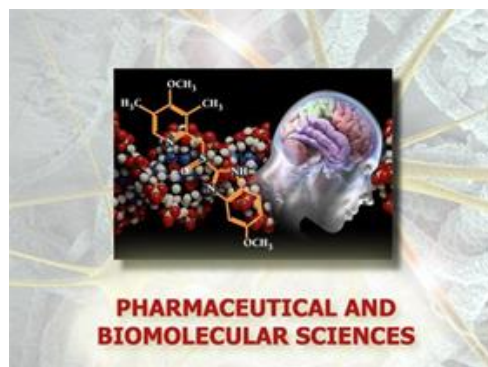




**UNIVERSITÀ
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Università degli Studi di Torino

Doctoral Program in Pharmaceutical and Biomolecular Sciences

Department of molecular biotechnology and health sciences

XXXVIIth Cycle

***In vitro* and *in vivo* efficacy of innovative integrin-targeted photosensitizers for photodynamic therapy**

Supervisor:

Prof. Enzo Terreno

Ph.D. candidate:

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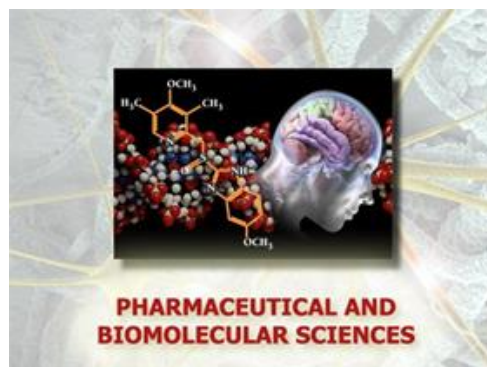
Coordinator:

Prof. Roberta Cavalli

Academic Years: 2024-2025



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Title of the Thesis: *In vitro* and *in vivo* efficacy of innovative integrin-targeted photosensitizers for photodynamic therapy

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The PhD was conducted within the framework of a collaborative research initiative,
supported by funding from the University of Turin,
the National Research Council (CNR), and Bracco Imaging S.p.A.



Consiglio Nazionale
delle **Ricerche**



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1 Photodynamic Therapy: insights into mechanisms and therapeutic potential

Photodynamic Therapy (PDT) is a medical technique used for treating malignant tumors and non-tumoral conditions. The use of PDT in clinical and preclinical settings has evolved considerably with regard to the development of drugs and mechanisms to be exploited to optimize the triggered reaction.

Clinical applications

In the clinical field, PDT is already approved as a treatment for various pathologies, especially its use is consolidated for solid tumors and dermatological diseases.¹ One of the main photosensitizers used is Photofrin, a formulation of hematoporphyrin derivative, and it is a standard treatment in some oncological indications such as bladder, esophageal and lung carcinomas.² In addition, wide clinical application has been demonstrated for superficial skin tumors such as basal cell carcinoma. In these pathologies PDT has led to complete regression thanks to the ability of light penetration into lesions, thus allowing the selective destruction of cancer cells without significant damage to surrounding healthy tissues.³ In deep tissue conditions such as esophageal and lung cancer, advanced endoscopic techniques have been developed to precisely direct laser light to tumors, increasing the effectiveness of treatment and reducing side effects. Another clinically relevant agent is 5-aminolevulinic acid (5-ALA). This, once administered, is converted in the cancer cells into protoporphyrin IX (PpIX), a highly photoreactive compound.⁴ Therefore, 5-ALA has a metabolic activation, where the precursor is only activated inside the cancer cell. 5-ALA has been successfully used for high-grade brain tumors, including glioblastomas.

In addition to cancers, PDT is applied to treat non-oncological conditions such as macular degeneration, the treatment of chronic inflammation and some antibiotic-resistant infections.⁵

Preclinical development

In the preclinical field, PDT is the subject of numerous research projects. The studies focus on the development of photosensitizers designed to provide greater selectivity for cancer tissues and reduce the photosensitivity of healthy tissues. For example, the derivatives of chlorine such as chlorine e6 and temoporfin, and thalaporfin have been developed with improved penetration properties and specificity. Temoporfine is already approved in clinical use for the treatment of squamous cell carcinoma of head and neck⁶; thalaporphine has shown significant potential in cancer models such as lung and pancreas.⁷

Other preclinical research concerns the combined use of PDT with nanoparticles and drug delivery platforms. The use of nanoparticles, for example, allows photosensitizers to be transported directly into cancer cells where they are selectively activated. In addition, another class of photosensitizers are developed to be activated by external stimuli such as pH or temperature, making it possible to control their action even more.⁸ Preclinical PDT is also being studied as a possible antibacterial therapy for the photoinactivation of pathogenic organisms (PDI), useful in the treatment of antibiotic-resistant infections. This application is particularly interesting because it allows to treat localized infections without the use of antibiotics, reducing the risk of developing bacterial resistance.⁹

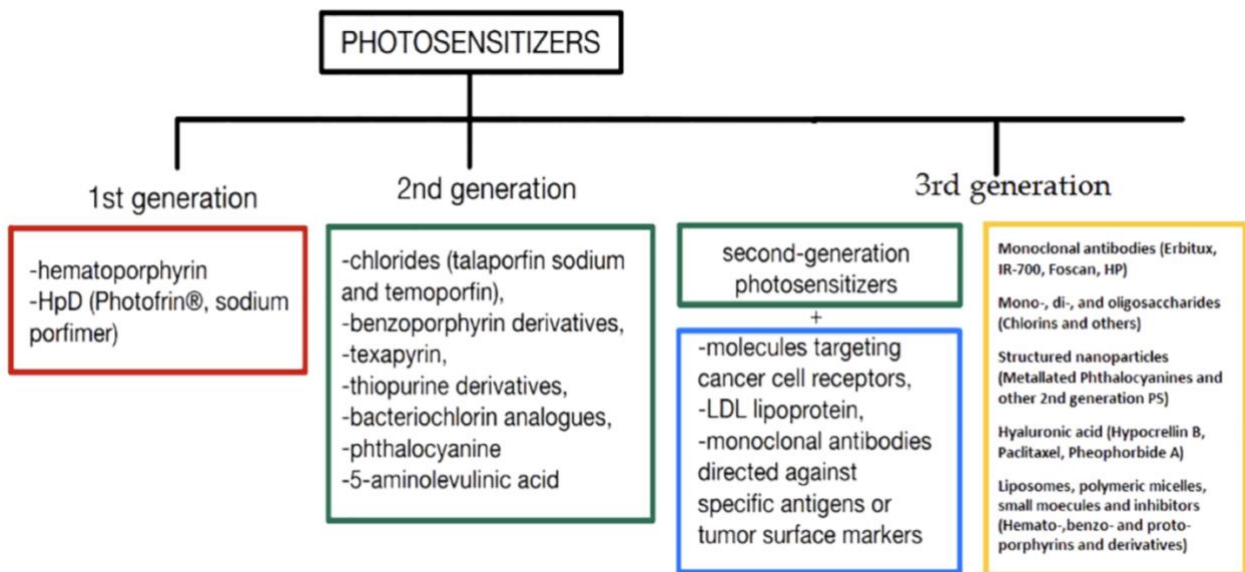


Figure 1. Photosensitizers are classified by generation, considering the difference in structure, selectivity of action, or accumulation in tissues. ¹⁰

However, in this study, PDT therapy will be explored in its application to tumor models. For decades, this therapy has been studied for its significant advantages in terms of safety, selectivity, and its non-invasive nature.¹¹ Despite ongoing efforts, this approach is still being explored to expand its applications and intensify its effectiveness.

1.1

Fundamental mechanisms of action

Three distinct components are essential in the PDT treatment for triggering the reaction responsible for the photodynamic effect: a molecule named photosensitizer, the molecular oxygen and the light characterized by a specific excitation and emission spectrum. First a photosensitizer (PS) is administrated and then it is activated by light. In the presence of molecular oxygen, this activated molecule initiates a photodynamic reaction.

Precise timing in the irradiation and the localization of the drug are required during the process, aiming to destroy the lesion while sparing the surrounding healthy tissue¹².

It is known that PDT is a complex treatment, and its effectiveness comes from several factors working together. However, some of the ways it works are still not completely understood.

Today, two main mechanisms are known to regulate the photodynamic effect, both of which are heavily based on the presence of oxygen molecules in the cells.

Once the photosensitizer reaches the cell, due to the light to which it is exposed, at a specific wavelength suitable for the absorption spectrum of the molecule, it is undergoing a shift from its normal singlet state (S_0) to an excited singlet state (S_1). Some of the absorbed energy is released as fluorescence, while the rest transitions the photosensitizer into an excited triplet state (T_1), which is the active form responsible for the therapeutic effect.¹³

The role of both the photosensitizer and oxygen in PDT is described in detail by the Jablonski diagram, given the energy transitions of the PS through various states during the photodynamic process, and distinguishing between these two pathways.¹⁴

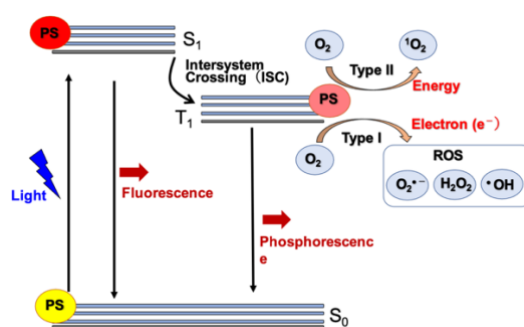


Figure 2. Schematic Jablonski diagram for Type I and Type II reactions in PDT from Professor Hirofumi Miyaji (Hokkaido University).

Type I reaction

In the excited triplet state (T_1), the PS transfers its energy to adjacent biomolecules. Thanks to this energy, a transfer of a hydrogen molecule or electron is made between the drug and the tumor tissue, resulting in the formation of free radicals. When the free radicals react with oxygen, reactive oxygen species (ROS) are generated. In detail the superoxide anion radical ($O_2^{\bullet-}$) drives a chain of reactions. This buildup of oxidative stress in the end causes the destruction of cancer cells.¹⁵

Type II of reaction

On the other hand, the second mechanism drives the direct transfer of energy from the excited PS to the molecular oxygen, present in its normal triplet state. This involves the production of singlet oxygen ($^1\text{O}_2$), a highly reactive and strong oxidizing agent. In contrast to reaction I, where superoxide anions play a key role, here singlet oxygen is the primary driver, causing oxidative damage to cellular components and leading to cell death.¹⁶ Generally, reaction II is often considered preferred because more effective for the potent oxidizing ability of singlet oxygen. The damage caused by ROS concerns photodamage to proteins, lipids, and other molecules in the photosensitized region.¹⁷ And the type of cell death is determined by where the drug acts in the cell.

