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Unlocking the AhR Therapeutic Potential for Cystic Fibrosis With an Integrated Mucosal Platform for Drug Screening

*Lorenzo Sardelli, Enrica Frasca, Valentina Olga Garbero, Cosmin Butnarasu, Alex Affricano, Claudio Medana, and Sonja Visentin**

Bacterial-derived molecules are at the basis of bacteria–bacteria and bacteria–host communication. In the context of cystic fibrosis (CF), they are considered possible therapeutic molecules for their natural binding capability on the immunomodulatory cytoplasmic aryl hydrocarbon receptor (AhR). An exponentially growing number of bacteria-derived molecules are identified as AhR activators, highlighting the need for systems to screen possible lead candidates. This challenge is addressed by applying an in vitro tool mimicking the two main barriers that potential AhR-targeting drugs must overcome: the cytoplasmic membrane and the CF pathological mucus. A small dataset of AhR ligands with potential therapeutic applications is selected. The apparent permeability of bacterial-derived molecules across a cellular membrane model is quantified and molecules capable of reaching the cytoplasmic target (AhR) are identified. In a second step, a CF in vitro mucus model is integrated with the phospholipid membrane and the impact of mucus on permeability is assessed. Overall, this study proposes an integrated mucosal platform as a suitable tool in the emerging field of postbiotics as a therapeutic strategy for CF. The mucosal platform can enable the rapid identification of molecules compatible with cytoplasmic targeting of AhR among candidate-drug representatives.

1. Introduction

Cystic fibrosis (CF) is one of the most common and severe genetic diseases in Europe (30% prevalence) and affects more than 150 000 people worldwide, reducing life expectancy with a median age of death between 24 and 37 years.[1] CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to a pathological secretion of mucus in terms of quality (e.g., dehydration) and quantity (e.g., overproduction), hampering mucociliary clearance and promoting the accumulation of bacteria that exacerbate chronic lung inflammation[2–6] Various approaches were developed to break this vicious circle and improve the symptomatology of CF patients through antibiotic treatments, mucus-loosening medications, anti-inflammatory drugs, and CFTR modulators.[7,8] Despite the remarkable therapeutic achievements in recent years, research persists in the quest for increasingly innovative treatments. In particular, bacteria-derived metabolites (postbiotics) gained the attention of researchers for their multiple advantages, such as quasi-unlimited variegation of chemical–physical properties, natural origin, and compatibility with possible mass production through fermentation pipelines.[9–11]

The bacteria-derived metabolites (postbiotics) have been successfully studied for their antimicrobial,[12,13] antibiofilm,[14] immunomodulatory activity,[15] and as neurotransmitters.[16] In the context of CF, some bacterial metabolites have been investigated for their ability to modulate both bacteria and host immune cells, leading to a potential innovative treatment.[17] Here, the cytoplasmic aryl hydrocarbon receptor (AhR) plays a pivotal function for immune homeostasis maintenance and infection resistance in physiological conditions.[18] Traditionally viewed as a xenosensor, more recently, it started to be considered a potential therapeutic target[17,19] (**Figure 1**). The number of molecules recognized as AhR modulators is increasing at a tremendous rate and encompasses a wide range of compounds, ranging from food derivatives, microbiome-derived molecules, and plant-derived metabolites to endogenous tryptophan metabolites.[20,21] Up to date, only a few classes of bacteria-derived molecules have been considered for CF treatment.[17,21,22] In particular, bacteria-derived tryptophan metabolites were appreciated for their capability to modulate AhR activity in in vitro and in vivo models,[23–25] opening up new multimodal therapeutic strategies with the potential to restore the impaired drug-metabolization capability and the bacterial resistance in CF-lung tissues.[26,27]

L. Sardelli, E. Frasca, V. O. Garbero, A. Affricano, C. Medana, S. Visentin
Department of Molecular Biotechnology and Health Science
University of Torino
via Nizza 44bis, Torino 10126, Italy
E-mail: sonja.visentin@unito.it

C. Butnarasu
Institute of Pharmacy Biopharmaceuticals
Freie Universität Berlin
SupraFAB, Altensteinstr. 23a, 14195 Berlin, Germany

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adtp.202400141

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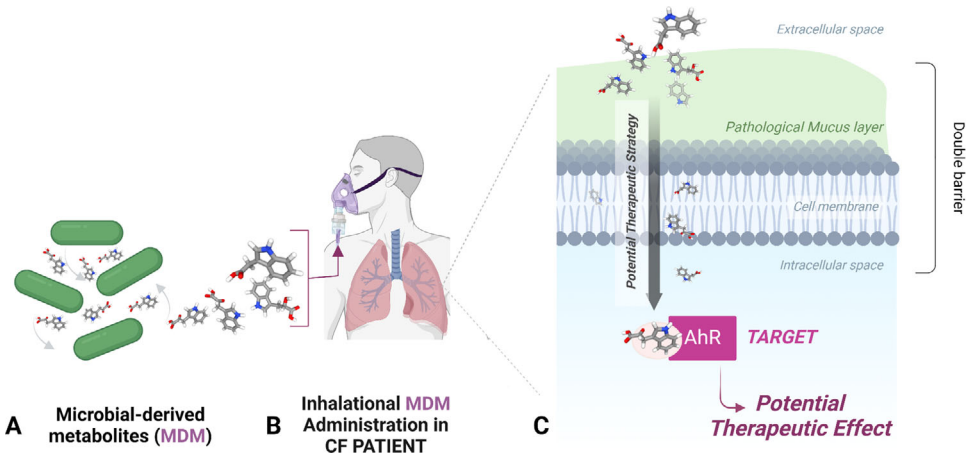

