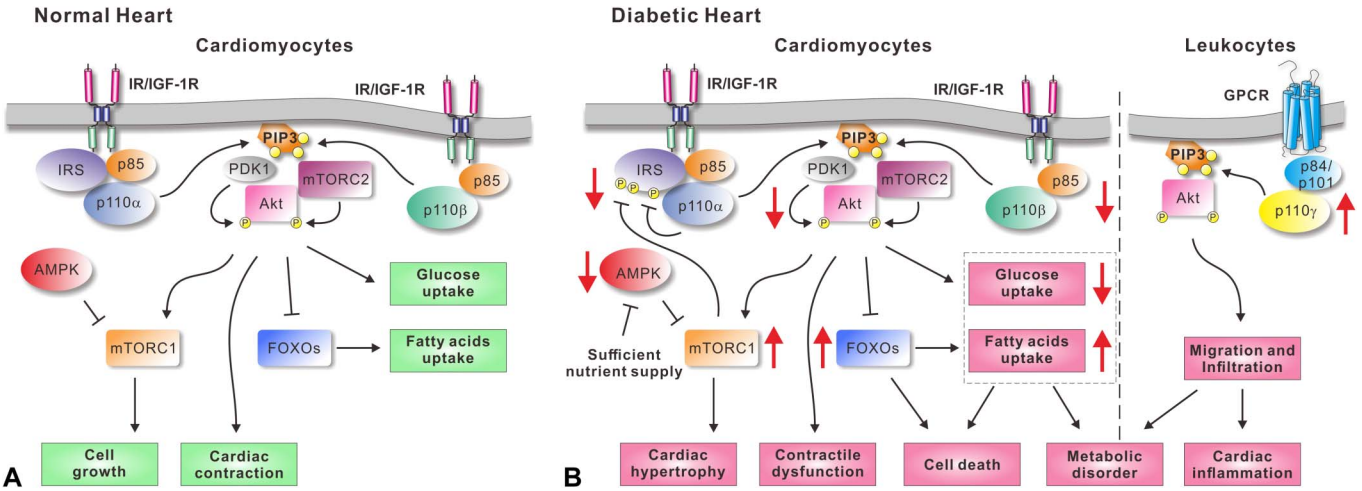

FIGURE 1. Distinct PI3K isoenzymes control specific aspects of the pathogenesis of diabetic cardiomyopathy. A, In normal hearts, IR or IGF-1R activates IRS, which assists p85 binding to activated IR/IGF-1R and consequently recruits and activates p110α. p110α then catalyzes the production of the intracellular second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which in turn recruits proteins carrying a pleckstrin homology domain, like phosphoinositide-dependent kinase-1 (PDK1), mechanistic TOR complex 2 (mTORC2), and protein kinase B (Akt). On phosphorylation by PDK1 and mTORC2, Akt is fully activated and can regulate a plethora of downstream cellular processes, such as promoting glucose uptake, maintaining cardiac contraction, regulating mTORC1-dependent cell growth, and inhibiting FOXO-mediated fatty acids uptake. mTORC1 activity is also inhibited by the energy sensor AMPK, which is suppressed by nutrient supply. p110β is also recruited by p85 downstream of IR/IGF-1R. Although PI3Kα is the only PI3K that responds to acute insulin stimulation, PI3Kβ is necessary for long-term insulin signaling, whereas its exact role in cardiac metabolism requires further investigation. B, In diabetic hearts, acutely activated PI3Kα phosphorylates and inhibits IRS, resulting in its downregulation. Low (downward red arrow) IRS, in turn, reduces PI3Kα/Akt signaling activation in late stage of diabetes. This results in reduced Ca^{2+} currents and cardiac contractile dysfunction. Furthermore, reduced Akt-dependent glucose uptake and increased FOXO-mediated fatty acid use, together, induce a switch in cardiac metabolism and cell death. mTORC1 is unexpectedly upregulated (upward red arrow), possibly because of sufficient nutrient-induced inhibition of AMPK, and may contribute to the pathological hypertrophy observed in diabetic hearts. Moreover, mTORC1, and its downstream signaling effectors, can phosphorylate IRS and downregulate PI3Kα/Akt signaling, further exacerbating insulin resistance. Conversely, p110γ is recruited and activated by p84 or p101 in response to GPCR activation in leukocytes. PI3Kγ thus regulates immune cells migration and infiltration in the heart, causing cardiac inflammation and metabolic disorder.

PI3Ks are the most studied and phosphorylate phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] to produce phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3 or PIP3], a key intracellular second messenger. Instead, class II and III PI3Ks are less characterized and catalyze the generation of phosphatidylinositol (3,4)-bisphosphate [PtdIns (3,4)P2] and phosphatidylinositol 3-phosphate (PtdIns3P or PI3P).

Depending on the form of adaptor subunit with which the catalytic subunit associates and the type of upstream membrane activating receptor, class I PI3Ks are further subgrouped into class IA and IB. Class IA PI3Ks, including PI3Kα, β, and δ, are heterodimers composed by one of the p110 catalytic subunits (including p110α, p110β, and p110δ) and an adaptive subunit belonging to the p85 family. In response to ligand stimulation of receptor tyrosine kinases (RTKs), the SH2 domain of the p85 adaptor binds to phosphorylated tyrosine residues, consequently recruiting and activating the p110 catalytic subunit. Some other adaptor proteins may be necessary for linking RTK to PI3K signaling. For instance, activated insulin receptor (IR) or insulin-like growth factor–1 receptor (IGF-1R) can phosphorylate IR substrates (IRS1 and IRS2), which serve as adaptors to further recruit PI3K close to IR/IGF-1R, ultimately initiating the regulation of metabolism and growth (Fig. 1).[14] However, PI3Kγ is the only member of class IB PI3K. It contains the p110γ catalytic subunit and either p101 or p84/87 regulatory subunit and has long been considered to be triggered by G protein–coupled receptor (GPCR) agonists.[15] However, recent studies show that PI3Kβ can also be activated by GPCRs,[16,17] whereas PI3Kγ can be engaged by RTKs in a Ras/p87-dependent way,[18] thus depicting new understanding of class I PI3Ks.

In response to extracellular ligands, RTKs and GPCRs activate class I PI3Ks and the ensuing production of PIP3, which accumulates at the plasma membrane. PIP3, in turn, recruits proteins carrying lipid-binding domains, primarily the pleckstrin homology domain, such as phosphoinositide-dependent kinase-1 (PDK1), mechanistic target of rapamycin complex 2 (mTORC2) subunit mSin1, and protein kinase B (Akt). The spatial vicinity of PDK1 and Akt facilitates the phosphorylation of Akt at Thr308 by PDK1, whereas recruitment of mSin1 by PIP3 releases the inhibition of mTORC2 and the ensuing Akt phosphorylation at Ser473.[19–21] Akt is thus fully activated and can regulate multiple downstream signaling pathways, involved in cell proliferation, metabolism, migration, and survival[19] (Fig. 1). Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) dephosphorylates PIP3 on the D3 position and thus represents

the major endogenous PI3K inhibitor. PI3Ks and PTEN thereby keep the balance of cellular PIP3 levels that, when deregulated by either amplification of PI3Ks or loss of PTEN, may lead to dramatic consequences such as cell transformation and cancer.[22]

Besides serving as lipid kinases, PI3Ks can also function as protein kinases that directly phosphorylate protein substrates.[23] For instance, PI3Kα phosphorylates both its p85 subunit and IRS1.[24] The phosphorylation of IRS1 by PI3Kα on one hand competitively reduces the phosphorylation of p85,[24] on the other hand results in its degradation,[25] thus contributing to insulin resistance during diabetes. In addition, the protein kinase activity of PI3Kγ is involved in the control of β-adrenergic receptor (β-AR) density in the heart. PI3Kγ-mediated phosphorylation of nonmuscle tropomyosin is a crucial step for agonist-dependent β-AR internalization.[26] Moreover, PI3Kγ can phosphorylate an intracellular inhibitor of protein phosphatase 2A (I2PP2A), resulting in the inhibition of protein phosphatase 2A (PP2A), an enzyme that dephosphorylates β-AR in early endosomes, ultimately inhibiting the resensitization phase of β-AR recycling.[27] Overall, the protein kinase activities of PI3Ks play additional roles to their well-known lipid kinase activities. Nevertheless, limited studies are currently available to fully understand the function of PI3K protein kinase activities.