Depending on the location of the drug when activated (inside the cell, in lysosomes, mitochondria, or bound to the cell membrane), it may lead to disruption of cell membranes or cause damage to mitochondria or lysosomes.¹⁸ In addition to the damage directly caused by ROS, PDT can also act on blood vessels, leading to oxygen and vital nutrient deprivation.¹⁹ The immune system can also activate the inflammatory cascade in response to treatment. The immune response can be either stimulated or suppressed by PDT, depending on different variables.²⁰ The efficiency of PDT is linked to the systemic anti-cancer immune response of the body. By disrupting the tumor structure, this technique acts as a direct bridge between immune system cells and cancer cells, triggering a strong inflammatory reaction and leukocyte infiltration into the tumor.²¹ A strong inflammatory response induced by PDT recruits the immune system, triggering an attack on the tumor through neutrophils, macrophages, dendritic cells, N cells, CD8^+ T cells, CD4^+ T cells, Tregs, and B cells.²² The specific mechanisms are not yet fully understood, and we will not delve into them.

1.2

Phthalocyanines as key photosensitizers in photodynamic therapy

A photosensitizer is a substance that absorbs light in a specific wavelength and initiates photochemical or photophysical reactions. The dye determines the wavelength of light, which then influences the kind of laser employed for activation. To ensure safety and effectiveness an ideal photosensitizer needs to be chemically pure, stable at room temperature, and activated by light in the 600-800 nm range without triggering photosensitivity from sunlight exposure. It should not interfere with natural molecules like hemoglobin, and it should have low toxicity in the dark and selectively target diseased tissues, staying longer in the diseased areas and quickly move over to the healthy ones.²³ The origin of these drugs may be natural or synthetic. In recent years, natural active compounds like pheophorbides, curcumin, anthraquinones, polyacetylenes, thiophenes, chlorophyllins, hypericins, and alkaloids have gained significant attention and have been widely studied for their promising photodynamic properties.²⁴ Despite many photosensitizers have been noticed, only a small number have been completely approved for clinical use.²⁵ Porphyrin family (HpD, BPD), 5-ALA (5-aminolevulinic acid), texaphyrins, the chlorin family (Temoporfin, Purlytin, NPe6, LS11, HPPH), and the dyes family (Phthalocyanine, Naphthalocyanine) are some of the primary families of PSs.²⁶

Similar in structure to porphyrins, the phthalocyanines belong to the second generation of molecules.²⁷

Phthalocyanines are complex molecules made up of tetrapyrrole units, recognized for their remarkable chemical and thermal stability. They are notable in PDT for their ability to absorb light in the far-red region of the visible spectrum, together with their excellent stability, durability against photobleaching, and efficient generation of both fluorescence and singlet oxygen.²⁸

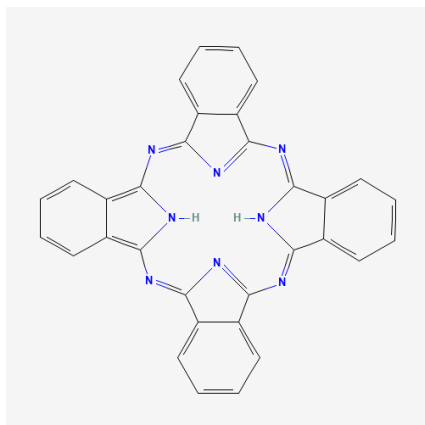


Figure 3. The basic structure belonging to the phthalocyanine group is shown. Phthalocyanine is a tetrapyrrole compound consisting of four isoindole units, with nitrogen atoms substituting for the linking carbon atoms in the macrocyclic framework (PubChem).

Metal Pc-based PS are recognized for their prominent photoelectrochemical characteristics. The alignment of their redox couple positions and the efficient light absorption abilities contribute to the high effectiveness of the dye.²⁹

The most prominent absorption band is known as the Q-band and it is detected around 700 nm.³⁰ The presence of this peak enables the use of light which therefore has the characteristic of having a high tissue penetration, reaching depths of up to 10 mm.³¹

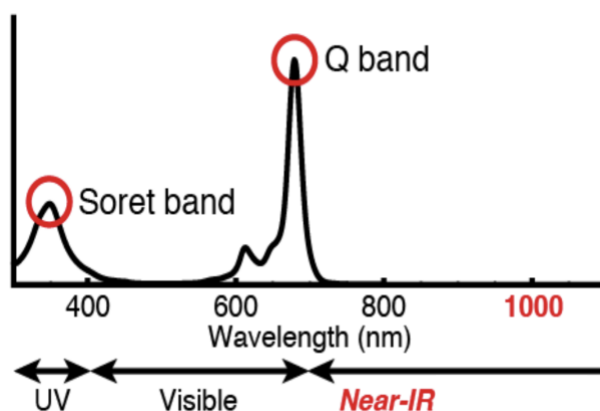


Figure 4. Typical absorption spectrum of a metal Pc.³²

1.3

Chemical modifications and their impact on photophysical properties

In phthalocyanine complexes with transition metals, the selection of the central metal ion is the key to adjust the excited-state properties of the compound. Among the various metal centers, silicon (Si) stands out as an optimal choice over zinc (Zn) due to its ability to coordinate axial ligands, which enhances solubility, reduces aggregation, and allows for precise modulation of electronic properties, making Si-phthalocyanines particularly advantageous for optoelectronic and photodynamic applications.³³

In addition to the central metal ion, the introduction of substituents, plays a crucial role in defining the photophysical properties of the drug.

These substituents can influence key properties like light absorption, solubility in different tissue compartments, and stability when exposed to light. Both the type and position of the substituents on the phthalocyanine molecule are crucial for precisely controlling how it interacts with light and surrounding substances, allowing its effectiveness to be tailored to the specific conditions of its use.³⁴ A significant issue due to their hydrophobic nature is their pronounced tendency to form dimers or aggregates, potentially impacting their performance.³⁵

Consequently, complex formulations are often required to improve cellular uptake.³⁶

Among the phthalocyanines two are in clinically approved: Photosens, a sulfonated aluminum phthalocyanine, which is used to treat esophageal cancer, and Pc-4, a silicon (IV) phthalocyanine derivative, which is used to treat cutaneous T-cell lymphoma.^{37,38}

1.4

Application of RGD peptides in integrin-mediated tumor targeting

Since tumor selectivity remains a significant issue for this therapeutic approach, recent efforts have focused on investigating both passive and active targeting strategies to aim these drugs at specific tumor biomarkers³⁹. Antibodies or small molecules such as peptides can be employed to develop an actively targeting PS. Photoimmunotherapy (PIT) specifically employs antibodies conjugated to photosensitizers to target cells. IR700Dx is the most used PS, it is notable for its strong water solubility and performs a dual function, in fluorescence imaging as well as photodynamic therapy. When stimulated by 690 nm light, it emits light at 700 nm.⁴⁰ NIR-PIT has demonstrated the capability to induce immunogenic cell death (ICD), which in turn activates the immune system of the body to fight tumors. This could potentially enhance its effectiveness in eradicating cancer.⁴¹ On the other hand, small organic ligands and peptides are highly effective for tumor targeting due to their ability to penetrate tumors better, bind selectively, and be rapidly internalized. They provide an excellent alternative to antibodies since they have a lower chance of inducing immune reactions and generally result in fewer adverse effects.⁴² Promising drugs for targeted delivery include folic acid derivatives that target the folate receptor, glutamic acid urea compounds that focus on PSMA, cyclic peptides designed to engage integrin $\alpha_v\beta_3$, somatostatin analogues for targeting the somatostatin receptor, and aromatic sulfonamides specific to carbonic anhydrase IX.⁴³ These ligands can either be directly attached to photosensitizers or linked to the delivery systems that transport the photosensitizers, enhancing the precision of targeted PDT.⁴⁴

Integrins

Integrins are crucial cell-adhesion receptors, essential to connect the extracellular matrix to the cytoskeleton. They are made up of two transmembrane subunits, α and β . Integrins act as bidirectional signaling molecules, reacting to external stimuli while simultaneously transmitting internal signals.⁴⁵

Despite being explored as pharmacological targets for almost forty years, their complexity has restricted the successful development of integrin-targeting drugs to only seven approved compounds, such as abciximab, eptifibatide, tirofiban, natalizumab, vedolizumab, lifitegrast, and efalizumab. Notably, cilengitide, an $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin inhibitor investigated for glioblastoma treatment, has not yet received regulatory approval.⁴⁶

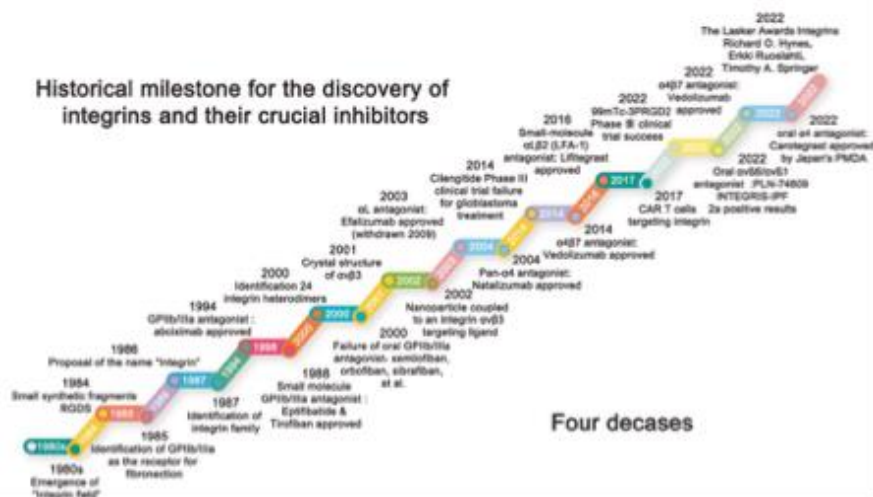


Figure 5. A timeline highlighting key milestones in the study of integrin receptors and the progression of their antagonists and agents over the last four decades.⁴⁷