Figure 1. A) Graphical representation of the potential therapeutic strategy of AhR-targeting by microbial-derived metabolites (MDM), promoting protective effects for the pathological scenario of inflamed and infected CF lung tissues. B) Some metabolites produced by bacteria (MDM) are promising new therapeutic compounds and pave the way for future inhalation administrations in CF patients. C) As AhR ligands, these metabolites have been observed to support defensive responses in CF lungs. However, to reach their target (AhR) in the cytoplasm, these molecules must pass through two barriers: the pathological mucus and the cell membrane.

Among them, new evidence demonstrated that the Indole-3-Carboxaldehyde (ICA) administered by inhalation exerts protective effects in the inflamed lung tissues of a murine model of CF.[22]

Although tryptophan metabolites are the main class of molecules considered for a possible AhR-targeting therapeutic strategy of CF, the same approach is, in principle, applicable to other bacteria-derived molecules that are active on AhR. Molecules derived from the bacterial metabolism of polyphenols, for example, are extensively studied as AhR ligands with different positive effects in pathological inflammation, such as reduction of neutrophil infiltration within tissues, diminished production of pro-inflammatory oxidants (including NFκB, TNF-α, COX-2, IL-1β, and IFN-γ), and restoration of IL-10 levels.[28–31] Similarly, quorum sensing (QS) molecules are bacteria-derived entries at the base of interspecies (bacteria–bacteria) and interkingdom (bacteria–host) communication.[18,27–31]

Tryptophan, polyphenolic metabolites, and QS molecules are just a few representatives of the vast source of naturally bioactive molecules represented by postbiotics.[11,32] Their molecular variety brings not only the advantage of a virtually unlimited library of drug candidates but also the urgent need for screening systems that can quantify key pharmaceutical aspects as rapidly and informatively as possible. From a therapeutic perspective, pharmacokinetic properties such as permeability have never been thoroughly explored for postbiotics, even if this aspect is a key factor in the design and development of new drugs. Poor membrane permeability often translates into poor or non-existent in vivo efficacy. In the specific context of CF, the molecular journey of postbiotics from the extracellular environment to the cytoplasm is further complicated by the pathological mucus, which can strongly reduce the permeability, and consequently, the bioavailability of drugs.[33–35]

Cellular models are extremely useful in quantifying active transport, but they lack standardization and speed, both key aspects for early-stage drug discovery. However, for the specific scenario of cystic fibrosis, they fail to recapitulate key in vivo elements of mucus, such as rheological properties and microstructure, which are essential in therapeutic development. Similarly, in vivo models are too complex to isolate the contribution that the cytoplasmic and mucosal barriers individually have on permeability of molecules.[36] These limitations are significant for drugs targeting a cytoplasmic receptor like AhR as molecules must cross both cytoplasmic and the mucus barrier to reach the target.

In light of that, a detailed assessment of the partitioning of a given species in the mucus/membrane system is vital from a pharmacokinetic standpoint. New therapeutic candidates targeting intracellular targets must be carefully assessed for their ability to overcome the CF mucus barriers.

This work addresses the challenge of the in vitro characterization of the permeation of bacteria-derived molecules as advanced AhR-targeting therapeutics for CF. In particular, we quantify the permeability of selected bioactive molecules through an in vitro non-cellular-based permeability system (i.e., PermeaPad)[33] and discuss possible implications for developing new AhR-targeting therapeutics. Further, we address the complex issue of the CF mucosal barrier by measuring the impact of a pathological mucus model on the passive diffusion of our dataset. We believe that our approach could be easily extended to many more bacteria-derived molecules with therapeutic potential. Nevertheless, the integration of our findings with efficacy data could pave the way for the development of treatments that effectively penetrate the CF mucosal barrier, ultimately improving patient outcomes.

2. Results and Discussion

2.1. Selection of Representative Postbiotic Molecules and Positioning in the Chemical Space of Approved Drugs

Since the AhR was first described, the number of compounds able to modulate the AhR has greatly increased.[20] Among them, we retrieved AhR ligands of host and microbial origin previously reported in the literature and investigated their