The 4 different class I PI3K isoforms show distinct expression patterns. Murine models with PI3Kα or PI3Kβ deficiency show embryonic lethality, whereas PI3Kγ or PI3Kδ knockout mice are viable and fertile, implying distinct and nonredundant roles of different PI3K isoforms in different cellular processes.[28] Although PI3Kα and PI3Kβ are ubiquitously expressed, PI3Kδ is enriched in leukocytes, and PI3Kγ functions in both leukocytes and cardiac cells.[29] In the heart, PI3Kα and PI3Kγ are key players of metabolism, survival, contractility, and stress response.[30]

Class II PI3Ks, including PI3K-C2α, PI3K-C2β and PI3K-C2γ, and the only member of class III PI3K, Vps34, are all monomers which are less characterized. Based on their ability to produce PtdIns(3,4)P2 and PI3P, they emerge as key regulators of vesicular trafficking. Recent studies reveal that PI3K-C2α controls an endosomal PI3P pool to promote primary cilium function, and contributes to the protection against kidney cyst formation, whereas PI3K-C2γ functions downstream of insulin signaling by regulating an endosomal PtdIns(3,4)P2 pool and Akt2 activity.[31–33] Furthermore, Vps34 critically controls the generation of PI3P and is mainly involved in the regulation of autophagy.[34] Nevertheless, the roles of class II and class III PI3Ks in cardiac health and disease still require further investigation. Thus, in the next paragraphs, we will focus on the role of class I PI3Ks in diabetic cardiomyopathy.

PI3Kα IN DIABETIC CARDIOMYOPATHY

PI3Kα Modulates Heart Metabolism in Diabetic Cardiomyopathy

In the heart, class I PI3Ks are primarily involved in the regulation of insulin signaling and utilization of glucose and/or fatty acids. Under normal conditions, the main substrate for adenosine triphosphate (ATP) production in the heart is constituted by fatty acids, and their oxidation is responsible for 50%–70% of total ATP.[35] Nevertheless, other substrates, such as glucose (20%) and lactate (10%), may provide additional fuel sources.[36] Cardiac myocytes use the glucose transporters GLUT1 and GLUT4, with GLUT4 being the most abundant, to uptake glucose, whereas fatty acids are able to pass through the cell membrane by passive diffusion or by exploiting transporters, such as fatty acid translocase (FAT/CD36), fatty acid–binding protein, and fatty acid transport protein 1.[37] The PI3K signaling is one of the pathways involved in GLUT4 and FAT/CD36 regulation.[37] Insulin binds its receptor on the cell membrane and activates its intracellular tyrosine kinase activity that catalyzes the phosphorylation of tyrosine residues of the IRS1. In turn, IRS1 transmits the insulin signaling by activating the PI3K/Akt pathway involved in cardiomyocyte metabolism. This signaling triggers GLUT4 translocation from intracellular vesicles to the sarcolemma, thus increasing the glucose uptake rate. Fatty acid translocase/CD36 is also able to translocate to the sarcolemma through the PI3K pathway, leading to an increase in fatty acid uptake.[38] In diverse animal models of type 2 diabetes mellitus, the heart shows altered glucose and fatty acid uptake.[39–41] In fact, it has been demonstrated that the uptake of fatty acids is increased, leading not only to enhanced fatty acid oxidation but also to an increase in myocardial lipid accumulation. This dysregulation of the uptake and, in turn, compartmentalization of fatty acids, is responsible for the cardiac lipotoxicity, characterized by cardiomyocyte apoptosis, myocardial fibrosis, and impaired mitochondrial function.[42–44] The diabetic heart is accompanied by a lower basal PI3Kα activity and a reduction in insulin-mediated PI3Kα activation,[45] which ultimately reduce glucose uptake, leaving the heart relying mostly on mitochondria-mediated fatty acid β-oxidation for energy production.[46] This metabolic imbalance of PI3K-mediated fatty acids and glucose homeostasis in diabetic heart finally results in diabetic cardiomyopathy pathological features, such as decrease in cardiac efficiency, pathological cardiac hypertrophy, fibrosis, and apoptosis. Overall, dysregulation of PI3Kα induces metabolic imbalance, leading to the development of diabetic cardiomyopathy.

PI3Kα Modulates Cardiac Contraction in Diabetic Cardiomyopathy

Diabetic cardiomyopathy is accompanied by contractile dysfunction. Studies have shown that, in cardiomyocytes of streptozotocin (STZ)-induced type 1 diabetic rats and obese db/db type 2 diabetic mice, Ca^{2+} inward current through the voltage-dependent Ca^{2+} channel (LTCC), a critical initiator of the contractile cycle, is reduced.[47,48] Indeed, LTCC protein expression is diminished with a consequent decrease in Ca^{2+} current (I_{Ca}) density in type 1 diabetic myocytes compared with control cells. Notably, I_{Ca} also shows an altered voltage dependence, with the steady-state activation and inactivation curves shifted toward more positive potentials in diabetic compared with nondiabetic myocytes.[49] This evidence

suggests that only a small proportion of LTCCs are available for opening during the action potential, reducing the net influx of Ca^{2+} through these channels. It is also known that in these models of diabetes, the maintenance of a normal cardiac LTCC function is critically regulated by the PI3K signaling pathway, more specifically by PI3Kα. Cardiac-specific ablation of p110α leads to a decreased number of LTCC on the cell surface of myocytes, causing in turn a decrease in myocyte contractility and fractional shortening.[50] Altogether, these observations suggest a protective role of PI3Kα in diabetic cardiomyopathy by preserving cardiac contractility.

PI3Kα Regulates Cardiac Hypertrophy in Diabetic Cardiomyopathy

Increased left ventricular mass is another feature of diabetic cardiomyopathy. This is characterized as pathological hypertrophy, which is associated with upregulation of fetal genes, interstitial fibrosis, and cardiac dysfunction, whereas the physiological hypertrophy that occurs in response to postnatal growth, exercise, and pregnancy is characterized by normal organization of cardiac structure and normal or enhanced cardiac function, and usually is reversible.[51] In type 2 diabetes, hypertrophy may occur independently of high arterial blood pressure and is a well-known risk factor for HF.[52] Moreover, the likelihood of having mass that exceeds the 75th percentile is greater in patients with type 2 diabetes, after adjusting for various covariates, including hypertension. Hypertrophy usually occurs in the late stage of diabetes, and may eventually lead to ventricular remodeling.[53] Pathological hypertrophy is also present in type 1 diabetes. Streptozotocin-treated mice display an increase in cardiomyocyte width, which is accompanied by increased levels of cardiac stress markers, such as atrial natriuretic peptide and brain natriuretic peptide, and cell death.[54] Different studies have shown that PI3Kα and its upstream signal IGF-1 are able to protect the heart from pathological hypertrophy induced by different cardiac stresses, such as pressure overload, dilated cardiomyopathy, and myocardial infarction.[55–61] This protection is beneficial from PI3K-mediated physiological hypertrophy which antagonizes pathological hypertrophy. Mice expressing a constitutively active form of PI3Kα (caPI3Kα) have hearts that are 15% larger but with normal cardiac function, whereas reduced PI3Kα activity [dominant negative PI3K(p110α), dnPI3Kα] results in 15% smaller heart size but with normal heart function.[62] The cardioprotective role of PI3Kα is extended to diabetic cardiomyopathy, with the finding that constitutive activation of PI3Kα prevents STZ-induced increase of cardiomyocyte size and diastolic dysfunction, whereas decreased PI3Kα activity exaggerates STZ-provoked pathological hypertrophy and cardiac fibrosis and apoptosis.[54] The reduction of pathological hypertrophy and cardiac fibrosis in caPI3Kα mice is also observed in mice overexpressing IGF-1R,[63] further confirming the cardioprotective effect of IGF-1R/PI3Kα signaling against diabetic cardiomyopathy.

In summary, PI3Kα signaling is downregulated in diabetic hearts and results in metabolic switch, insulin resistance, contractile dysfunction, and pathological hypertrophy. Reboosting of PI3Kα signaling by overexpression of p110α or of its upstream partner IGF-1R is beneficial against diabetes-induced cardiac injury.