Integrin $\alpha_v\beta_3$ was the first integrin studied, it is recognized as the vitronectin receptor due to its interaction with vitronectin. It has been found to interact also with other ligands, such as TGF- β , fibronectin, osteopontin, fibrinogen, and tenascin. This integrin is extensively discovered in various tissues including mesenchyme, blood vessels, smooth muscle cells, fibroblasts, and platelets.⁴⁸ It plays an important role in angiogenesis, extracellular matrix regulation, vascular smooth muscle cell migration, and osteoclast adhesion to bone. Moreover, integrin $\alpha_v\beta_3$ contributes to the immune response by controlling the migration and phagocytic activity of immune cells.⁴⁹ Integrin $\alpha_v\beta_6$ is essential for activating TGF- β , which helps regulate immune responses and inflammation in the lungs, skin, and gastrointestinal tract.^{50,51} Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_6$ have high affinity to the RGD motif. For this reason, they are selectively bound and recognized by peptide vectors containing this amino acid sequence. It has been noted that the expression levels of specific integrins may rise in cancer cells, acting as significant markers of tumorigenesis. Integrin $\alpha_v\beta_3$ is typically conveyed at low levels in adult epithelial cells but is significantly increased in many cancers. It is involved in multiple stages of tumor progression: tumorigenesis, epithelial-mesenchymal transition, angiogenesis, metabolic reprogramming, immune evasion, bone metastasis, and drug resistance.⁵² Because of the differing expression levels in healthy versus diseased tissue, it may act as a potentially valuable target for tumor imaging and clinical research, assisting in early detection.⁵³ ⁵⁴ Considering the role of integrin-mediated adhesion in regulating various physiological processes as well as pathological conditions, there is a strong need for the development of antagonists that are specific to integrin subtypes.⁵⁵ They act both as inhibitors of cancer angiogenesis and as blockers of excessive blood clotting.⁵⁶ However, recent advancements have led to the development of ligands that possess adequate activity and selectivity to distinguish effectively between closely related integrin subtypes, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ or $\alpha_v\beta_6$.⁵⁷ ⁵⁸ ⁵⁹

In this context, employing a RGD-containing peptide as a vector targeting integrins to specifically reach tumor cells has been adopted as a strategy to deliver the photosensitizing agent.⁶⁰

2 Objective of the study aims and research hypotheses

Considering the current scenery of photodynamic therapy, the objective of this work is to develop two photo sensitizing agents ad hoc for two selected integrin targets. This objective is to meet the increasing need for specific treatments, to optimize efficacy and improve the toxicity profile of the drug. In detail, the study included a first phase of synthesis of two silicon phthalocyanine conjugated with an integrin-targeting moiety. During a second phase these compounds are tested in three-dimensional cell cultures and *in vivo* to determine their cytotoxicity, when activated by the laser. Two different cancer models were identified to develop this study.

In chapter 3 the application of the first photosensitizer was studied on glioblastoma model using stem cells. Different Glioma Stem Cell (GSCs) lines were selected, considering the variation on expression of $\alpha_v\beta_3$. The photodynamic activity of the drug was studied in GSCs spheroids and in a murine model.

In chapter 4 the second photosensitizer was synthesized and its efficacy investigated in a pancreatic adenocarcinoma model, using the BxPc3 cells characterized by an overexpression of integrin $\alpha_v\beta_6$.

Chapter 5 reports the general discussion that emphasizes the common aspects between the two studies, showing a broader and more general discussion perspective.

The study focuses on the development of integrin-targeting PDT probes to better convey the photodynamic effect.

3 Integrin targeted photodynamic therapy in patient-derived glioblastoma spheroids and in a murine model

Abstract

PDT is an emerging localized treatment for cancer that utilizes light-activated compounds to produce reactive oxygen species, effectively targeting and destroying tumor cells. In the case of glioblastoma multiforme (GBM), the most aggressive form of primary brain cancer with a median survival of just 14–17 months, PDT presents a promising approach to address the infiltrative tumor margins that drive recurrence. GBM remains incurable largely due to its highly invasive nature, with glioblastoma stem cells (GSCs) contributing significantly to therapy resistance and tumor relapse after standard treatments. This study explores an innovative PDT technique using near-infrared light and a dual-c(RGDfK)-silicon phthalocyanine conjugate. This conjugate specifically targets integrin $\alpha_v\beta_3$, a protein highly expressed by GBM cells and their associated vasculature. The deeper tissue penetration of the NIR light enhances the precision of the treatment, minimizing damage to healthy brain tissue while effectively addressing invasive tumor margins. The approach was preclinically evaluated using patient-derived GBM spheroids and a mouse model to test its effectiveness in infiltrative GBM cells. By combining NIR light with precise targeting, this PDT strategy aims to reduce local recurrence by eradicating residual tumor cells at the infiltrative margins.

3.1

Introduction

GBM is an aggressive brain tumor with limited treatment success, it is a heterogeneous disease and the presence of cancer stem cells (CSCs) stimulates a rapid tumor growth which hampers an effective response to traditional treatments.⁶¹

In the case of GBM, around 80% of tumour recurrences happen within 2 cm of the boundary of the surgical resection.⁶² This proximity to the resection cavity makes post-surgical PDT a compelling option for targeting the residual cancer cells that prove a challenge to visualize and remove during surgery. Precision PDT could help improve GBM surgery and lower the chances of recurrence by eliminating these remaining cancer cells in the affected region. By combining the deeper penetration with localized targeting, this method offers a promising approach for improving treatments for conditions like GBM.

The cancer stem cells theory identifies cancer stem cells (CSCs) as key drivers of tumor growth, treatment resistance, and relapse. In glioblastoma, CSCs can self-renew, differentiate, and spread through cerebrospinal fluid after incomplete surgery, promoting recurrence and therapy resistance.⁶³

⁶⁴ Interestingly, GSCs, isolated from solid tumours, have been demonstrated to form three-dimensional neuro spheres when grown *in vitro*, that we can call spheroids. GS spheroids, which functionally recapitulate the heterogeneity of the original tumor, can be used to study the nature of this tumor better than adherent cells.⁶⁵

Integrins, especially integrin $\alpha_v\beta_3$, are significant contributors to GBM progression. The overexpression of $\alpha_v\beta_3$ integrin is observed in tumor cells and their connected vasculature, facilitating tumorigenesis and angiogenesis.⁶⁶ The expression of the $\alpha_v\beta_3$ integrin is notably prevalent in astrocytic tumors, whereas a weaker expression has been observed in oligodendrogliomas. In general, the upregulation of this integrin in GBM, compared to the low levels observed in healthy tissue, is well established, and its presence is closely associated with tumor malignancy. Notably, a pronounced overexpression of $\alpha_v\beta_3$ is observed at the tumor periphery, where GBM exhibits a more invasive and aggressive phenotype.⁶⁷

Targeting the integrin $\alpha_v\beta_3$ is therefore considered a promising strategy to enhance surgical outcomes by addressing residual cells in the tumor margin which could improve the specificity and effectiveness of cancer treatment.^{68 69}

Tumor targeting employs various strategies to enhance the accuracy of diagnostics and therapies. Passive targeting takes advantage of the Enhanced Permeability and Retention (EPR) effect, which enables larger molecules to accumulate in tumor tissue due to the abnormal permeability of tumor vasculature. Active targeting increases specificity by utilizing agents such as antibodies or peptides that bind to overexpressed tumor markers, like EGFR or VEGFRs. Regarding glioblastoma the metabolic targeting, as exemplified by 5-aminolevulinic acid (5-ALA), exploits the altered metabolism of cancer cells. In detail, 5-ALA is metabolized into protoporphyrin IX (PpIX) at a higher rate in tumors,

facilitating its use in PDT to selectively destroy cancer cells. These approaches aim to improve treatment efficacy while minimizing damage to healthy tissues.⁷⁰

Here we focus on a well-known class of photosensitizers represented by phthalocyanines, including silicon phthalocyanine (SiPc), which share structural similarities with porphyrins, such as protoporphyrin IX (PpIX). These molecules feature an expanded macrocyclic ring system, enabling absorption of longer wavelengths of light and thereby deeper tissue penetration.⁷¹ Silicon phthalocyanines have demonstrated potent photosensitizing activity in both preclinical and clinical studies.⁷² In fact, photosensitizers that absorb light in the red to near-infrared (NIR) spectrum (600-800 nm) are especially advantageous because they can be excited more deeply in the tissue while minimizing light scattering.⁷³ As a result, this innovative therapeutic approach is particularly effective for reaching tumour cells beyond the resection cavity in the infiltrative edge. The study focuses on this approach, which aims to optimize targeted PDT by developing a dual-c(RGDfK)-silicon phthalocyanine conjugate and by testing its efficacy in patient derived spheroids and in a murine model with variable integrin expression as representative models of GBM.⁷⁴

3.2

Materials and Methods

Synthesis and conjugation of the SiPc with 2 unit of c(RGDfK)

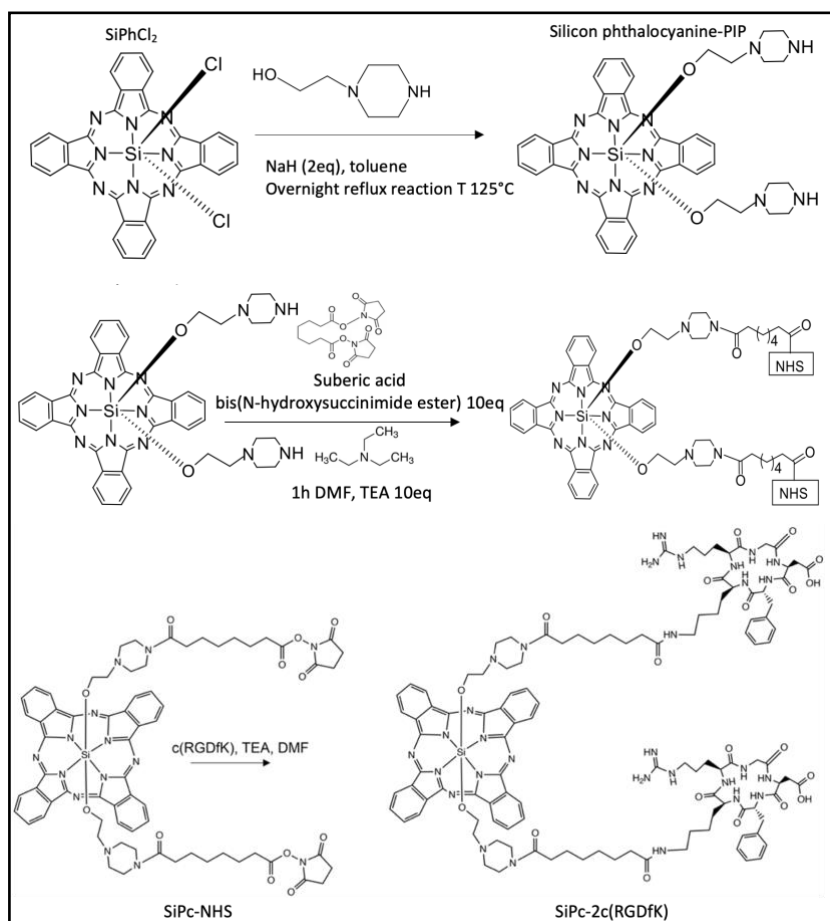
Compound SiPc-NHS was prepared by a previously reported method.⁷⁵

Silicon phthalocyanine dichloride (611.51 g/mol) was used as the starting material. 200 mg (0.65 mmol) of SiPcCl₂ reacted with NaH (2 eq.) and 400 µL of 1-(2-hydroxyethyl) piperazine (130.19 g/mol) in 40 mL of toluene under reflux at 125°C overnight. The product was analyzed by HPLC, using waters XTerra C18 column) and a gradient reported below. Eluents: MilliQ water + 0.1% TFA (Phase A) and acetonitrile (Phase B).