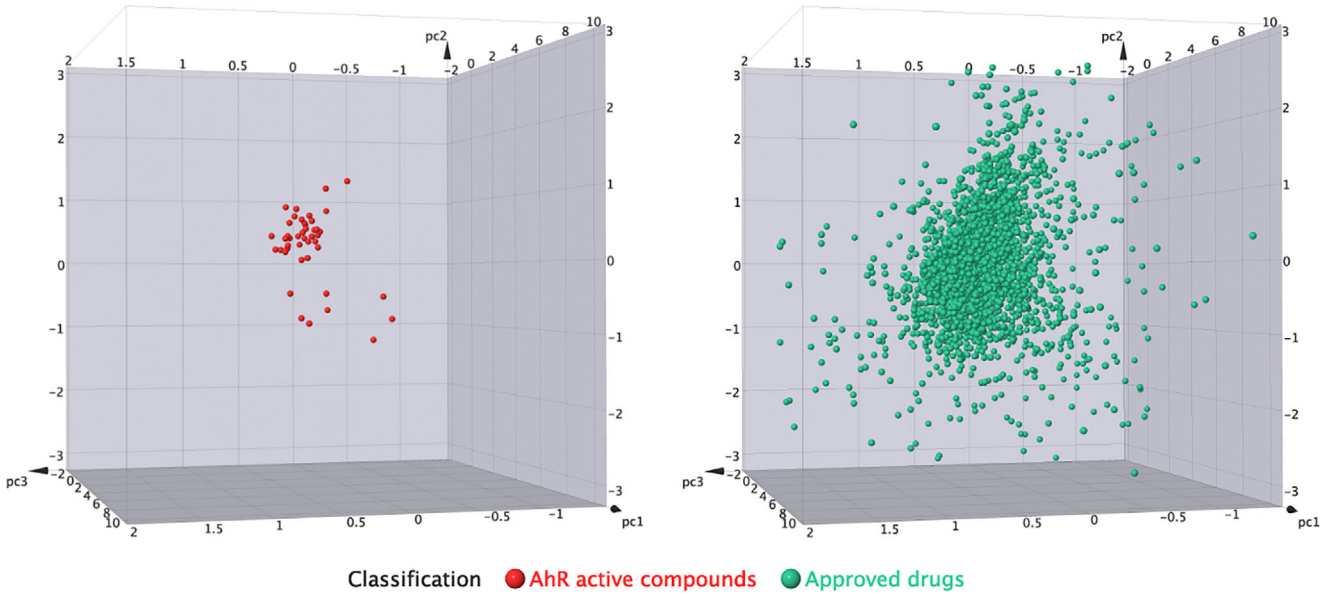

Figure 2. AhR ligands of host and microbial origin occupy a limited portion of the chemical space of the approved drugs. Principal component analysis of AhR ligands of host and microbial origin within the chemical space constructed on the Lipinski (MW, cLogP, HBA, HBD) and Veber (TPSA, NRotBs) descriptors. AhR ligands are retrieved from ref. [20].

distribution within the chemical space of the approved drugs.[20] We defined the chemical space based on the molecular descriptors of the Lipinski (i.e., MW, cLogP, HBA, and HBD) and Veber (i.e., TPSA and NRotBs) rules (**Figure 2**). Molecules exhibiting activity on AhR tended to occupy a relatively narrow region of the chemical space when compared to the broader landscape occupied by the approved drugs. Despite many AhR-targeting ligands having already shown important in vitro results, such as in CF models,[22] atopic dermatitis,[20] cancer,[37,38] viral infections[39] and tissue repair,[40] their observed in vitro activity often is not an indicator of transferable in vivo activity. Indeed, in addition to in vitro activity, the identified hits must have an optimal pharmacokinetic (PK) profile to be able to overcome biological barriers and reach their target in vivo. Thus, early-stage screening of identified hits based on in vitro activity is critical for identifying lead candidates with optimal PK profiles.

To shed light on this aspect, we selected three families of postbiotics, each able to bind the AhR, and investigated their ability to overcome biological barriers (**Figure 3**).

The first family is composed of four different compounds representing the postbiotic molecules derived from the tryptophan metabolism:[20] indole (IND), indole-3-acetic acid (IAA), indole-2-lactic acid (ILA), and indole-3-carboxaldehyde (ICA). The indole and derivatives are considered interesting molecules as new potential therapeutics of CF for their multifaceted role in both bacteria–host communication through their capability to bind to AhR[17] and to suppress the production of *Pseudomonas aeruginosa* virulence factors,[41] biofilm formation,[42] and swimming motility,[43] as well as to positive immune activation.[44] Among the tryptophan metabolites, we consider ICA as particularly relevant for its promising therapeutic potential for CF in two proof-of-concept studies.[22,27] The second family of postbiotics is composed of molecules derived from the bacterial metabolism of polyphenols. We select as representative molecules the 3,4-dihydroxyphenylacetic acid (DOPAC) and gallic acid (GA) as they are AhR-binders exhibiting potent antioxidant properties and promoting the expression of drug-metabolizing enzymes in an AhR-dependent mechanism.[20,45] Lastly, we consider quorum sensing molecules (QS), such as 2-heptyl-3-hydroxy-4-quinolone (HQ) and *N*-(3-oxododecanoyl)-L-homoserine lactone (HOL), which are reported to be strong AhR-binders,[46] whose activation is a key step for triggering the host defences thus countering early-stage infections without devolving into persistent inflammation.[18]

2.2. Overcoming the Cellular Membrane Barrier: Permeability Across the Biomimicking PermeaPad System

Permeability is a key property to be considered in the early phases of drug discovery as it defines, together with solubility, the bioavailability of a drug. In an attempt to provide a tool that is suitable for fast quantification of the key pharmaceutical aspect of the permeability of AhR-binding compounds, we measured the P_{app} of the selected molecules through an in vitro model of the cellular membrane.