PI3Kβ IS POTENTIALLY INVOLVED IN DIABETIC CARDIOMYOPATHY

Although PI3Kα is known to be the main PI3K isoform involved in insulin signaling, recent findings suggest that PI3Kβ is also necessary for insulin metabolism. PI3Kβ is not required for the short-term response to insulin, instead PI3Kα is the only PI3K that responds to acute insulin stimulation and activates downstream Akt signaling.[64] However, mice expressing a catalytically inactive form of p110β or treated with selective PI3Kβ inhibitors show increased levels of blood glucose and peripheral insulin resistance, implying that the kinase activity of PI3Kβ is necessary for long-term insulin signaling.[16] Whether PI3Kβ activity affects cardiac metabolism as well is still unexplored. However, recent evidence unveils a critical role of cardiac p110β in mediating cardiac development and regeneration through the activation by yes-associated protein (YAP), a key player of the Hippo pathway.[65] Yes-associated protein requires p110β-dependent activation of Akt to promote cardiomyocyte proliferation and survival. Accordingly, adeno-associated virus–mediated p110β expression in the heart rescues heart function impairment in YAP-deficient mice.[66] These findings highlight a protective role of PI3Kβ against heart disease and the potential involvement of this enzyme in the regulation of diabetic cardiomyopathy.

AKT AND THE DOWNSTREAM SIGNALING DIRECTLY REGULATE THE PATHOGENESIS OF DIABETIC CARDIOMYOPATHY

PI3Ks control multiple biological processes through the regulation of Akt and its downstream effectors. Recent evidence uncovers the direct participation of this pathway, including Akt, mTORC1, and FOXOs, to cardiac injury in diabetic cardiomyopathy.

Akt

In the heart there are 3 known Akt members, Akt1, Akt2 and Akt3, with Akt1 and Akt2 being the most abundant. Studies with transgenic rodent models reveal that distinct Akt isoforms control different biological processes in the heart.

Akt1 functions mainly downstream of IGF-1/PI3K signaling in regulating postnatal cardiac growth.[67] Mice with acute cardiac-specific Akt1 overexpression display reversible cardiac hypertrophy, whereas a constant Akt1 activation induces extensive hypertrophy and cardiac dysfunction.[68] By contrast, Akt1 deleted mice ($Akt1^{-/-}$) are resistant to exercise-induced cardiac growth, but develop aggravated cardiac hypertrophy in response to pressure overload. This implies a role of Akt1 in promoting physiological cardiac hypertrophy, whereas antagonizing pathological hypertrophy.[67] The less studied and mysterious Akt3 shows partial functional overlap with Akt1, as Akt3 overexpression in the

heart induces cardiac hypertrophy with preserved contractility at early age, but impairs contractile function when mice are at 20 weeks of age. However, a better understanding of Akt3, which is expressed only at low levels in the heart, still relies on future studies.

However, Akt2, but not Akt1, is the main modulator of insulin/PI3K-stimulated glucose uptake and metabolism in the heart.[69] This process is believed to function through Akt2-dependent regulation of GLUT4 translocation from intracellular vesicles to plasma membrane, ultimately facilitating glucose uptake, as insulin-stimulated glucose uptake is impaired in Akt2 deleted mice ($Akt2^{-/-}$),[70] or in cells in which Akt2 is downregulated by RNAi.[71] Moreover, Akt2 knockout mice are sensitized to cardiomyocyte apoptosis induced by ischemic injury.[69] These findings highlight Akt2 as the regulator of cardiac metabolism and cardiomyocyte death in diabetic cardiomyopathy.

However, whether Akt is activated or suppressed in diabetic heart is still controversial. Some studies report that Akt is upregulated in rodent diabetic hearts and in ventricular tissues from type 2 diabetic patients,[72,73] whereas others find that diabetes reduces Akt activity.[45] Riehle and Abel[74] hypothesize that high insulin signaling at early stage of diabetes may upregulate Akt and contribute to diabetic cardiac hypertrophy, whereas low Akt activity due to insulin resistance at late stage of diabetes results in lipotoxicity and cardiomyocyte apoptosis. In addition to this point, distinct Akt isoforms may function at different stages of diabetic hearts and contribute to different pathogenesis of cardiomyopathy.

In summary, the present studies reveal the distinct roles of different Akt isoforms in cardiac biological processes, with Akt1 and Akt3 mainly regulating cardiac growth and Akt2 controlling metabolism and cell death. Nevertheless, diabetic cardiomyopathy studies based on cardiac-specific modification of Akt are still required to fully understand its role in the diabetic heart.

mTORC1

The mechanistic TOR complex 1 (mTORC1) is activated by PI3Ks/Akt, but also inhibited by adenosine monophosphate–activated protein kinase (AMPK), through direct phosphorylation of raptor subunit and indirect phosphorylation of mTORC1 inhibitor tuberous sclerosis complex 2 (TSC2).[75,76] mTORC1 modulates cardiac biological processes such as upregulating protein synthesis through the activation of S6 kinase and inhibition of translation repressor 4E-BP. Interestingly, mTORC1 activity is upregulated in diabetic organs, including the heart,[72,77,78] possibly because of the inhibition of AMPK by increased ATP/adenosine monophosphate ratio during high nutrient supply. Accordingly, high-fat diet (HFD) in mice decreases AMPK activity and upregulates mTORC1 phosphorylation, whereas mice with kinase-inactive form of AMPK display enhanced mTORC1 activity and exaggerated HFD-induced cardiac hypertrophy and contractile dysfunction.[78] Overactivated mTORC1 may contribute directly to pathological hypertrophy observed in diabetic hearts, because rapamycin, a well-known mTOR inhibitor, efficiently moderates the hypertrophy phenotype in the murine model of type 2 diabetes (db/db mice).[72] However, the activation of mTORC1 and S6K triggers the phosphorylation of IRS1 on Ser-307 and Ser-270 and disrupts the response of PI3K/Akt signaling to insulin, thus exacerbating insulin resistance.[79,80] Finally, inhibition of mTORC1 shows protection against diabetic cardiomyopathy.[72,81] A study in drosophila demonstrates that systemic inhibition of the corresponding TOR pathway, by mutating TOR, overexpressing its negative regulators TSC1-2 or enhanced expression of its effector 4E-BP, protects against HFD-induced diabetic cardiomyopathy. Intriguingly, fat body-specific blockade of TOR prevents both obesity and heart dysfunction, whereas myocardial-specific inhibition of TOR protects the heart despite developing obesity in response to HFD, thus implying an essential role of TOR signaling in the process of both obesity and diabetic cardiomyopathy.[81] Similar effect is detected in type 2 diabetic db/db mice with the observation that rapamycin significantly reduces serum glucose, insulin level, body weight gain, and more importantly preserves heart function.[72]

FOXOs

The family of forkhead box O (FOXO) transcription factors is another well-studied PI3Ks/Akt downstream effector that has been demonstrated as one of the major players in the pathogenesis of diabetic cardiomyopathy. FOXOs are a group of transcription factors that regulate the expression of genes involved in cell growth, proliferation, differentiation, and longevity. Akt phosphorylates FOXO1 on Ser256 and FOXO3 on Thr32 and Ser253, which promote the interaction of FOXOs with 14-3-3 and the translocation from the nucleus to the cytoplasm, thus inhibiting their transcription activities. The role of FOXOs in different types of heart injury has been studied during the past decades, leading to the general conclusion that FOXOs are detrimental in the heart under different stress conditions (reviewed in ref. 82).