- 0 min: 95% A, 5% B
- 25 min: 20% A, 80% B
- 28 min: 0% A, 100% B

Purification involved precipitation in diethyl ether, followed by silica gravity column chromatography (DCM/MeOH 95:5 +0.1%TEA → DCM/MeOH 5:5 + 0.1%TEA elution) yielding Silicon phthalocyanine-PIP (799 g/mol) with a 40% yield.

20 mg (0.0250 mmol) of purified SiPh-PIP was then reacted with suberic acid bis(N-hydroxysuccinimide ester) (10 eq., 368 g/mol) and triethylamine (10 eq., 101 g/mol, density 0.72) in 4 mL of DMF. SiPhPIP was added dropwise to the pre-dissolved reagent solution. After 1.5 h, the final product (0.00375 mmol, 1305 g/mol, 15% yield) was analyzed via HPLC and purified by cold diethyl ether wash (15 min at 5500 rpm) with 13% final yield.



Scheme 1. Scheme of the synthesis of Silicon Phthalocyanine-PIP (SiPh-PIP) and its conjugation with the peptide. Silicon phthalocyanine dichloride (SiPhCl₂) reacts with 1-(2-hydroxyethyl) piperazine to form SiPh-PIP. After purification, SiPh-PIP is conjugated with the peptide using suberic acid bis(N-hydroxysuccinimide ester) in the presence of triethylamine, followed by purification and verification of the final conjugate.

This final compound was conjugated with 2 molecules of the c(RGDfK) (supplied by Bracco Imaging Spa). HPLC analysis was performed on a Waters AutoPurification system (3100 Mass Detector, 2545 Pump Gradient Module, 2767 Sample Manager, and 2998 PDA detector).

In detail a solution of SiPc-NHS (10 mg, 0.0075 mmol), TEA (10 μ L, 0.068 mmol) in 10 mL of DMF were added to a solution of c(RGDfK) (9 mg, 0.015 mmol) in 10 mL of DMF and stirred at room temperature for 3 hours. The final mixture was precipitated and washed with ethyl ether three times. The solid was then purified by preparative RP-HPLC by using a Water XTerra™ Prep RPdC8 19/100 column, applying a gradient of CH₃CN in CH₃COONH₄ 0.1 M, pH=7 from 30% to 80% in 15 min (20 mL/min). The purity of the compound was determined by UPLC using peptide BEH C18 column, 1.7 μ m, 2.1×150 mm, applying a gradient of CH₃CN in H₂O (0.1% TFA) from 30% to 80% in 10 min and 100% of B in 3 min (1 mL min⁻¹, final yield 13%, purity 98,7 %).

Optical Characterization of the photosensitizer

The absorbance spectrum of the compound SiPc-2c(RGDfK) was acquired in DMSO at the concentration of 2 μ M using the SpectraMax spectrophotometer.

Glioblastoma Stem Cell culture

Glioblastoma stem cells were cultured as described previously.⁷⁶ In short, the cells were cultured in DMEM-F12 medium (Gibco™, Thermo Fisher Scientific, Catalog number 11320033) supplemented with penicillin/streptomycin (1%) (Gibco™, Thermo Fisher Scientific, Catalog number 15140122), fetal bovine serum (FBS, 10%) (Gibco™, Thermo Fisher Scientific, Catalog number 10270106), B27 supplement (2%) (Gibco™, Thermo Fisher Scientific, Catalog number 17504044), basic fibroblast growth factor (bFGF, 10-20 ng/mL) (Recombinant Human FGF-basic, PeproTech, Catalog number 100-18B), epidermal growth factor (EGF, 10-20 ng/mL) (Recombinant Human EGF, PeproTech, Catalog number AF-100-15), and heparin (5 μ g/mL) (Heparin Sodium Salt, Sigma-Aldrich, Catalog number H3149). The final medium is so called Neurospheres Medium (NS medium). Culture maintenance involved regular medium changes and incubation under optimal conditions of temperature and CO₂ concentration. After washing the cells with PBS, they were detached using Accutase (Sigma-Aldrich, Catalog number A6964). To prepare adherent cells, the cell culture plates were coated with Cultrex (Trevigen, MD, USA) allowing attachment and monolayer formation. A 1:100 dilution of the ExtraCellular Matrix (ECM) solution in NS medium was then applied to the flask in the appropriate volume. The flask was then incubated at 37 °C for 30 minutes. Coated flasks could be stored for up to 7 days at 37 °C or for up to three weeks at 4 °C. Regular monitoring of cell morphology and viability ensured key traits were preserved, maintaining experimental reproducibility.

Determination of the target expression (integrin $\alpha_v\beta_3$)

For the patient derived glioma cell lines (GS756, GS359, GS741, GS401) integrin $\alpha_v\beta_3$ expression was determined based on RNA sequencing data. GS741 was determined to have a low gene expression while GS359 was found to have a high expression. Starting from the screening performed with RNA-seq, three cell lines were selected for integrin evaluation through In-Cell Western: Gs359, Gs756, and Gs401. The Gs741 cell line was excluded as its integrin expression level was intermediate between the others, whereas our goal was to select two lines with significantly different expression levels.⁷⁷ In-Cell Western assays were conducted according to the standard protocols provided by LI-COR Bio, Lincoln, Nebraska, USA. In short, 1×10^4 cells were plated in p96 plates, then treated, fixed, permeabilized, and exposed to primary antibody (AbI LM609 Abcam) and IRDye800CW Goat anti-Mouse IgG secondary antibody (LICORbio). The fluorescence signals were detected and quantified using the Odyssey Imager. Every step, including cell fixation and permeabilization, blocking, antibody titration, and normalization, was fine-tuned to fit the specific experimental conditions (LI-CORbio).⁷⁸

GS756, GS359 and GS401 Spheroids formation

GSCs were plated (day 0) in 96-well (transparent) round bottom plates (BioFloat F202003) at a density of 5×10^2 cells/well (GS756) or 1×10^3 cells/well (GS359 and GS401). Spheroid growth was monitored under a microscope until spheroids reached a homogeneous size with diameters around 200-300 μm by day 3 for each cell line.

In Vitro uptake evaluation in adherent cells and spheroids

After incubation, 1×10^4 cells from each cell line were seeded into 96 well plates. The following day, the medium was aspirated, and the cells were washed once with 1x PBS. The cells were then treated with a 5% DMSO solution of SiPc-2(cRGDfK) 500 nM prepared in NS medium, with 70 μL added per well. Treatments were administered for 2, 4, and 6 hours. After the incubation period, the cells were washed with PBS to remove any unbound SiPc-2(cRGDfK). Fluorescence intensity in PBS was measured using an Odyssey scanner, utilizing the 700 nm channel. The fluorescence was recorded while the cells remained adhered to the plate, following a washing step with PBS and the subsequent addition of fresh PBS for the measurement. Fluorescence data were subsequently analysed to quantify uptake across different time points and cell lines. For spheroid cultures, the protocol was the same, except for the omission of the ECM coating step. Spheroids were seeded with either 1×10^3 cells per well (GS359, GS401) or 5×10^2 cells per well (GS756), following the same treatment and measurement process as with monolayer cultures.

Staining of GCS cells in adhesion

2×10^4 cells per well were grown in adhesion in chamber slides (Nunc), the next day these were stained with ready-to-use DAPI and with the fluorophore SiPc-2 c(RGDfK) 500 nM for 2 hours. After washing with PBS, they were acquired under a fluorescence Olympus microscope.

Affinity assay of the photosensitizer with competitor

Cells were seeded at a density of 1×10^4 cells per well on day 0. On day 1, the cells were incubated with 100 μL of the drug at various concentrations (500 nM to 1 nM), in the presence of the competitor (peptide cRGDfK) at 50 μM , for 2 hours at 37 °C. After the incubation, the treatment was removed, and the cells were washed once with PBS. Fluorescence was measured at 700 nm using an Odyssey scanner (exc: 680 nm).

Confocal acquisition of GS756 cell spheroids

Acquisition of spheroids composed of GS756 cells was performed following incubation at 500 nM concentration of the drug. After a 2-hour incubation time, the spheroids had been washed extensively with PBS to remove any unbound drug. The nuclei were then stained with DAPI. Imaging was performed in the Opera Phenix Plus confocal microscope, where the drug is labeled in red. This protocol enables high-resolution assessment of drug distribution and cellular uptake within the three-dimensional tumor model.

Laser Specifications for PDT

In this study, the Modulight ML6500 laser was used, set to a wavelength of 690 nm and controlled via a PC-based controller program, offering customizable power settings and uniform beam delivery to ensure precise and consistent light dosing for effective photodynamic therapy.

Photodynamic therapy on GS756, GS359 and GS401 Spheroids

1×10^3 GS401 and GS359 cells, and 5×10^2 GS756 cells were seeded in round bottom plate (BioFloat F202003). After 48 hours spheroids were treated with 500 nM SiP-2(cRGDfK) (5% DMSO in medium). After 6 hours of incubation at 37 °C, photodynamic therapy was performed at 20 J/cm² using a 690 nm wavelength laser on an plate shaker. A control plate, under the same conditions but not exposed to the laser, was always included. 24 hours after the photodynamic therapy, the LDH assay (LDH Glo Promega J2380) was used to quantify the cytotoxicity of the treatment.⁷⁹ Bioluminescence measurements were taken using the GloMax Discovery (Promega). The treated spheroids were concomitantly observed under the microscope to study changes in morphology.