The PermeaPad bio-mimetic system was used as a permeability model because it consisted of an advanced mimetic barrier made of vesicles of phospholipids (**Figure 4A**). As a result, the permeability measured on PermeaPad was the combined result of passive and paracellular diffusion.[47] Seeking standardization, we performed a permeability-based classification of the postbiotic molecules with a control reference molecule (caffeine, CA) and set a permeability threshold (i.e., $1.15 \pm 0.32 \times 10^{-6}$ cm s^{-1}) that we previously reported to be useful to discriminate between high- and low-permeable compounds.[33] The permeability was maximum for ICA, reaching the P_{app} of $54.21 \pm 18.12 \times 10^{-6}$ cm s^{-1}, and minimum for GA, which showed a P_{app} of 0.09 ±

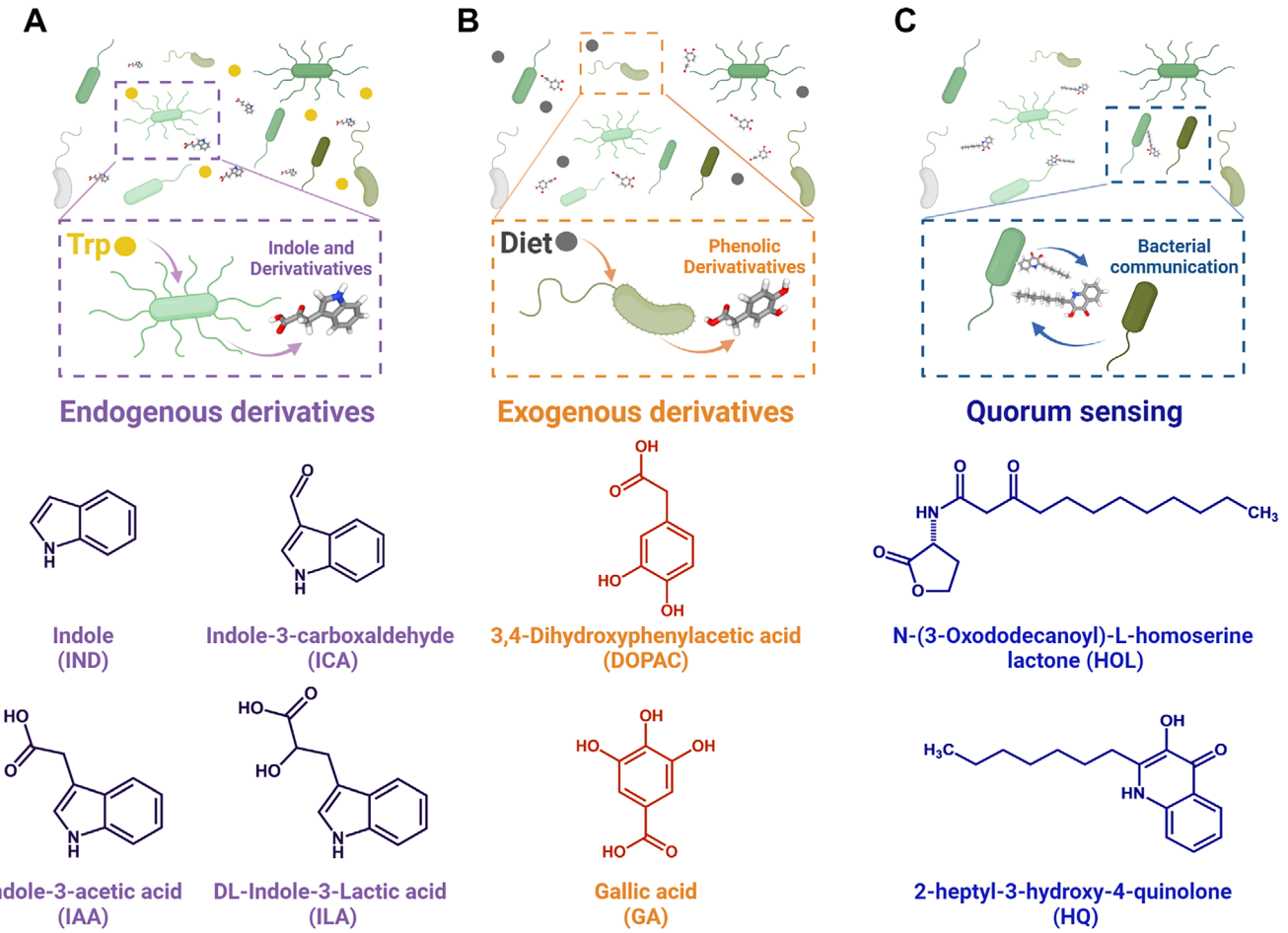

Figure 3. Classes of postbiotic molecules, emerging potential AhR-targeting therapeutics, selected for permeability investigation. A) Tryptophan metabolites (purple) are represented by indole (IND) and its derivatives indole-3-acetic acid (IAA), indole-2-lactic acid (ILA), and indole-3-carboxaldehyde (ICA). B) Poly-phenol-derived postbiotics (orange) are represented by 3,4-dihydroxyphenylacetic acid (DOPAC) and gallic acid (GA); while, C) 2-heptyl-3-hydroxy-4-quinolone (HQ) and *N*-(3-oxododecanoyl)-L-homoserine lactone (HOL) represent the family of postbiotics derived from quorum sensing molecules (blue). Caffeine (CA) is selected as a standard molecule with high permeability, as well as for its AhR-binding properties.