FOXO1 is a key mediator of diabetic cardiomyopathy, as it is activated in rodent models of both type 1 and type 2 diabetes.[83,84] FOXO1 directly participates to the cardiac insulin resistance mediated by IRS1, 2 during the process of diabetic cardiomyopathy. In diabetic hearts, IRS1 and IRS2, and their downstream effector Akt, are inactivated resulting in the activation and nuclear translocation of FOXO1. FOXO1-dependent transcription of genes involved in glucose metabolism, inflammation, and cardiac cell death is thus initiated, causing the alteration of cardiac metabolism and finally insulin resistance.[85] Qi et al[85] find that nuclear FOXO1 interacts directly with the promoter of the fetal β-MHC gene, stimulating its expression in cardiomyocytes and facilitating cardiac dysfunction. FOXO1 also promotes cardiac cell death through the upregulation of inducible nitric oxide synthase, which induces protein nitrosylation of glyceraldehyde 3-phosphate dehydrogenase and caspase 3, eventually accelerating the activation of poly (ADP-ribose) polymerase 1 (PARP1) and programmed cell death.[84] However, FOXO1-dependent activation of inducible nitric oxide synthase promotes the translocation of CD36, the fatty acid transporters, to the plasma membrane, which enhances fatty acid metabolism, but reduces glucose metabolism, leading to lipid accumulation in diabetic heart.[83,86] Moreover, FOXO1 activation

further downregulates IRS1 and Akt signaling, exacerbating insulin resistance in the heart.[83] In line with these findings, cardiac-specific deletion of FOXO1 significantly abolishes cardiac hypertrophy, preserves contractile function and increases the survival of HFD and db/db mice, without altering systemic diabetic phenotypes, as body weight gain, glucose tolerance, and serum insulin level are comparable between cardiomyocyte-restricted FOXO1 KO and control mice.[83,85]

FOXO3 is also involved in the regulation of diabetic cardiomyopathy. The nuclear translocation of FOXO3 increases in the heart of diabetic rodent models, including STZ-induced type 1 diabetic rats, spontaneously type 2 diabetic Goto-Kakizaki rats, HFD-induced, and db/db type 2 diabetic mice.[83,87,88] The nonredundant role of FOXO3 and FOXO1 in diabetic cardiomyopathy may be due to the different transcription targets. FOXO3a induces the upregulation of caspase-1 and proinflammatory cytokines, like IL-1β and IL-18, thereby increasing pyroptosis, a highly inflammatory process that finally results in programmed cell death of cardiomyocytes during diabetes. Moreover, high glucose may induce oxidative stress and Bim-mediated apoptosis in cardiac microvascular endothelial cells in FOXO3a-dependent manner, contributing to cardiovascular complications in diabetes.[89] Nevertheless, a study investigating the effects of cardiac-specific deletion of FOXO3 in diabetic cardiomyopathy is still missing, leaving the mechanisms underlying FOXO3-mediated cardiac dysfunction largely unknown. Overall, FOXOs contribute to the development of diabetic cardiomyopathy downstream of PI3Ks/Akt signaling and may serve as targets for the prevention of cardiac dysfunction during diabetes.

PI3Kγ REGULATES CARDIAC INFLAMMATION IN DIABETIC CARDIOMYOPATHY

Another critical cause of diabetic cardiomyopathy is inflammation, which induces cardiomyocyte death, cardiac fibrosis, and insulin resistance in diabetic hearts.[90–92] Studies show that reduction of cardiac inflammation, by strategies such as inhibition of TNFα or caspase 1, displays beneficial effects against diabetic cardiomyopathy.[90,91] PI3Kγ is enriched in leukocytes and regulates immune cell migration and infiltration, suggesting a potential but indirect role of PI3Kγ in diabetic cardiomyopathy.

Previous studies demonstrate that leukocyte PI3Kγ has a detrimental role in HF. Mice expressing a kinase-inactive PI3Kγ (PI3Kγ-KD) or receiving a PI3Kγ-selective inhibitor (AS605240) are protected against pressure overload-induced cardiac remodeling and contractile dysfunction. In these mice, cardiac function preservation is accompanied by a significant reduction of leukocyte infiltration in the heart, emphasizing the maladaptive role of cardiac inflammation in pressure overload-induced HF. Importantly, wild-type (WT) mice that are transplanted with PI3Kγ KD bone marrow display reduced immune cell infiltration and preserved cardiac contractility under pressure overload induced by transverse aortic constriction. Conversely, PI3Kγ KD animals carrying a WT immune system show prominent leukocyte infiltration and cardiac fibrosis.[93] These findings imply that PI3Kγ is important in guiding leukocyte infiltration in the heart, and that inhibition of PI3Kγ may be beneficial.

A recent study uncovers that PI3Kγ is upregulated in the heart in a model of STZ-induced type 1 diabetes. Streptozotocin-mediated leukocyte infiltration, cardiac fibrosis, and finally cardiac contractile dysfunction are prevented in PI3Kγ$^{-/-}$ and PI3Kγ KD mice, and in WT mice treated with the PI3Kγ selective inhibitor GE21,[94] thus revealing a negative role of PI3Kγ in diabetic cardiomyopathy. Nevertheless, whether the protection observed in PI3Kγ$^{-/-}$ and PI3Kγ KD mice stems only from dampened leukocyte infiltration or also from a blunted cardiac response is still not fully understood. In addition, given the role of cardiac PI3Kγ in the regulation of receptor internalization,[95] an open question is whether PI3Kγ is also involved in the internalization of IR, which results in insulin resistance in type 2 diabetes. These issues are still unresolved and require further investigation. Overall, class IB PI3K plays a maladaptive role during the natural history of HF, including diabetic cardiomyopathy, by regulating cardiac inflammation and targeting PI3Kγ activity could be therapeutically exploited to ameliorate heart disease.

CONCLUSION REMARKS AND FUTURE PERSPECTIVES

In summary, different isoforms of class I PI3Ks play distinct and nonredundant roles during the pathogenesis of diabetic cardiomyopathy (Fig. 1). PI3Kα/β-Akt signaling is downregulated in the heart and results in reduced contractility, increased cardiomyocyte death, and dysregulation of cardiac metabolism. By contrast, leukocyte PI3Kγ is activated in diabetes and plays a detrimental role by facilitating cardiac inflammation. Thus, targeting different isoforms of class I PI3Ks selectively can be a valuable approach to reduce diabetic cardiac injury by impacting on different molecular aspects of the pathology. Since long-term downregulation of insulin/PI3Kα/Akt signaling impairs cardiac contractility and metabolism, blood glucose reduction by monotherapy with hypoglycemic agents may not be sufficient to reverse the cardiac injury. By contrast, a combination treatment of hypoglycemic agents with insulin, to reboost PI3K/Akt pathway, might be required for cardiac recovery. In line with this view, 25% of type 2 diabetic patients treated with oral hypoglycemic agents require the addition of insulin therapy, which improves glycemic control and lowers microvascular complication risk.[96] Whether this is also true in the therapeutic aspect of diabetic cardiomyopathy is worth for further investigation. However, inhibition of PI3Kγ with new clinically available selective inhibitors may represent a novel potential strategy to treat diabetic cardiomyopathy through the inhibition of cardiac inflammation.

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EXPERIMENTAL THERAPEUTICS (L S MAIER, SECTION EDITOR)

Therapeutic Targeting of PDEs and PI3K in Heart Failure with Preserved Ejection Fraction (HFpEF)

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Abstract
Purpose of Review Heart Failure with preserved Ejection Fraction (HFpEF) is a prevalent disease with considerable individual and societal burden. HFpEF patients often suffer from multiple pathological conditions thatcomplicate management and adversely affect outcome, including pulmonary hypertension and chronic obstructive pulmonary disease (COPD). To date, no treatment proved to be fully effective in reducing morbidity and mortality in HFpEF, possibly due to an incomplete understanding of the underlying molecular mechanisms.
Recent Findings The emerging view proposes chronic systemic inflammation, leading to endothelial dysfunction and interstitial fibrosis, as a prominent cause of HFpEF, rather than a mere co-existent disease. In the last decade, efforts from pharmaceutical companies attempted to target pharmacologically enzymes which play key roles in systemic and lung inflammation, such as the cyclic nucleotide-degrading enzymes phosphodiesterases (PDEs) and phosphoinositide-3 phosphate kinases (PI3Ks), especially to limit COPD.
Summary In this review, we will summarize major successes and drawbacks of hitting these enzymes to tackle inflammation in HFpEF-associated co-morbidities, with a major focus on the results of completed and ongoing clinical trials. Finally, we will discuss the potential of repurposing and/or developing new PDE and PI3K inhibitors for HFpEF therapy.

Keywords Phosphodieserases · PDE inhibitors · Phosphoinositide-3 kinase inhibitors · Chronic obstructive pulmonary disease

Introduction

The incidence of heart failure (HF) is growing worldwide, mainly due to the overall aging of the population and to the pandemic diffusion of cardiovascular risk conditions, such as hypertension, diabetes, dyslipidemia, overweight, and physical inactivity. Standard treatments for HF include neuroendocrine antagonists (i.e., beta-adrenergic receptor blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and mineralocorticoid receptor antagonists), diuretics, and digitalis, which have contributed in the last decades to improve the outcomes for HF patients. Nonetheless, HF is still associated with high mortality, reduced quality of life, and repeated hospitalizations.