Study of the growth curve in the murine model for GS756 and GS401 cell lines

GS756 and GS401 cells were inoculated in NOD SCID mice by preparing the cell suspension with 3 million cells in a mixture of PBS/Matrigel® in equal parts (n=5 in each group of mice). The total volume injected is 200 µL and it was administered subcutaneously. Matrigel® is prepared in advance by thawing it on ice overnight. The cells are detached from culture using Accutase, washed with PBS, counted, and adjusted to concentration. During all these steps, all the Matrigel® and cells are kept on ice until injection to maintain viability.

Drug biodistribution in the NOD SCID mouse model after inoculation of GS756 cells, as high express the target

The biodistribution assessment protocol of SiPc-2(cRGDfK) in NOD SCID mice bearing subcutaneous tumors derived from GS756 cells therefore employs a precisely dosed 10 nmol of SiPc, prepared in a 5% DMSO solution in PBS. This photosensitizer solution was administered via a single intravenous tail vein injection, and the fluorescence signal was collected at the designated time points of 4, 6, and 24 hours post-injection (n=4 mice per group).

Following injection, mice were placed in dark housing to minimize light-induced activation of PS until time of imaging. At each time point, the mice were anesthetized with isoflurane and visualized using Ivis Imager. After imaging at each time point, the mice were euthanized, followed by collection of a set of tissues from tumor, liver, kidneys, spleen, and additional organs for ex vivo fluorescence measurements, quantifying the SiPc-2c(RGDfK) accumulation.

Ex vivo analysis: quantification of tumor tissue fluorescence

The list of organs analyzed in the biodistribution study included the kidney, skin, muscle, lung, heart, spleen, liver, stomach, pancreas, colon, small intestine, brain, and tumor tissue. Each organ was carefully collected, processed, and analyzed to assess the accumulation and distribution of the administered compound within different tissue types. Tumor analysis in the biodistribution study was

initiated with careful dissection and collection from treated NOD SCID mice. The tumors were frozen immediately after collection to preserve their integrity and were stored at -80 °C until analysis. Each tumor was weighed for processing and then transferred into a 2 mL tube kept on dry ice. For this, RIPA buffer and TRIS buffer at pH 8 should be added in volumes that had previously been calculated based on the weight, so that a similar condition of lysis can be obtained. Tissues from all organs were lysed by the action of a tissue lyser working at 50 Hz for three minutes to totally homogenize them. After the lysis, the centrifuge should cool in a refrigerator at 4 °C at 10×g for 5 minutes. Then, 150 µL aliquots of all samples were transferred to a 96 well fluorescence quantification plate. On the plate reader Odyssey, the fluorescence intensity was measured and compared to a standard curve from 1 µM or 2.5 µM prepared from known concentrations of the dye. For the Odyssey settings were configured as follow: a wavelength of 700 nm, with the lowest intensity setting, a focus offset of 3.0, and a resolution of 169 µm.

PDT efficacy in murine model

Photodynamic therapy efficacy in animals was elaborated by inoculating 3×10^6 GS756 cells, showing the development of a subcutaneous tumor. Animals were either started when the tumors reached a volume of 60-90 mm³. All animals were exposed to a fixed dose of 10 nmol of photosensitizer, photoactivated with a 690-nm wavelength laser after 6 hours from the drug administration. Animals were divided into three groups: one was left untreated, one was treated at 100 J/cm², and another one was treated at 150 J/cm². Long-term tumor response was monitored with 2 females and 2 males, while 2 were sacrificed 24 hours post-treatment for histological evaluation for a total number of 6 animals each group. Tumor samples were formalin fixed and paraffin embedded to allow detailed cellular and tissue response assessment.

Group	Growth monitored over time		Sacrificed 24 hours post-treatment	
	Male	Female	Male	Female
CTR	2 mice	2 mice	1 mouse	1 mouse
PDT dose: 100 J/cm ²	2 mice	2 mice	1 mouse	1 mouse
PDT dose: 150 J/cm ²	2 mice	2 mice	1 mouse	1 mouse

3.3

Results

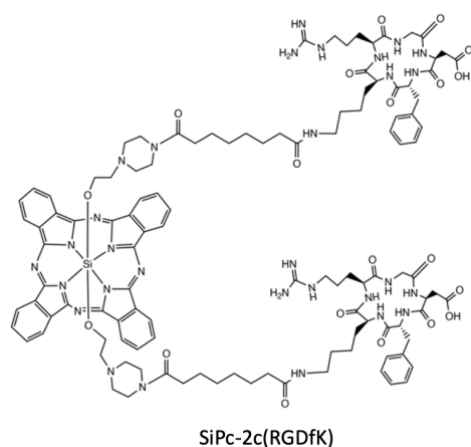


Figure 1. Chemical structure of silicon phthalocyanine conjugated with 2 molecules of c(RGDfK).

The final molecule (Fig. 1) consists of a planar aromatic macrocycle made up of four isoindole units, with a central silicon atom (Si) coordinated at the core. The silicon atom is bonded to two axial ligands, which contribute to the stability of the structure. Attached to the axial ligands we have inserted two units of c(RGDfK) peptide, a cyclic peptide with the sequence arginine-glycine-aspartic acid-D-phenylalanine-lysine. The pure product was obtained as blue powder (Fig. 2a; 2.5 mg, yield 20%). The identity of the compound was verified by mass spectrometry [MS (ESI): m/z calculated for $C_{114}H_{144}N_{30}O_{20}Si$ $[M + 2H]^{2+} = 1141.54$, found 1141.75; fragments derived from one Si–O bond cleavage: 872.23 $[M + H]^+$ (calculated for $C_{41}H_{65}N_{11}O_{10} = 872.03$, corresponding to the axial ligand), 705.57 $[M + 2H]^{2+}$ (calculated for $C_{73}H_{80}N_{19}O_{10}Si = 705.80$, corresponding to SiPc with one axial ligand).] (Fig. 2b).

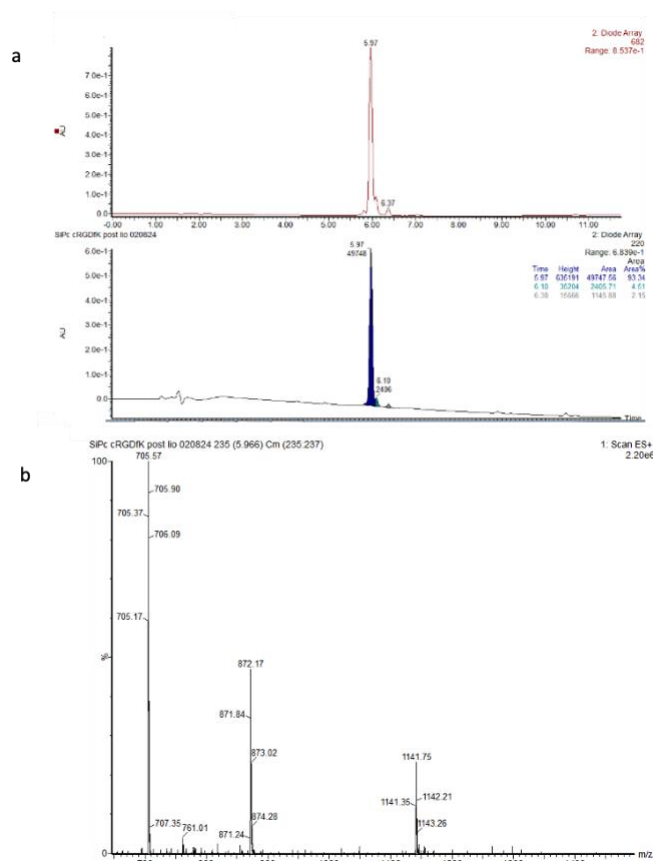


Figure 2. (a) Chromatogram of compound SiPc-2c(RGDfK) recorded at 682 nm (top) and 220 nm (bottom). The compound shows a retention time of 5.97 min and purity of 93.3%; (b) Mass spectrum (ESI+) of the peak at 5.97 min .

The absorbance spectrum of the photosensitizer shows a characteristic peak at 682 nm, typical of phthalocyanines. This peak corresponds to the Q-band absorption, reflecting the extended π -conjugation in its macrocyclic structure.⁸⁰

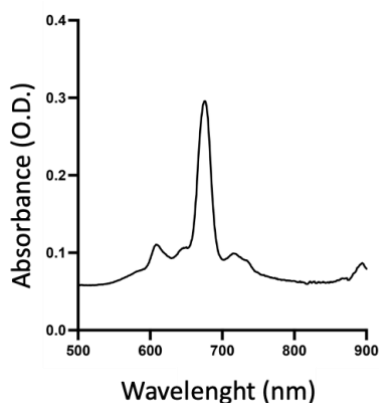


Figure 3. The absorbance spectrum of conjugated photosensitizer at 2 μ M in DMSO.

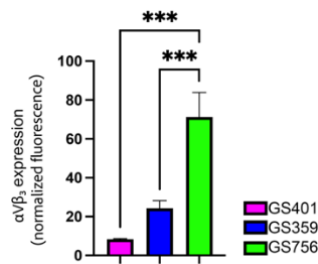


Figure 4. Characterization of integrin expression in GSCs. Statistical significance is shown as follows: **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Gs401 and Gs756 exhibit a significant difference in receptor expression, with one (Gs756) showing notably higher level compared to the other (Gs401). This difference in expression was quantified by the normalized fluorescence intensity, highlighting the distinct integrin profiles of these cell lines.

Once the cells are grown, they form spheroids, which show different sizes according to plate density and specific cell line. The results show that Day 2 is the ideal time for peak spheroid formation and growth in a homogeneous manner when the spheroid is approximately 300 μm in diameter (Figure 5a).

The internalization of the selective probe in spheroids is appreciated over time and it is particularly present in the highly demanding target cell line (Figure 5b).