0.03×10^{-6} cm s^{-1} (Figure 4B). One molecule, HQ, resulted in being completely non-permeable with null P_{app} values. Generally, in our experimental setup, the indole and derivatives resulted to be more permeable than the QS molecules and phenolic metabolites (**Figure** 4C). For example, the average P_{app} measured for indole derivatives (17.99×10^{-6} cm s^{-1}) was ≈141× higher than the average permeability of QS molecules and 44× higher than phenolic metabolites. Among indole and its derivatives, ICA doubled the P_{app} of indole (IND), exceeded at least 10× the permeability of the other indole-derivatives (ILA, IAA) and by a factor of 7× the P_{app} of caffeine, considered a highly-permeable standard molecule in PermeaPad ($P_{app} = 7.18 \pm 2.65 \times10^{-6}$ cm s^{-1}, comparable to literature data[33]).

The higher permeability of ICA and IND compared to the other derivatives can be explained as a result of molecular charge, which is known to decrease drug permeability.[48] At the experimental pH tested (i.e., 7.4 pH), the carboxylic group present in IAA and ILA is deprotonated, resulting in a structure with an overall negative charge (Figure 4D). Mechanistically, the permeability of negatively charged indole derivatives through the phospholipid vesicles of the membrane might be strongly suppressed, while the resulting observed permeability might be accounted for only by para-vesicular diffusion.

The permeability values demonstrate that indole and derivatives can easily permeate through the in vitro cellular membrane, supporting the evidence of these probiotics as potential bioactive molecules targeting the AhR. However, from a pharmaceutical point of view, limiting the selection of drug candidates at a class level is insufficient for reliable outputs. A more refined chemical description of same-class molecules is indeed needed to improve a more effective selection of new therapeutic compounds. The presence of charged functional groups, for example, induced a non-negligible reduction in permeability in acid indole derivatives; while, a neutral charge was associated with higher P_{app}.

Importantly, the best-performing molecules in terms of permeability (ICA) found here corresponded to the indole-derivative already applied in vivo as a new therapeutic agent for CF.[22] These findings were also consistent with those reported for other indolic derivatives, whose high permeability through Caco-2 cell monolayer was further associated with

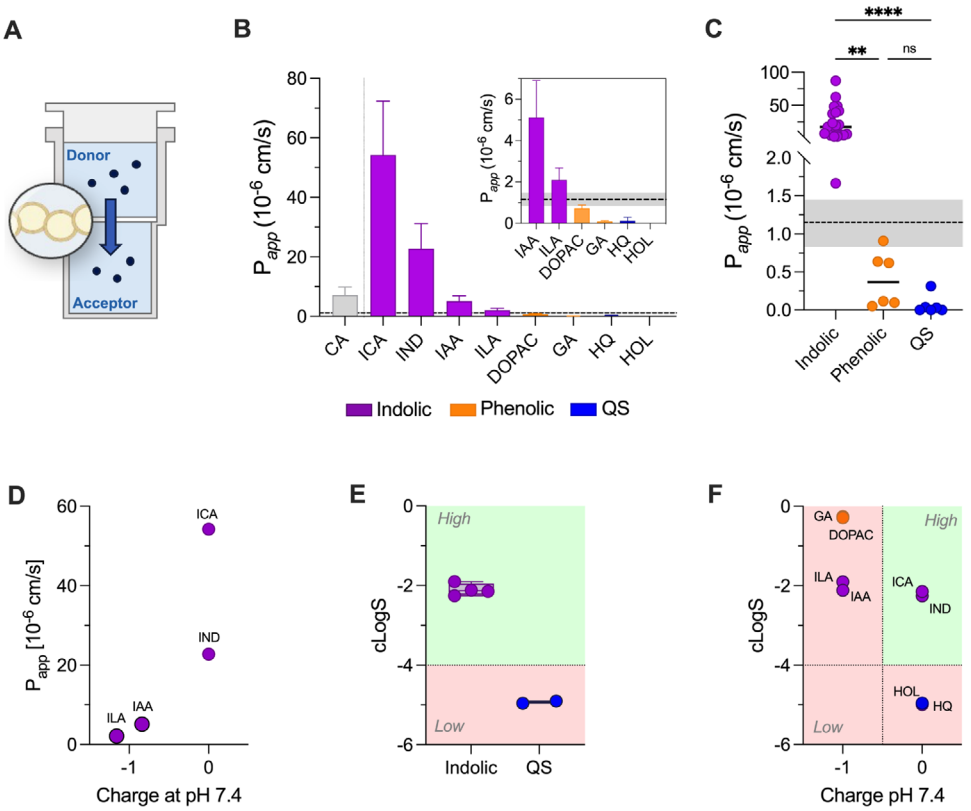

Figure 4. A) Graphical representation of the PermeaPad setup for quantification of the permeability. B) Apparent permeability (P_{app}) of the dataset (purple for indole and derivatives, orange for phenolic derivatives, blue for QS molecules, and grey for caffeine, respectively). The insert represents a magnification of the permeability values of the molecules with P_{app} lower than 10×10^{-6} cm s^{-1}. The threshold 1.15 ($\pm$0.32) $\times 10^{-6}$ is represented by the grey area. C) P_{app} of the three classes of bacteria-derived compounds considered. D) Relation between charge and permeability of indoles. E) Comparison of the solubility (cLogS) of indoles and QS compounds. F) Scatter plot of charge at pH 7.4 and solubility. The red and green areas in (E,F) highlight regions of low and high permeability, respectively.