In the past years, improved understanding of cardiac disease and the results of large HF outcome trials [1] have enabled investigators to identify two distinct forms of HF: HF with reduced ejection fraction (EF, HFrEF) and HF with preserved EF (HFpEF) [2–6]. While HFrEF strongly responds to standard treatments for HF, HFpEF appears more refractory to traditional approaches. This is possibly derived from distinct pathophysiological and molecular mechanisms for HFrEF and HFpEF, both in myocardial and in systemic signaling. A key aspect is that HFpEF constitutes a heterogeneous and syndromic disease, with strictly interlaced comorbidities. For instance, HF and pulmonary disease frequently coexist, sharing risk factors, coincident disease mechanisms, and therapeutic targets [7, 8•]. Of

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Topical Collection on *Experimental Therapeutics*

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note, HFpEF currently accounts for >50% of all HF cases, and its prevalence, relative to HFrEF, keeps rising [9]. This fact is critically worrisome, since, despite the preserved left ventricle (LV) systolic function, HFpEF patients experience high morbidity and mortality [10].

In HFrEF, a hypocontractile LV acquires an eccentric configuration and ultimately dilates, owing to cardiomyocyte loss, decreased myofilament density, and replacement fibrosis (for a review [1]). Instead, HFpEF is essentially defined by a concentrically remodeled and stiff LV, with increased tele-diastolic filling pressures. Within the myocardium, key pathological events are represented by cardiomyocyte hypertrophy and stiffness, coupled with capillary loss. However, the pathophysiology and molecular biology of HFpEF are yet to be fully understood. A recently developed paradigm [11•] proposes that the primary cause of HFpEF lies in a systemic proinflammatory state, which prompts coronary microvascular endothelial cells to produce reactive oxygen species (ROS). These limit nitric oxide (NO) bioavailability and lead to the decreased activity of protein kinase G (PKG) in cardiomyocytes. Reduced PKG activity, in turn, removes the brakes on concentric cardiac remodeling and cardiomyocyte stiffening. Both stiffened cardiomyocytes and increased collagen deposition by myofibroblasts ultimately cause diastolic LV dysfunction. Indeed, several studies have supported a causal relationship between endothelial dysfunction/chronic low-grade systemic inflammation and diastolic dysfunction in HFpEF (for a review [12]).

In this scenario, hopes for new efficient therapies for HFpEF rest on drugs directed at myocardial cell remodeling, vascular remodeling, and local and/or systemic inflammation as potentially targetable clinical entities. Indeed, it is more and more evident that HFpEF cannot be treated as a cardiac disease but rather as a syndrome. Many experimental and clinical efforts have been focused on the development of drugs targeting key enzymes involved in the control of both the heart function as well as the concomitant systemic inflammatory response and the regulation of vascular tone, including phosphodiesterases (PDEs) and the p110δ/γ isoforms of class I phosphoinositide-3 kinases (PI3Ks). In this review, we will highlight the most relevant and recent discoveries in the development of PDE-related therapies for HFpEF-associated comorbidities (Table 1). The most promising potential for the development of new therapies for HFpEF relies on the targeting of pulmonary, endothelial, and systemic inflammation. We cannot however overlook the cardiac specific aspects of this pathology; hence, the failures and successes of PDE inhibition in the failing heart will be briefly summarized. Some hits on PI3Ks as new therapeutic targets for HFpEF-associated comorbidities will also be provided.

Molecular Signaling Pathways at the Basis of HFpEF

Irrespective of the considered cause underlying HFpEF, tuned signaling pathways mediated by second messengers play a key role. Among these, pools of cyclic nucleotides, including cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), have been involved in the regulatory mechanisms leading to systemic inflammation and, ultimately, heart failure (for a review see [13•]). Physiologically, cGMP production results from the

Table 1 Pros and cons of the use of specific PDE inhibitors for the treatment of HFpEF- and HFpEF-related comorbidities

PDE family/subfamily	Substrate	Pros of inhibition in HFpEF	Cons of inhibition in HFpEF
PDE1 PDE3	cAMP > cGMP	Blockade of vascular smooth muscle cell growth occurring in pulmonary hypertension.	The efficacy in HFpEF has to be determined.
PDE2	cAMP = cGMP	Protection against cardiotoxic cAMP signaling from substained β-AR stimulation.	Myofibroblast activation with a stiffer connective tissue.
PD3	cAMP > cGMP	Increased myocardial contractility.	Proapoptotic and prohypetrophic activity.
PDE4	cAMP	Potent cAMP-mediated anti-inflammatory responses (effective in COPD).	Severe side effects in the heart.
PDE4B PDE7A	cAMP	Suitable to target systemic inflammation.	Possible secondary effects in the heart.
PDE5	cGMP	Pulmonary vascular dilation regulation.	No effectiveness in large trials including HFpEF patients with and without pulmonary hypertension.
PDE9	cGMP	Suppression of hypertrophy, fibrosis, and chamber dysfunction.	To be defined.

activation of either a soluble guanylate cyclase (sGC), stimulated by NO, or a particulate membrane-located enzyme, coupled to the natriuretic peptide (NP) receptor. Once generated, cGMP activates PKG, which is responsible for the phosphorylation of a number of target proteins and exerts anti-fibrotic and anti-hypertrophic effects in a wide set of experimental in vitro and in vivo cardiac disease models [14]. Of note, cAMP and cGMP pathways influence the dynamics of each other, a process which is referred to as cN crosstalk [15].

cAMP is produced by adenylyl cyclases (AC), which are stimulated by activated β-adrenergic receptors (β-ARs). This second messenger in turn activates cAMP-dependent kinase A (PKA). Signal termination is mediated by the action of both phosphatases and PDEs. Recently, PI3Kγ has emerged as a key player of cAMP compartmentalization within β2-AR/PDE4 microdomains. In healthy cardiomyocytes, PI3Kγ primarily serves as a scaffold protein and negatively regulates cAMP levels downstream of β2-ARs; indeed, PI3Kγ acts as an anchoring protein for PKA (AKAP) and PI3Kγ-bound PKA activates PDE3 and PDE4, resulting in cAMP degradation [16, 17]. In a pathological setting, acute activation of β-ARs, leading to increased cAMP signaling, favors contractility; whereas, chronic β-AR activation elicits detrimental prohypertrophic and proapoptotic effects. By contrast, adenosine-derived cAMP signaling can attenuate many of these effects [18]. Indeed, different pools of cAMP exert opposing effects in pathological cardiac remodeling [19]. Importantly, both cGMP and, in minor part, cAMP critically influence cardiomyocyte compliance through PKG/PKA-mediated phosphorylation of the sarcomeric protein titin [11•, 20].

Both cAMP and cGMP levels are regulated in level and subcellular distribution in a specific and stimulus-dependent manner by PDEs. The functional impact and therapeutic utility of blocking cyclic nucleotide hydrolysis by acting on PDEs have long been recognized and were recently reviewed in [13•]. The molecular diversity of PDEs was first recognized in the 1970s, paving the way to the current organization of 11 structurally-related and functionally-distinct PDE gene families. PDE3 and PDE4 were the first candidates for specific therapeutic targeting in cardiac failure and COPD, respectively. Unfortunately, the hopes arising from preclinical studies were not realized in clinical trials, and many PDE-targeting compounds were put aside, due to lack of efficacy and safety. Actually, only a limited number of agents are approved for widespread clinical use; as an example, the PDE3 inhibitor milrinone is now only approved for the acute treatment of patients with decompensated and refractory heart failure and those waiting for heart transplantation, due to the high mortality associated with its chronic administration. Hopefully, new optimized inhibitors, with more favorable risk-benefit profiles, will be developed in the future.