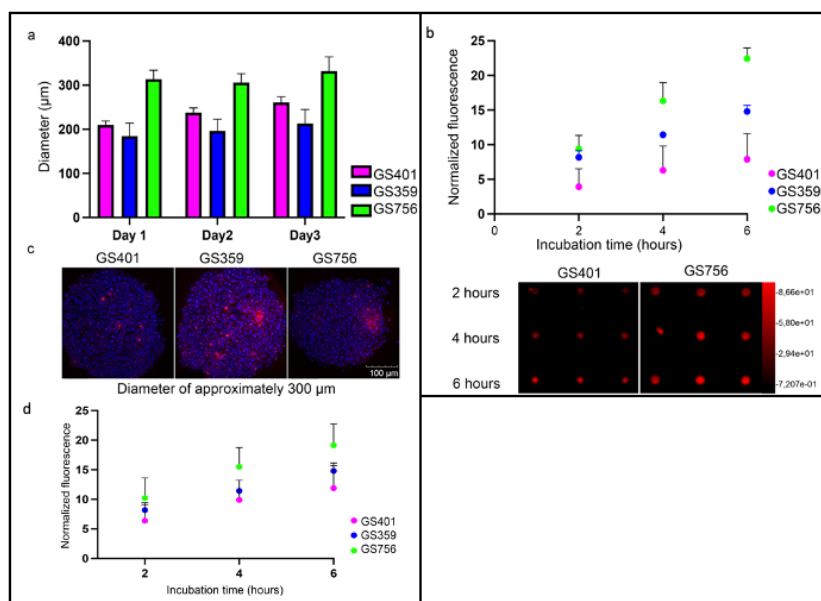


Figure 5. Spheroid characterization and temporal uptake dynamics

a) Diameter of the spheroids was measured according to growth from day 0 (plate seeding) to day 3, with the aim of obtaining spheroids approximately 300 μm in diameter. b) Spheroids were acquired over time using a 700 nm emission filter following drug treatment to monitor the uptake dynamics from 2 hours to 6 hours incubation time. c) Confocal imaging of spheroids, approximately 300 μm in diameter, was performed after a 2-hour incubation with SiPc-2 c(RGDfK). The red signal indicates the location of the probe, while the nuclei are stained in blue. d) The increased uptake of the photosensitizer is also evident in adherent cells. Drug uptake measurements were normalized to an internal control to ensure accuracy. The error bars in the graph represent the standard deviation ($\pm\text{SD}$), illustrating the variability within each group compared to the mean.

The spheroids were monitored for changes in size and drug uptake over time to assess the dynamics of photosensitizer internalization. The 300 μm spheroid size was achieved by controlling growth conditions over 3 days. Drug uptake was evaluated using both fluorescence microscopy and confocal imaging, with normalized data to control for any internal variations, ensuring consistency across experimental conditions. The increased uptake observed in spheroids and adherent cells highlights the efficiency of the drug in penetrating cellular structures.

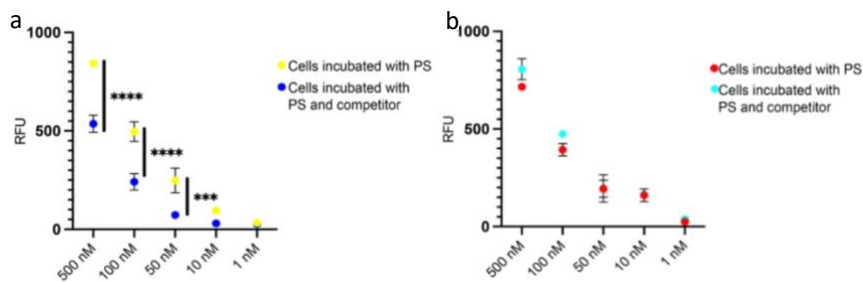


Figure 6. Competitive assay with only peptide in increasing concentrations in GS756 a) and in GS401 b). The error bars in the graph represent the standard deviation ($\pm$ SD). Statistical significance is shown as follows: **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

In this competitive assay, the effect of increasing concentrations of peptide alone on integrin binding was assessed in GS756 (a) and GS401 (b) cell lines. The data indicate how varying peptide concentrations influence the interaction between the photosensitizer and integrins, with significant differences observed between the two cell lines. The results are quantified with statistical analysis, highlighting the concentration-dependent effects of the peptide and demonstrating the degree of specificity and competition with the integrin binding sites.

After photodynamic treatment on the spheroids, they appear different under the microscope (Figure 7a). Those from the GS756 cell line that have been treated with PDT appear visibly disintegrated (Figure 7b), with drug aggregates around the spheroid, unlike the control spheroids which are still vital and compact, as well as those derived from other cell lines that do not or moderately express the target.

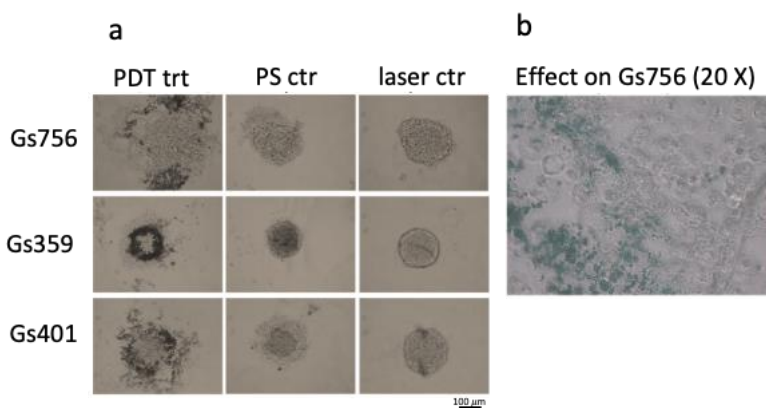


Figure 7. a) Bright-field images of spheroids after PDT treatment in the three cell lines. b) Magnification of the PDT treatment effect on GS756 cells.

The GS756 cell line, which has high $\alpha_v\beta_3$ expression, displayed much greater sensitivity to PDT than other GBM cell lines with lower integrin expression. The increased sensitivity to the laser was qualitatively observed through microscopy images of probe internalization and quantitatively confirmed via the lactate dehydrogenase (LDH) assay, which indicated increased bioluminescence signals, reflecting significant cell damage (Figure 8).

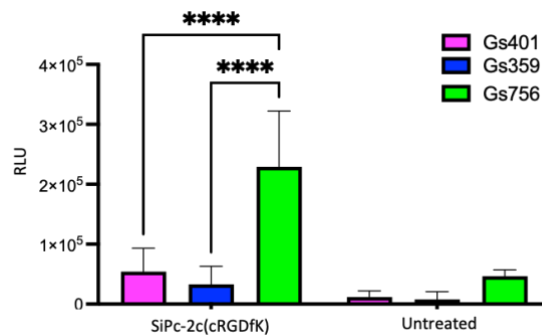


Figure 8. The bioluminescent signal of LDH released by Gs756 spheroids treated with the drug and exposed to 20 J/cm² laser is statistically significant compared to signals observed in other cell lines and untreated controls.

An additional control, measuring the response of spheroids treated with either the drug alone or the laser alone, showed no difference from untreated controls (data not shown). Tumor growth curves obtained with the two GS cell lines GS756 and GS401 are presented in Figure 9.

A delayed development of tumors was observed in mice injected with GS401 cells when compared with GS756 cells. In this latter group, the growth was very heterogeneous: striking variability could be observed in time and speed of tumor development between the five animals. On the contrary, the tumors issuing from GS401 cells grew in a more homogeneous way, with less variability among the animals, except for one animal.

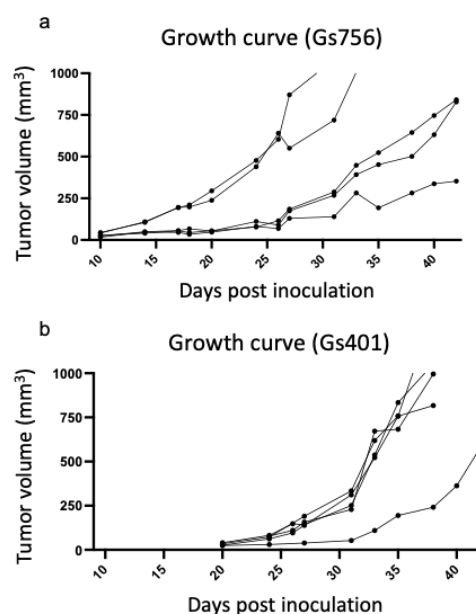


Figure 9. Growth curve of GS756 (a) and GS401 (b) in a subcutaneous NOD SCID mouse model: tumor volume was measured three times a week using a manual caliper to follow growth dynamics and compare the tumor development rates of the two cell lines.

Tumor volume reached the range of 60-90 mm³ in all GS756 tumor-bearing animals between 14- and 24-days post-inoculation. For subsequent treatment, the range of the target tumor volume was set at 60-90 mm³ because at this stage, growth curves for GS756 cells are still linear rather than exponential.

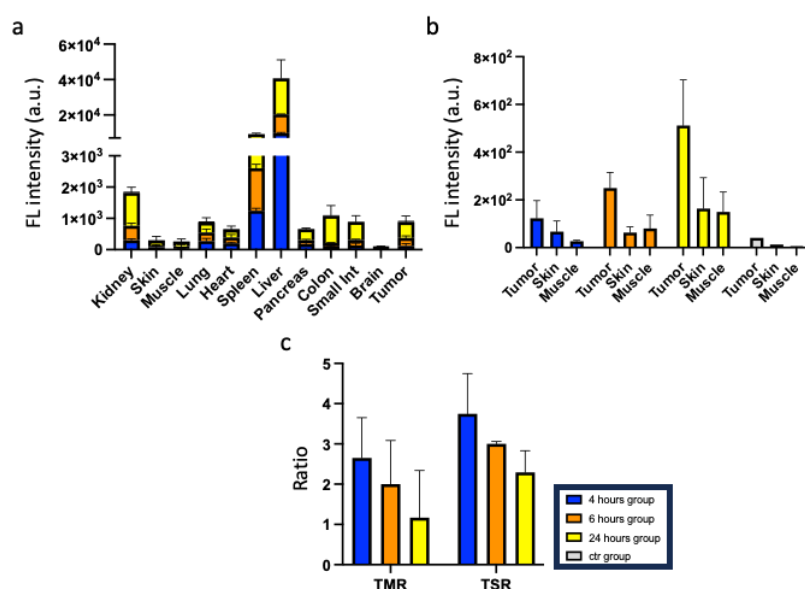


Figure 10. (a) Fluorescence measured by the Odyssey plate reader for all collected organs at each time point (4, 6, and 24 hours) (n=4 per group). (b) Time-course fluorescence measurements for tumor, skin, and muscle (gram of organ), enabling the calculation of Tumor-to-Skin and Tumor-to-Muscle ratios, as shown in graph (c).

This figure presents the fluorescence intensity data collected from various organs at different time points following drug administration. Panel (a) shows the fluorescence measurements for all collected organs at 4, 6, and 24 hours, revealing the biodistribution profile of the photosensitizer. Panel (b) focuses on the tumor, skin, and muscle, highlighting temporal changes in fluorescence over time. From these data, tumor-to-skin and tumor-to-muscle ratios were calculated, as depicted in panel (c), providing insights into the preferential accumulation of the photosensitizer in tumor tissue compared to surrounding tissues.