good potency on AhR, demonstrating favorable drug-likeness properties.[40]

Differently from what was observed for indole and derivatives, phenolic-derivatives (DOPAC and GA) and the QS molecules (HQ and HOL) were far below the permeability threshold, showing a max P_{app} of $0.72 \pm 0.16 \times 10^{-6}$ cm s^{-1} (DOPAC). In our previous work, we identified solubility (cLogS) as a key molecular descriptor for predicting permeability in the PermeaPad system.[33] We showed that a cLogS threshold of ≈ -4 can be used to discriminate between high (cLogS > −4) and low (cLogS < −4) permeable molecules. Following this evidence, we can infer that the different permeabilities of the indoles and QS molecules can be explained by their high and low solubility, respectively (**Figure** 4E). Phenolic compounds are highly polar; yet, highly soluble (**Figure** 4F). Despite the high solubility, they show low permeabilities. This evidence suggests that for relatively small molecules (i.e., MW < 300 g mol^{-1}), the charge and polarity rather than solubility can strongly limit the diffusion through the membrane. Based on our results, QS and phenolic molecules are less appealing than indole and its derivatives as new potential drugs for CF. From a therapeutic perspective, phenolic-derivatives and QS molecules would be less efficient in reaching the cytoplasmic target by only passive diffusion and would require other diffusion mechanisms, such as facilitated or active transport.[49–51] However, it is important to underline that the low permeability of other molecules in the PermeaPad system does not necessarily indicate a lack of biological activity in vivo. For instance, the P_{app} of DOPAC corresponds to a concentration of ≈5 µM in the acceptor chamber, which is sufficient to induce nuclear translocation of the AhR.[45] Nonetheless, based on our results, QS and phenolic molecules are less appealing than indole and its derivatives as new potential drugs for CF.

2.3. Integrating the Cystic Fibrosis Mucus Barrier in PermeaPad: The Effect of CF Mucus Models on Permeability

Although informative about cell permeability, the in vitro system PermeaPad models exclusively pertain to the cellular membrane, disregarding the impact of mucus.

Mucus is a natural hydrogel with complex filtering properties due to its peculiar features. From a chemical point of view, the main gel-forming constituents of mucus are linear, high molecular weight glycoproteins, called mucins, harboring numerous O-linked oligosaccharide branches. The complex chemical structure of mucins imparts negative charges via carboxyl and sulphate groups and contributes to the formation of hydrophobic domains, facilitating the self-assembly of mucin polymeric

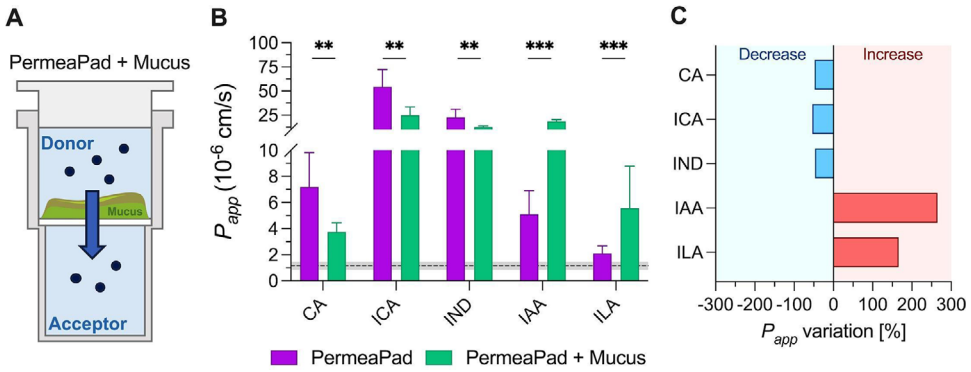

Figure 5. A) Graphical representation of the integrated system PermeaPad + mucus to evaluate in vitro the permeation of selected molecules through both CF mucus and cellular membrane (modeled by PermeaPad). B) Apparent permeability (P_{app}) of indole and indole derivatives in the absence (purple) and presence (green) of the CF mucus model. C) Percent variation (%) of the P_{app} of molecules after the addition of mucus.

networks.[36] These features make the prediction of drug passage through the mucus particularly challenging due to our limited understanding of how molecules, especially if charged and with hydrophobic surface characteristics, interact with the mucus barrier. This complexity contributes to the difficulty of forecasting in vitro the permeation of substances through the mucus layer, particularly in CF, where the pathologically thick mucus layer is characterized by aberrant properties that impair molecule diffusion and make CF patients susceptible to chronic infections and dysregulation. Although the pathological role of mucus is ubiquitously recognized, it is scarcely considered when addressing new potential therapeutic treatments in the early stages of the drug development pipeline, including the case of postbiotics and in particular indole and derivatives.

We then examined how a pathological CF-mucus model may affect the permeation of the bacteria-derived molecules by integrating a cystic fibrosis mucus model[52] with the PermeaPad 96-well. We used the integrated system (PermeaPad + Mucus) as a tool to perform a second screening, in a further level of complexity, of the molecules that resulted to be permeable through the cellular membrane (**Figure** 5A).

In the integrated system, mucus behaved like an active barrier for AhR-targeting molecules. Five of the six postbiotic molecules were impacted by the presence of the CF-mucus model.