Therapeutic PDE Inhibition for Myocardial Stiffening in HFpEF

HFpEF patients typically show pathological ventricular hypertrophy, with interstitial fibrosis and diastolic chamber stiffening, this latter being linked to the reduced phosphorylation of the giant cytoskeletal protein titin by PKA and PKG [21]. Overall, the response to physiopathological stimuli is differentially mediated by AC and GC enzymes and by different PDE isoforms, with specific and sometimes opposing functions [13•, 22•]. PDE4, 7, and 8 isoforms are cAMP specific, while PDE1, 2, 3, 5, and 9 (of which both PDE5 and PDE9 are selective for cGMP) regulate cGMP in the heart. Notably, PDE5 and PDE9 are not redundant but target different intracellular pools: PDE5 mainly affects NO-sGC-derived cGMP, whereas PDE9 regulates NP-receptor GC-derived pools [22•].

LV biopsy analysis from HFpEF patients reported low levels of cGMP and reduced PKG activity, particularly when compared with that of HFrEF patients [23]. Administration of sGC activators has been suggested to provide a downstream correction to the low myocardial NO-cGMP availability which characterizes HFpEF. However, the use of sGC activators is seriously hampered by hypotension [24], which is an especially worrisome side effect for HFpEF patients, where LV stroke volume critically depends on adequate filling. Other therapeutic approaches aimed at targeting specific PDE isoforms were therefore pursued.

PDE3 and PDE2 Inhibitors

The first PDE being identified as a possible target in heart failure was PDE3. In the short term, PDE3 inhibitors can be therapeutically exploited to raise intracellular cAMP content in cardiomyocytes and, hence, increase myocardial contractility. Unfortunately, chronic administration leads to an increased cardiovascular mortality due to proapoptotic and prohypertrophic actions; this is especially problematic in elderly patients, in whom cardiomyocyte loss and hypertrophy are major pathologic features (for a complete review on PDE3, see [25]). The adverse consequences of long-term treatment with conventional PDE3 inhibitors in adults with heart failure discouraged further extensive development of therapeutic agents. However, recent discoveries suggested that individual PDE3 isoforms could be targeted through their protein-protein interactions to yield cardioprotective actions without adverse consequences [26]. Nevertheless, at present, drug-like compounds for specific PDE3 isoforms still have to be developed.

Unlike PDE3, myocardial PDE2 is upregulated in human HF [27], possibly as a protective mechanism to counteract cardiotoxic cAMP signaling from sustained β-AR stimulation. Since PDE2 is a central component of the cGMP/cAMP crosstalk, its direct activation has been suggested as an alternative strategy for HF therapy, but this

possibility is still a matter of debate due to opposing results [19, 28]. Notably, PDE2 upregulation is reported to activate myofibroblasts even in the absence of profibrotic stimuli, resulting in stiffer connective tissue [29].

PDE5 and PDE9 Inhibitors: Past Failure and Future Hopes

In the attempt to increase cGMP levels, and hence PKG activity, another attempt was to expand the application of powerful PDE5 inhibitors, like sildenafil, also to HFpEF. The great hopes on the exploitation of sildenafil were however dampened, since PDE5 inhibition was effective only in HFrEF but not in HFpEF [30]. Among potential reasons for such a failure, the lack of PDE5A upregulation and low myocardial cGMP attributed to depressed NO signaling have been proposed [23].

As a further alternative, the inhibition of PDE9 was suggested, in reason of the recent finding of marked upregulation of PDE9 protein in LV biopsies from HFpEF patients [22•]. This suggested that the low cGMP levels might be related to enhanced expression of PDE9; if so, inhibiting this isoform should have beneficial effects. Indeed, in mice subjected to sustained pressure overload, blocking PDE9 by gene deletion or by selective pharmacological inhibition suppressed hypertrophy, fibrosis, and chamber dysfunction [22•]. PDE9A inhibitors appear well tolerated in humans and are being studied for neurocognitive disease (NCT00930059); these new data may arouse interest also in HFpEF and other forms of heart failure. The recent success of LCZ696 (sacubitril/valsartan), a combined angiotensin receptor blocker and neprilysin inhibitor (the latter blunting natriuretic peptide proteolysis) that is being tested in HFpEF (NCT01920711), offered exciting opportunities for a combined treatment [31]. Recent results further support exploring these agents as new tools to treat the failing heart and potentially other organs, in which PDE9A and the natriuretic peptide signaling have a key role [22•].

Comorbidities

HFpEF patients have a significantly higher burden of non-cardiac comorbidities and mortality arising from non-cardiovascular causes compared with those of HFrEF, such as obesity, metabolic syndrome, diabetes mellitus type 2, hypertension, atrial fibrillation, chronic obstructive pulmonary disease (COPD), anemia, and renal dysfunction [32]. Systemic inflammation and endothelial dysfunction are important hallmarks of these comorbidities and, according to the new HFpEF paradigm (Fig. 1), are also importantly involved in HFpEF pathophysiology [11•, 33, 34]. Myocardial inflammation was shown to contribute to extracellular matrix changes in HFpEF, and both myocardial collagen and the amount of inflammatory cells correlated with diastolic LV dysfunction [34]. Of note, endothelial dysfunction is highly prevalent in HFpEF and is correlated to reduced exercise capacity and worse outcome [33].

Intensive management of comorbidities has not been formally tested in HFpEF-specific clinical trials; nevertheless, practice in HFpEF programs is to manage comorbidities aggressively [35], given that these conditions are very common and compete with HFpEF to worsen prognosis [32].

Chronic Microvascular Inflammation, Fibrosis, and Systemic Inflammation

All HFpEF comorbidities are associated with chronic systemic inflammation, as underlined also by the profiling of circulating biomarkers [36]. The emerging paradigm proposes that HFpEF-associated comorbidities are responsible for driving both structural and functional remodeling through systemic endothelial inflammation [11•]. Indeed, in HFpEF patients, systemic inflammation affects multiple organs, including the skeletal muscles and kidneys [35]; this leads to reduced tolerance to effort, due to inadequate skeletal muscle perfusion and to impaired renal microcirculation, with the consequently reduced ability to excrete sodium. Hence, targeting systemic inflammation emerges as a primary target to prevent the deleterious cascade of events, which finally engenders diastolic dysfunction and heart failure.

Containment Strategies for Myocardial Fibrosis

Besides cardiomyocyte intrinsic defects, myocardial remodeling stems from microvascular inflammation and endothelial expression of adhesion molecules, which attracts infiltrating leukocytes. These, in turn, secrete transforming growth factor β (TGF-β), finally leading to enhanced interstitial collagen deposition, which leads to diastolic dysfunction [11•]. Moreover, endothelial inflammation results into increased ROS and reduced NO bioavailability. This, in a vicious cycle, reduces sGC activity and cGMP content and depletes the favorable effects of PKG on adjacent cardiomyocyte stiffness and hypertrophy.

Recently, lysyl oxidase-like 2 (LOXL2) was found to be upregulated in the interstitium of HFpEF human hearts. In HF experimental models, increased LOXL2 expression leads to increased TGF-β production, triggering the formation and migration of myofibroblasts, with the consequent enhanced collagen deposition and crosslinking in hypertrophic regions [37]. Notably, LOXL2 promotes TGF-β production through the PI3Kα-Akt pathway [37]. This finding paves the way to the possibility of targeting specific PI3K isoforms to prevent matrix remodeling. However, due to its ubiquitary expression and multiple roles in growth, proliferation, and metabolism,