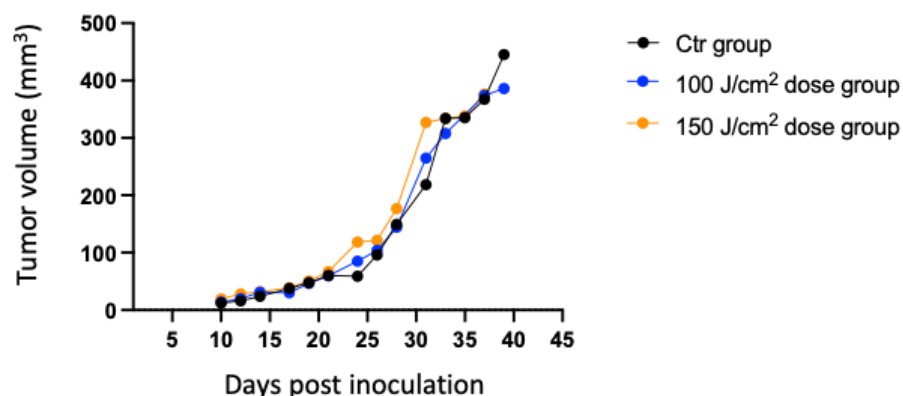


Figure 11. The tumor size was averaged as a function of time since inoculation (n=4 per group). No differences in tumor growth rates were observed between the three groups, indicating no noticeable effect from the treatments. No differences in growth timing were detected.

In this study, the growth curves of GS756 tumors were monitored following photodynamic therapy (PDT) treatment 6h after injection of PS. The tumor sizes were measured over time and averaged for each group. Comparison of the three treatment groups showed no significant differences in tumor growth rates, suggesting that the chosen treatment with a drug/light interval of 6 hours and a dose of 100 J/cm² and 150 J/cm² did not impact tumor progression. Additionally, the timing of tumor growth was consistent across all groups, further reinforcing the conclusion that the treatments did not exert a detectable effect on tumor development.

3.4

Discussion and conclusions

In the present study, we report, for the first time, the efficacy of PDT treatment in three-dimensional patient-derived models of GBM using a newly synthesized photosensitizer targeting integrin $\alpha_v\beta_3$. Second, we calculated drug biodistribution with the goal of optimizing its internalization time within the tumor to enable us to conduct an *in vivo* study on its photodynamic efficacy. Although PDT has been approved by the FDA as a clinical treatment for various precancerous conditions its use in GBM is still being explored.⁸¹

The use of PDT for the treatment of GBM has been extensively investigated but it has yet to demonstrate a well-established therapeutic application.⁸² Unlike in dermatology, where laser light is applied locally and directly to the lesion, various approaches have been tested for brain treatments, including fiber optic diffusers, optical fibers, and cavitation balloons, and interstitial applications.⁸³ Clinical trials are currently ongoing using 5-ALA and demonstrated the safety of the procedure. Among the factors contributing to the therapeutic shortcomings of PDT in GBM is the concentration of oxygen in the brain. In fact, oxygenation levels drop from 5%–15% in healthy brain tissue to as low as 0.1% in necrotic brain tumors.⁸⁴

In our research, the emphasis transitions from addressing the entire tumor mass to specifically targeting the remaining positive infiltrating tumor cells. In particular, we investigate an approach aimed at eradicating glioma stem cells, which, while not constituting the bulk of the tumor, are found in regions with a normal oxygen concentration. This elevated oxygen level makes these areas suitable for the effective application of photodynamic therapy, leaving surgery as the treatment of choice for eradicating the primary tumor mass. Targeting an overexpressed receptor specific to the cells that remain in the positive margins represents an emerging strategy at this time.⁸⁵ The combination of these two elements, maintaining normal oxygen levels in the treated area and ensuring the treatment selectively spares healthy tissues, forms the foundation of how effective this approach can be in defined conditions.⁸⁶

Unlike monolayer cell cultures, that do not mimic the complexity of *in vivo* conditions, GS spheroids offer a more accurate three-dimensional representation of the tumor biology. This three-dimensional setup enhances the relevance and reliability of experimental results by better reflecting cell-to-cell interactions. GS spheroids have proven to be valuable in *in vitro* cancer models, particularly because of their ability to reveal differential integrin expression, which correlates with how the probe is internalized *in vivo*.⁸⁷ By combining a silicon phthalocyanine with cRGDFK, we aimed to improve effectiveness of PDT through enabling the targeted delivery of the photosensitizer to the tumor. SiPcs have demonstrated potent photosensitizing activity in both preclinical and clinical studies.⁸⁸

In our study, through employing three different cell lines expressing varying levels of $\alpha_v\beta_3$ integrin, we assessed the effectiveness of targeted PDT in different integrin expressing states. As is well known,

inhibiting specific integrins involved in cell migration can significantly reduce tumor malignancy, lowering invasiveness and disease progression. This targeted approach increases the cytotoxic impact on GBM cells while minimizing damage to surrounding healthy tissue.⁸⁹

The strong relationship between PDT efficacy and $\alpha_v\beta_3$ integrin expression highlights the important role this integrin plays in influencing the therapeutic response. The elevated expression of $\alpha_v\beta_3$ in GSCs promotes tumor angiogenesis and contributes to the aggressive behavior of GBM. Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are highly expressed not only in glioma cells but also in the surrounding tumor vasculature, playing key roles in GBM progression and angiogenesis. Targeting the tumor vasculature with PDT deprives tumor cells of necessary nutrients and oxygen while also increasing vascular permeability, which improves the delivery of subsequent therapeutic agents.⁹⁰ This dual effect, direct cytotoxicity and vascular disruption makes PDT a promising treatment.⁹¹ To better study this effect, future research may benefit from the development of vascularized organoid models, incorporating endothelial cells to replicate blood vessels. Organoids can grow endothelial cells and form blood vessels using co-culture techniques that include both endothelial cells and angiogenic factors like VEGF. These methods mimic the environment of the tumor, allowing vascular networks to form approaches like implanting organoids in tissues with existing blood vessels or using microfluidic systems can encourage integration of blood vessels from the host or stimulate the growth of new vessels within the organoid itself. These vascularized organoids are particularly useful for studying blood vessel formation and testing treatments that target tumor blood supply. Such models would enable a more precise investigation of how treatments such as PDT impact both tumor cells and vasculature, offering critical insights for improving therapeutic outcomes.⁹²

Although clear photodynamic efficacy was observed in the three-dimensional model, the effectiveness did not entirely translate to *in vivo*.

The tumor growth curve for both cell lines shown heterogeneity in the growth rate and time, particularly in the GS756 model. In addition, the tumors of GS401 grew very slowly and belatedly; thus, this negative model was excluded in further experiments because it was badly fitted.

The complexity of glioma stem cells highlights the significant role of nitric oxide synthase-2 (NOS2) in their proliferation and tumor growth. Glioma stem cell typically exhibit high NOS2 expression, which supports cell survival, resistance to apoptosis, and continued proliferation. This elevated nitric oxide production in GS401 cell line could account for their slower but resilient growth pattern, in contrast to the GS756 line, which demonstrates more variability in tumor development, likely a result of greater heterogeneity within its cell population.⁹³

The *ex vivo* biodistribution analysis also revealed that the optimal Tumor-to-Muscle and Tumor-to-Skin signal ratio occurred at 6 hours post-treatment. This post-treatment time provided the best balance since it minimized skin fluorescence that may interfere with the treatment accuracy.⁹⁴ Optimization of this ratio is very crucial, as high skin fluorescence can mask the tumor signal and lead to loss of information, low effectiveness, and low accuracy during PDT.⁹⁵ The values measured with IVIS Imager are not shown, as they were deemed unreliable compared to results obtained from *ex vivo* samples. The selected laser dose and drug concentration in the therapeutic efficacy study did not result in a measurable tumor growth reduction among the treated groups. This suggests that the

administered drug dose may have been too low to initiate an effective photodynamic response. Effective tumor inhibition requires a higher concentration of the drug to enhance higher generation of ROS, important in the induction of cytotoxicity in tumor cells via photodynamic therapy. When the drug dose is too low, ROS production may be insufficient because there are not enough molecules in the tumor to respond effectively to light activation. This limited ROS generation is unable to produce the necessary cytotoxic environment, leading to reduced PDT effectiveness and minimal impact on tumor.⁹⁶

In summary, targeted delivery systems could offer a new therapeutic strategy for local targeted therapy of GBM.⁹⁷ This is underlined by the specific integrin-binding affinity, the deep tissue penetration capabilities of the photosensitizer, and the targeting mechanisms of PDT, which collectively render this therapeutic approach a promising option to prevent or delay GBM relapses.⁹⁸ The study of spheroids derived from glioma stem cells provides a good grounding for the optimization of conditions in subsequent *in vivo* efficacy studies. The spheroid model allows a more realistic estimate of drug penetration and its cytotoxic consequences in tumor-like structures, having value in understanding how such a treatment might perform in a more complex system. *In vivo* environments introduce additional variables, including metabolic degradation, which may affect drug behavior and efficacy, preventing the initiation of a robust PDT response. Further research is needed to better confirm the potential of the targeted therapy to improve outcomes for GBM patients.

4 Targeted photodynamic therapy in pancreatic adenocarcinoma: efficacy in spheroids and in a murine model

Abstract

Photodynamic therapy offers a selective and effective method for diagnosing and treating cancer, leveraging photosensitizers activated by light to destroy cancer cells in the presence of oxygen. Despite its promise, efficacy of PDT has been limited by the low specificity of traditional photosensitizers for targeting tumors. This study aims to address this limitation by creating a targeted PDT approach for pancreatic ductal adenocarcinoma (PDAC) using a peptide-conjugated photosensitizer specifically binding to integrin $\alpha_v\beta_6$, a receptor prevalent in PDAC cells and linked to tumor growth. Given limited treatment options of PDAC, targeting $\alpha_v\beta_6$ offers a new pathway for precision treatment. The cyclic peptide cyclo[FRGDIAFp(NMe)K], recognized for its high affinity and specificity for $\alpha_v\beta_6$, was chosen to guide a phthalocyanine-class photosensitizer directly to PDAC tumors. By developing 3D spheroid models and *in vivo* BxPc3 xenograft models in NOD/SCID mice, we aimed to refine this PDT approach and assess its therapeutic promise.