The impact of the CF-mucus barrier was evaluated by quantifying the changes in the apparent permeability measured with and without mucus (**Figure** 5B). In all the bacteria-derived molecules considered, mucus caused a significant change in apparent permeability values, with three out of five molecules exhibiting a P_{app} reduction. The major permeability drop was found for ICA, whose P_{app} with the CF-mucus resulted in half (−54%) of the value measured without the mucus (**Figure** 5C). Indole (IND) and caffeine (CA), instead, showed slighter reduction in P_{app}, which was reduced by −44% and −48%, respectively.

Under a therapeutic assessment point of view, the reduction of permeability of about half the original values of these molecules is particularly relevant as it indicates that the evidence of in vitro permeation through the simple cellular membrane may not represent a complete criterion for screening the best performing AhR-targeting drug candidate. For its significant effect on permeability, the inclusion of CF mucus barrier assumes a critical importance in the early-stages of the drug-discovery pipeline such as pharmacokinetic assessments, rational drug design, and drug candidate screening. It may serve, indeed, as the pivotal factor capable of significantly impairing the efficacy of the drug candidate in terms of targeting. Significantly, we find that the molecule with the highest permeability through the dual barriers of mucus and cellular membrane (ICA, P_{app} of $25.04 \pm 8.32 \times 10^{-6}$ cm s^{-1}) is the one already applied in in vivo studies showing promising bioactivity.[22] This finding suggests that the permeability assessment within the integrated system (PermeaPad + Mucus) aligns with observations obtained with animals, indicating that indole (with a P_{app} of $21.65 \pm 1.25 \times 10^{-6}$ cm s^{-1}) can also serve as a viable candidate for in vivo investigation and further consideration in the development of novel postbiotic therapeutics for CF.

Mucus had a different effect on the other indole derivatives. Indeed, an increase of the P_{app} was observed for acid derivatives of indole IAA and ILa (+256% and +166%, respectively). This trend can be explained as a result of calcium complexation, as quantified by identification through LC-MS/MS quantification. The presence of calcium ions in the mucus model leads to the neutralization of the negative charge of the carboxylic acid substituent present in ILA and IAA, forming calcium complexes. The complex formation induces a transit of negatively charged molecules to molecules with no net charge. This neutralization leads to a reduction in polarity, favoring an increase in lipophilicity, which in turn, enhances the diffusion through the artificial phospholipid membrane of PermeaPad (**Figure** 5).[34] Although this aspect represents an artefact due to the presence of calcium ions in the CF mucus model, it can be interpreted as a hidden advantage of the integrated platform (PermeaPad + mucus) in the perspective of initial assessment of new potential molecules for AhR-targeting therapeutics. Indeed, the integrated system has the potential to provide indications in a simple and rapid way of which molecules are prone to calcium complexes formation. This can help to understand whether, from a therapeutic point of view, the transition of a potential drug molecule from its free form to its complexed form can be considered a useful strategy to improve permeability, as already observed for indomethacin, naproxen, and ketoprofen.[34,53]

3. Conclusion

Novel therapeutic bacteria-derived candidates, aimed at treating cystic fibrosis (CF) by targeting the intracellular AhR, require thorough evaluation regarding their ability to overcome biological barriers such as the cytoplasmic membrane and CF mucus. In this study, we selected postbiotics from various families, including tryptophan metabolites (indole and its derivatives), polyphenol derivatives, and quorum sensing (QS) molecules. For the first time, we presented a comprehensive assessment of their interaction with the mucus/membrane system using biomimetic in vitro tools (PermeaPad and PermeaPad + Mucus model). The permeability studies indicated that indole and its derivatives exhibited favorable permeation through the cellular membrane, suggesting their potential as bioactive molecules targeting the AhR. However, a more detailed chemical characterization is necessary to enhance the selection of therapeutic compounds. Chemical descriptor analysis reveals that charged indole derivatives, QS, and phenolic molecules exhibit lower efficacy compared to indole and the neutral indole derivative indole-3-carboxaldehyde (ICA), primarily due to passive diffusion limitations in reaching the cytoplasmic target.

From a pharmaceutical efficacy perspective, the proposed system addressed the limitations of existing models by providing an intermediate solution between simplified cell-free cytoplasmic barrier models and cell-based in vitro models. In particular, the integration of an in vitro mucus model within the PermeaPad system was revealed to be of paramount importance in the early stages of drug discovery. Mucus significantly impacted the permeability of indole and its derivatives, affecting pharmacokinetic assessments, rational drug design; and, hence, possible efficacy in screening suitable candidates.

These findings highlight the critical role of mucosal barriers in cystic fibrosis and the need to consider cellular membrane and mucus barriers when developing novel therapeutic strategies for CF. In this context, the integrated platform (PermeaPad + CF-mucus model) emerges as a valuable tool, enabling rapid quantification of the permeability of bacteria-derived molecules from diverse origins within a single in vitro setup, countering the complexities associated with animal models and their possible confounding variables.