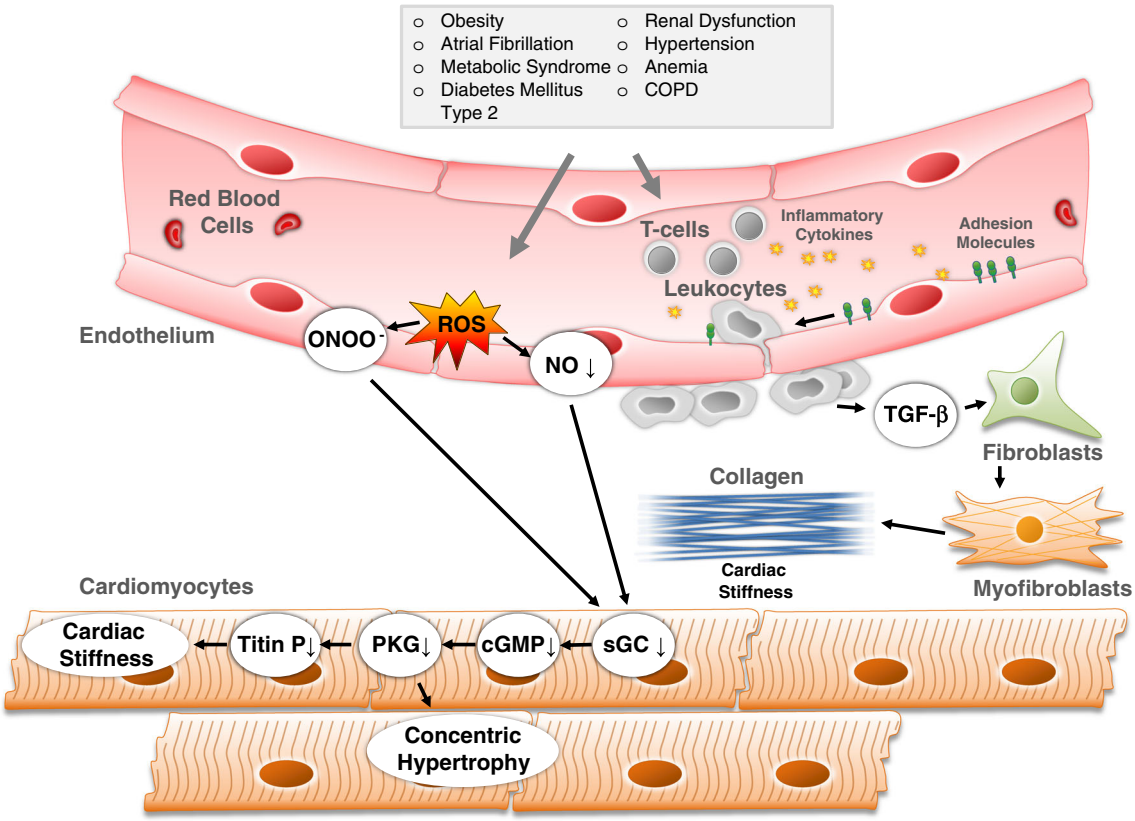

Fig. 1 From comorbidities to heart failure with preserved ejection fraction (HFpEF). According to the HFpEF paradigm, the systemic proinflammatory state stimulates cardiac microvascular endothelial cells to produce reactive oxygen species (*ROS*) and cell *adhesion molecules*. Production of ROS leads to formation of peroxynitrite (*ONOO−*) and to reduced nitric oxide (*NO*) bioavailability, which both lower the activity of soluble guanylate cyclase (*sGC*) in the adjacent cardiomyocytes. Reduced sGC activity decreases cyclic guanosine monophosphate (*cGMP*) levels and protein kinase G (*PKG*) activity. This results in the hypophosphorylation of the giant cytoskeletal protein *titin* and removes brakes on hypertrophic stimuli, ultimately leading to increased cardiomyocyte stiffness. At the same time, *ROS* favor the migration of leukocytes in the subendothelium. These release transforming growth factor-β (*TGF-β*), which stimulates the conversion of *fibroblasts* into collagen-producing *myofibroblasts*, contributing to further stiffening of the myocardium

the feasibility of a chronic systemic targeting of PI3Kα is scarce.

Besides LOXL2, mechanisms regulating extracellular matrix remodeling include PKG suppression of TGF-β signaling [38] and PDE1/PKA-regulated crosstalk between cardiomyocytes and cardiac fibroblasts [39]. PDE1 represents a major PDE activity in the human myocardium; in particular, PDE1C, whose expression is increased in failing human and mouse hearts [39], is the dominant isoform in human hearts; therefore, cAMP and cGMP may both be impacted by its inhibition. To date, however, synthesis of PDE1 inhibitors has been insufficient for chronic in vivo studies and its role in cardiac biology and disease remains elusive.

PDE Inhibitors to Reduce Systemic Inflammation

Given their potent anti-inflammatory effects, PDE4 inhibitors are gold candidates for treating diseases involving aberrant immune responses. Apremilast, a second-generation PDE4 inhibitor, is actually in Phase II (NCT02576678) and Phase III (NCT01212770) trials for use in psoriasis and psoriatic arthritis, respectively, possibly representing a suitable target for systemic inflammation in HFpEF. However, caution should be used when using PDE4 inhibitors since PDE4B has been shown to regulate Ca^{2+} current to protect against ventricular arrhythmias in mice [40].

Besides PDE4, the PDE7A isoform is also widely expressed in inflammatory cells, especially in T lymphocytes [41]. The coexpression of PDE4 and PDE7 in immunoinflammatory cells and the synergistic effects of PDE7- and PDE4-selective drugs in the suppression of inflammation suggest that dual PDE4/7 inhibitors could be particularly effective to treat diseases characterized by a generalized proinflammatory phenotype [42].

Hopes and Doubts on PI3K Inhibitors

Besides PDE enzymes, PI3Ks have been identified as potential targetable entities for the treatment of inflammation-related comorbidities. Of the eight isoforms of the PI3K family, class I

PI3Ks have been implicated in the pathogenesis of various diseases, including inflammation and autoimmunity, with emerging roles in cardiovascular disorders [43]. Both PI3Kδ and PI3Kγ are highly expressed in leukocytes, and multiple immune-related disease indications for these PI3K isoforms have been recognized [44, 45]. Notably, PI3Kγ is also expressed at relatively low levels in cardiomyocytes, smooth muscle cells, and endothelial cells. Such an interplay between PI3Kγ-expressing cells implicates that interfering with p110γ activity could be beneficial in cardiovascular disease by simultaneously relieving myocardial cell-autonomous defects, alleviating systemic inflammation, and preventing maladaptive remodeling [46].

A wide range of inhibitors that target single or multiple PI3K isoforms has been developed. Given the importance of PI3Ks in cell biology, a challenge has been the toxicity profile of pan-class I PI3K inhibitors. Promising isoform-selective inhibitors for PI3Kδ and PI3Kγ were developed only recently [47]. In 2014, an inhibitor of the p110δ isoform of PI3K was approved for the treatment of specific blood malignancies [48]. A paper showing increased genomic instability in normal and neoplastic B cells due to PI3Kδ inhibitor [49], however, recently dampened the great enthusiasm on the potential use of PI3Kδ inhibitors. This effect should be carefully considered in long-term follow-up, since such inhibitors can be administered to patients for years. It remains to be explored whether novel and safe approaches to treat systemic inflammation might be effective in HFpEF.

Pulmonary Hypertension

A significant proportion of HFpEF patients suffers from concomitant pulmonary hypertension, which complicates the clinical course [50]. In HFpEF, pulmonary hypertension, both at rest and in response to exercise, might result from the development of pulmonary vascular disease [51]. Increased pulmonary artery pressure and vascular resistance, resulting from vasoconstriction and remodeling of the small pulmonary arteries, finally lead to reduced blood oxygenation, dyspnea, heart failure, and eventually death. Moreover, changes in the lung parenchyma, albeit less characterized, also play an important role in HFpEF [52].

PDE5, which has a relatively high expression in the airway and vascular smooth muscle, is thought to be a key regulator of pulmonary vascular dilation and remodeling via its modulation of cGMP-PKG signaling. PDE5 inhibitors, such as sildenafil, are currently approved for treatment of pulmonary arterial hypertension [53]. An early single-center trial in patients with HFpEF and pulmonary hypertension reported salutary effects on pulmonary hemodynamics following treatment with sildenafil [54]. However, as discussed above, subsequent larger trials including both HFpEF patients with and without pulmonary hypertension failed to corroborate this finding [55, 56].

PDE1 and PDE3 may also be a crucial regulator of pathological vascular and airway smooth muscle remodeling. Thus, a dual-targeting approach might block the growth of vascular smooth muscle cells and reduce the vascular remodeling that occur in pulmonary hypertension. Whether this approach could also be suitable for HFpEF still has to be determined.

More promising is the oral sGC stimulator riociguat, which improved exercise tolerance or quality of life in pulmonary arterial hypertension, chronic thromboembolic pulmonary hypertension, and pulmonary hypertension attributable to HFrEF [57]. Actually, riociguat is in Phase II clinical trial (DYNAMIC trial, NCT02744339) for the treatment of HFpEF, and results are expected to be published in 2019.