4. Experimental Section

Materials: Sodium chloride (NaCl), calcium carbonate ($CaCO_3$), alginic acid sodium salt, D−(+)-gluconic acid lactone (GDL), and mucin from pig stomach (PGM Type III, bound sialic acid 0.5–1.5%) were used to produce the CF-mucus models (Merck–Sigma); while, the permeability assays were performed using PermeaPad 96-well plates purchased from Phabioc (Espelkamp, Germany). High purity (>98%) acetonitrile, ammonium acetate (AA), dimethyl sulfoxide (DMSO), formic acid (FA), 2-picolinic acid (PA), and trifluoroacetic acid (TFA)were acquired from Merck and used as reagents and solvents for pharmacological studies and liquid chromatography-mass spectrometry quantification. All bacteria-derived molecules were purchased from MedChemExpress or Merck and resuspended according to manufacturer indications prior to use.

Structure and Molecular Descriptors: The structure of AhR ligands of host and microbial origin was retrieved from the literature (Table S1, Supporting Information).[20] The drug simplified molecular-input line entry system (SMILES) codes were obtained with MarvinSketch (v. 23.16, ChemAxon). The charge at pH 7.4 and intrinsic solubility (cLogS) were predicted with MarvinSketch. Molecular weight (MW), partition coefficient (cLogP), hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), topological polar surface area (TPSA), and number of rotable bonds (NRotBs) were computed in Datawarrior (v. 06.01.01, Openmolecules). The csv file of the approved drugs was downloaded from the DrugBank (last updated on February 2024). Principal component analysis (PCA) implemented in Datawarrior was used to investigate the relationship between the approved drugs and AhR ligands of host and microbial origin and to visualize the chemical space.

Evaluation of the Impact of Biological Barriers on the Permeability of Microbial-Derived Metabolites-Permeability Assay: The permeability assay was carried out with the bio-mimicking permeability platform PermeaPad. Drug stock solutions were prepared at a concentration of 10 mg mL^{-1} in DMSO. Working solutions at 100 or 500 µM were prepared from the stocks by diluting in 10 mM phosphate buffer (PB) (pH 7.4, 5% DMSO). 400 µL of PB (5% DMSO) was added to the acceptor compartments of the PermeaPad plate, and 200 µL of the drug working solution was added to the donor compartments. Donor and acceptor plates were coupled, and the donor plate was sealed with a plate lid and incubated for 5 h at room temperature. After incubation, aliquots from each acceptor solution were sampled and molecule concentration was quantified by HPLC-ESI-MS/MS. The apparent permeability coefficient (P_{app}) of each molecule was computed from Fick's law for steady state conditions according to Equation (1):

$$P_{app} = \frac{dQ/dt}{C_0 \times A} \quad (1)$$

where dQ is the amount of drug expressed in moles permeated into the acceptor compartment at time dt (18.000 s), C_0 is the initial concentration in the donor well, and A is the area of the well membrane (0.15 cm^2). Caffeine was used as a reference standard to check the integrity of different PermeaPad plates. P_{app} was used as the average of three independent measurements.

Permeation Through the Mucus Layer: The CF mucus was produced as previously described.[52] Briefly, hydrogels were prepared by mixing PGM (43.8 mg mL^{-1}), alginate (21.0 mg mL^{-1}), $CaCO_3$ (7.0 mg mL^{-1}), and D−(+)-glucono-lactone (70.0 mg mL^{-1}) solutions in a volume ratio of 4:1:1:1, respectively. Solutions were prepared to include a NaCl content; thus, reaching a final salt concentration in the hydrogel of 7 mg mL^{-1}. Immediately after production, 20 µL of the CF mucus model was poured in the donor plate of PermeaPad, which was gently shaken for 1 min to ensure that the mucus was evenly distributed over the well's surface and to avoid bubble formation. The mucus-including PermeaPad plates was stored for one night at 4 °C for complete gelation of the model prior to permeation measurements being performed (Paragraph 2.3.1). The percent variation (%) between the P_{app} values obtained in PermeaPad and in the integrated system (PermeaPad + Mucus) were computed as described by Equation (2):

$$\text{Variation [\%]} = \frac{P_{app}\ (\text{PermeaPad + Mucus}) - P_{app}\ (\text{PermeaPad})}{P_{app}\ (\text{PermeaPad})} \times 100 \quad (2)$$

Quantification by HPLC-MS/MS: A liquid chromatograph-mass spectrometer LCMS-8045 (Shimadzu, Kyoto, Japan) was employed for the quantitation method. A binary gradient mode was used in all of the methods and gradients, columns, and solvents details reported in Table S1, Supporting Information. In all the methods a Luna C18 column (150 × 2.1 mm, 3 µm, Phenomenex, Torrance, CA, USA) was employed. The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in the positive or negative ion mode. Multiple reaction monitoring (MRM) tandem mass spectrometry acquisition mode was employed.

Statistical Analysis: Experiments were conducted in triplicates. Results were presented as the mean value ± standard deviation, unless otherwise specified. The normality of the data distribution was assessed using

the D'Agostino–Pearson test. Subsequently, comparisons between data pairs or groups were made using Student's *t*-test/Mann-Whitney test or ANOVA/Kruskal–Wallis test, depending on the outcome of the normality assessment. GraphPad Prism v.10 (GraphPad Software, USA) was used to perform the statistical evaluation. Statistical differences were represented as follows:$P \geq 0.05$ (n.d.)≥ 0.05 (n.d.); $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***); and $P < 0.0001$ (****).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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immune system, indole, mucus, permeability, postbiotics

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