Chronic Obstructive Pulmonary Disease (COPD)

The number of patients with HF and obstructive pulmonary disease is likely to increase worldwide [58, 59]. For HF and COPD, it was estimated that about 20–35% of patients have both diseases [60]. Prevalence of COPD in HF seems to be equally distributed between patients with reduced and preserved EF; however, patients with preserved EF have more severe COPD. Vice versa, cardiovascular comorbidities are common in patients with COPD, influencing approximately half of all hospitalizations and 20–30% of all deaths [61]. Clinically, COPD is characterized by progressive airway inflammation and obstruction, as well as a decline in lung function, with acute recurrences and exacerbations that lead to a markedly reduced quality of life and eventual death. Despite the significant interest of the scientific community and industry, no relevant therapy to improve outcome in COPD and HFpEF is available to date [62, 63]. In fact, about half of the patients remain undiagnosed, and more than half of those with diagnosis receives symptomatic relief only.

Currently, the most advanced line of research for COPD treatment focuses on the development of PDE inhibitors for both parenteral and inhaled administration.

PDE4 Inhibitors to Dampen Airway Inflammation

PDE4 isoforms have a relatively high level of expression in cells that regulate immunoinflammatory responses and tissue remodeling. Because early PDE4-selective inhibitors (especially rolipram) exhibited potent cAMP-mediated anti-inflammatory responses in cellular and animal models, PDE4 inhibitors were developed for clinically important, inflammation-related pulmonary diseases, including COPD. Early PDE4 inhibitors were hampered by gastrointestinal side effects; hence, more advanced PDE4 inhibitors were developed, including cilomilast and roflumilast, and reached phase III trials (NCT00103922, NCT01973998). Notably, roflumilast is now approved as an oral treatment for reducing the risk of exacerbations in patients with COPD [64]. In

COPD, roflumilast can be used alone or together with standard bronchodilator treatments, such as salmeterol (a long-acting β2-adrenergic receptor agonist) or tiotroprium (a short-acting anticholinergic drug) [65]. Nevertheless, the severe side effects associated with systemic and isoform-unselective PDE4 inhibition often require dose-lowering, with a consequent negative impact on clinical efficacy. Change in the route of administration from enteral to inhaled, together with isoform-selective blockade, might likely increase efficacy and reduce adverse events.

As a second approach to overcome major disadvantages, new PDE4 inhibitors with reduced emetic effects need to be developed (the PDE4D allosteric modulator D159687 is an emerging example) [66]. Moreover, inhibition of PDE4D in particular, rather than other PDE4 isoforms, is associated with the well-known gastrointestinal side effects of PDE4 inhibitors, whereas PDE4B seems to be pivotal in airway inflammation and hyperactivity. Such studies provided the rationale for the development of PDE4B-selective inhibitors as promising therapeutics to reduce inflammation.

Combined Isoform-Specific PDE Inhibitors: Two Hits, Multiple Benefits?

Xanthine derivatives like theophylline and caffeine, which inhibit all PDE families (except PDE8 and PDE9), have been clinically used primarily as bronchodilators in the treatment of pulmonary diseases, including COPD. These drugs, however, are challenged by a narrow therapeutic window and considerable toxicity (nausea, emesis, and arrhythmias). Nevertheless, several PDEs may likely need to be simultaneously targeted for the effective treatment of complex diseases such as COPD. Novel PDE inhibitors should possibly provide simultaneous inhibition of multiple specific PDEs, thereby producing additive or synergistic beneficial effects. The major impetus to develop PDE inhibitor therapy (despite past failures) mainly relates to the desire to take advantage of the powerful anti-inflammatory effects of PDE4 inhibitors, by concomitantly enlarging the narrow window between anti-inflammatory and side effects that greatly limit their application.

Notably, the anti-inflammatory effects of PDE4 inhibitors are not identical in all immune inflammatory cells. Consistently, inhibitors of PDE7 and PDE3 have been shown to enhance the effects of PDE4 inhibitors in T cells and macrophages, respectively. Moreover, since PDE4 inhibitors do not exert bronchodilator effects, an combinatorial approach could maximize the efficacy of PDE4 inhibitors without adversely affecting risk-to-benefit ratios. However, evidences indicating that combined PDE1 and PDE4 inhibitors (like KF19514) could be beneficial in pathological airway remodeling (mediated by PDE1) and pulmonary inflammation (driven by PDE4) [67] were not confirmed with clinical studies. PDE3 inhibitors are bronchodilators and, similar to PDE7 inhibitors, increase the effects of PDE4 inhibitors in inflammatory cells. Several inhibitors with dual selectivity for PDE3 and PDE4 have been developed as potential therapeutics for COPD. Two early dual PDE3-PDE4 inhibitors (zardaverine and benafentrine) only had modest and short-lived bronchodilatory effects in humans, while the trequisin analog RPL554 is currently undergoing trials for asthma and COPD [65].

Inhalatory Formulations for COPD Therapy

Inhaled medications are the cornerstone of the pharmacological treatment for patients with pulmonary diseases, including COPD. Several inhaler/nebulizer devices exist, and each of them has specific characteristics, as well as pros and cons [68]. Alternative approaches for targeting PDE4 in pulmonary disease might reasonably include inhaled formulations, as well as combinations of inhaled PDE4 inhibitors and corticosteroids or β-adrenergic receptor agonists. This strategy would enable specifically targeting airways, thereby possibly reducing dosage and off-target side effects. Of note, the inhaled PDE4B inhibitor GSK256066, characterized by a particularly high target affinity, is one of the most potent PDE4 inhibitors compared with roflumilast and multiple other inhaled PDE4 inhibitors. Nevertheless, GSK256066-induced increase in lung function did not meet the threshold of clinical significance in a Phase II trial in patients with moderate COPD [69].

Another inhaled PDE4 inhibitor, CHF6001, is currently in Phase II trial for moderate and severe COPD (NCT03004417). Moreover, the nebulized PDE3-PDE4 inhibitor RPL554 is entering the Phase II trial for COPD in combination with tiotroprium (NCT03028142).

Intriguingly, the recent development of inhaled formulations for PDE4 inhibitors by GlaxoSmithKline (GSK) was included in a double-research program, including inhaled PI3Kδ inhibitors. As in the case of PDE inhibitors, indeed, drug tolerability could also be an issue for systemic PI3K inhibitors, due to mechanism-based on-target side effects. Again, a potential way to overcome the hurdle is through topical applications. Of note, an inhaled PI3Kδ inhibitor GSK2269557 is undergoing a Phase II trial for the treatment of COPD subjects with acute exacerbation (NCT02522299).

Conclusions

Despite past failures dampening the enthusiasm of PDE inhibition as an efficient strategy to treat cardiac diseases at large, the important number of recent reviews [70] and clinical trials registered on the ClinicalTrials.gov website indicate that the interest in PDE inhibitors is still vivid. Many of these trials involve US Food and Drug Administration (FDA)-approved

PDE3, PDE4, and PDE5 inhibitors and are expected to prove the feasibility of a repurposing of these inhibitors in HFpEF and related comorbidities. In parallel, preclinical efforts are focused on the functional characterization of previously unappreciated PDE isoforms, such as PDE9, which are emerging as key regulators of exquisitely compartmentalized signaling domains. These findings envisage the possibility of targeting these specific isoenzymes to interfere with local and discrete signaling events and to provide higher therapeutic benefits and reduced side effects. At the same time, other strategies, such as development of PDE and PI3K inhibitors suitable for topical administration, are under consideration and are expected to further contribute to effective therapies with a more favorable risk-benefit profile. We feel that some of these approaches show promise to succeed where traditional drugs and the parenteral delivery route failed.

Nevertheless, considering the complexity of the HFpEF syndrome, it is likely that hitting a unique, specific enzyme may not produce the desired therapeutic effectiveness, and combinatorial treatments targeting, for example, different PDE/PI3K enzymes, responsible for distinct pathological manifestations or comorbidities, may be required. It is clear that this is an exciting field, which is worthy of future investigations and investments. As preclinical and clinical studies with PDE and PI3K inhibitor progress, we will understand whether PDE/PI3K inhibitors are definitively condemned or deserve another chance.

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Compliance with Ethical Standards

Conflict of Interest Valentina Sala, Jean Piero Margaria, Alessandra Murabito, Fulvio Morello, and Alessandra Ghigo each declare no potential conflicts of interest.

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