4.1

Introduction

Photodynamic therapy is a well-established medical technology used for the diagnosis and treatment of malignant tumors. PDT relies on the use of photosensitizers, light and oxygen to eliminate cancer cells.⁹⁹ PDT is based on the dynamic interaction between a photosensitizer, light with a specific wavelength, and molecular oxygen, promoting the selective destruction of the target tissue. However, despite the big potential, PDT remains limited in its application, due to the fact that traditional photosensitizers have a relatively low selectivity for the target of interest. In order to maximize PDT efficacy, a good strategy could be to conjugate photosensitizers with molecular vectors that target overexpressed receptors on tumors. In particular, a great target of interest is the integrin subtype $\alpha_v\beta_6$ integrins, which is widely expressed in Pancreatic Ductal Adenocarcinomas (PDAC). PDAC is the fourth most common cause of cancer-related deaths with a 5-year survival rate of less than 5%, predominantly due to clinical signs of disease occurring late in disease progression, often after metastasis has occurred. Current treatment options are extremely limited, with surgical resection and chemotherapy only effective in cases that are detected early and when disease is restricted to the pancreas.¹⁰⁰

Such integrin expression reveals a great variability in the human pancreatic adenocarcinoma cell lines, like BxPC3, MIAPaCa2, AsPC1, and PANC1. Namely, BxPC3 and PANC1 express very high levels of $\alpha_v\beta_3$ integrin, while this subtype is relatively low expressed in tumor cell lines MIAPaCa2 and AsPC1. Because of that fact, these lines are most useful models for investigation of integrin-related pathways in pancreatic cancer.¹⁰¹ Although this subtype, $\alpha_v\beta_6$, has assumed increasing importance.¹⁰² The BxPc3 is a cell line exhibiting epithelial morphology that was isolated from the pancreas tissue of a 61-year-old, female patient with adenocarcinoma.¹⁰³

The primary role attributed to integrin $\alpha_v\beta_6$ is the activation of transforming growth factor β (TGF β), a potent growth inhibitor.¹⁰⁴ It is secreted into the extracellular space in an inactive latent complex associated with the protein LAP. $\alpha_v\beta_6$ facilitates the activation of TGF β through interaction with the RGD sequence on LAP, which results in the release of active TGF β . Under normal circumstances, TGF β maintains tissue growth in check by repressing various signaling cascades.¹⁰⁵ However, in the context of cancer, overexpression of $\alpha_v\beta_6$ drives tumor invasion and metastasis in epithelial cancers. In the area of integrin inhibitors with the RGD sequence, one of the most valued representatives is the peptide cyclo[FRGDIAFp(NMe)K]. The peptide has been designed for increased binding specificity and stability, partly due to the fact that the RGD motif represents a binding epitope for specific interaction with integrin receptors.¹⁰⁶ While the peptide in fact exhibited high affinity for the $\alpha_v\beta_6$ integrin, highly specific molecular design is required for it due to the likelihood of nonspecific binding in targeting contexts. This fact has an important implication for designing targeted therapies and imaging because this effect of unintended binding due to excessive nonspecific interactions can undermine therapeutic and imaging resolution precision.

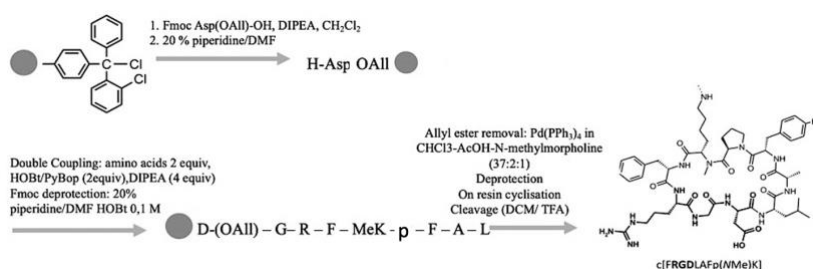
The aim of this study is to establish and optimize both spheroid and *in vivo* xenograft models of BxPc3 pancreatic adenocarcinoma in NOD/SCID mice for testing a targeted photodynamic therapy photosensitizer from the phthalocyanine class, specifically targeting the $\alpha_v\beta_6$ integrin.

4.2

Materials and Methods

Synthesis of cyclo[FRGDLAfp(NMe)K]

The schematic synthesis protocol of cyclo[FRGDLAfp(NMe)K] is reported in scheme 1.¹⁰⁷



Scheme 1. Synthesis process of the cyclic peptide in solid phase.

The synthesis of the cyclic peptide c[FRGDLAfp(NMe)K] was conducted on a 2-chlorotrityl chloride resin (Sigma-Aldrich, 8.55017, Novabiochem) via solid-phase peptide synthesis. The process began by loading Fmoc-Asp-OAlI (Sigma-Aldrich, 47578, CAS 144120536) on 0.500 g of resin in the presence of DIPEA and dichloromethane, with stirring for 1 hour. The loading capacity of the resin was verified by weight difference, yielding a calculated capacity of 0.6 mmol/g. To cap the resin, a solution of methanol, dry dichloromethane, and DIPEA (2:17:1) was applied, followed by Fmoc deprotection using 20% piperidine in DMF in two 15-minute intervals.

Following DMF washes, amino acids were sequentially coupled using a double-coupling method, with 5 equivalents of DIPEA and 2.5 equivalents per amino acid. Protected amino acids were sourced from Novabiochem, including Fmoc-D-Pro-OH (Sigma-Aldrich, CAS 101555-62-8) and Fmoc-N-ME-LYS(BOC)-OH (Sigma-Aldrich, 773107-250MG, CAS 197632-76-1 from IrisBiotech). Each coupling step lasted 30 minutes, while coupling for N-methyl-lysine was extended to 2 hours.

After completing the linear peptide sequence, the allyl group was removed using tetrakis(triphenylphosphine)palladium(0) (Sigma-Aldrich, 216666, CAS 14221-01-3) with N-methylmorpholine in DCM, incubating overnight in the dark. Subsequent Fmoc deprotection was performed, and a mini-cleavage verified the correct synthesis of the linear sequence. On-resin cyclization was achieved with 1 mmol PyBop and 5 equivalents of DIPEA in DMF, allowing the reaction to proceed overnight.

For analytical verification, UPLC was conducted using an Acquity UPLC BEH C18 1.7 μ m column. The gradient employed mobile phases A (water + 0.05% TFA) and B (acetonitrile), beginning with 80% A and 20% B for transitioning to 40% A and 60% B over 12 minutes, and reaching 0% A and 100% B at 13 minutes.

Analytical study of the two peptide species

The two species, A and B, with identical peptide mass, were obtained after solid phase peptide synthesis. They were analyzed to identify the correct compound using UPLC and NMR spectroscopy. A 1 mg/mL solution of the peptide in water was prepared and initially analyzed by UPLC. The sample was then heated at 50 °C for 1 hour and re-analyzed by UPLC to observe any changes in retention time or peak area distribution.

Two-dimensional NMR was conducted in both water and D₂O to further explore the structural characteristics of the two observed species. TOCSY experiments were utilized to establish correlations among all protons within each spin system, aiming to confirm the presence and relative abundance of the species. Additionally, ROESY experiments were performed to assess through-space dipolar interactions, with a focus on the configuration of key residues and potential structural shifts.

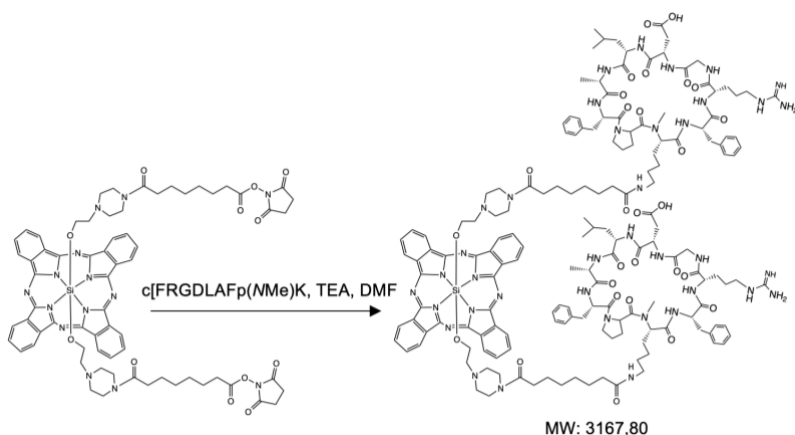
The separation of the two species, A and B, was achieved using an AKTA chromatography system.

Indirect assay to determinate IC₅₀ of the peptide

A competition-based assay was conducted to determine the IC₅₀ of the peptide for integrin $\alpha_v\beta_6$. The assay employed a well-characterized fluorescent inhibitor with excitation and emission wavelengths of 755 and 820 nm, respectively. The method specifications are reserved and supported by Bracco, not detailed.

Conjugation of the SiPc with 2 c[FRGDIAFp(NMe)K]

Compound SiPc-NHS was prepared by previously reported method and it was conjugated with 2 molecules of cyclo[FRGDIAFp (NMe)K].¹⁰⁸



Scheme 3. Conjugation reaction of SiPc-NHS with the peptide cyclo- [FRGDIAFp (NMe)K].

A solution of SiPc-NHS (10 mg, 0.0075 mmol), TEA (9.5 μ L, 0.068 mmol) in 10 mL of DMF were added to a solution of cyclo- [FRGDIAFp (NMe)K] (10 mg, 0.00956 mmol) in 10 mL of DMF and stirred at room temperature for 2 h. The final mixture was precipitated and washed with ethyl ether three times. The solid was then purified by preparative HPLC by using a Water XTerra™ Prep RPdC8 19/100

column, applying a gradient of CH₃CN in CH₃COONH₄ 0.1 M, pH=7 from 30% to 80% in 15 min (20 mL/min).

Optical characterization of the photosensitizer SiPc-2c[FRGDIAFp (NMe)K]

The absorbance of the photosensitizer was measured using a quartz cuvette in a UV/VIS spectrophotometer. For the analysis, the drug was prepared as a 4 μ M solution in two different solvents: 5% DMSO in PBS and 5% DMSO in serum. The serum solution was then analyzed over time at intervals of 2, 4, 6, and 24 hours to assess its stability.

PDAC cell cultures

The cell lines BxPc3 and Capan1, both originating from human pancreatic adenocarcinoma, were selected for their high expression of $\alpha_v\beta_6$ integrin. MiaPaca2 and PANC1, which exhibit low $\alpha_v\beta_6$ expression according to the literature, were chosen as negative controls. Capan1, MIApaca2 and PANC1 were supplied by the research team at the Department of Molecular Biotechnology and Health Sciences, Via Nizza 52. The BxPc3 cells were purchased from ATCC by Bracco Imaging Spa.

All the cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. The absence of mycoplasma contamination was verified using the EZ-PCR Mycoplasma Test Kit. Cells were maintained in T175 flasks, with a maximum subculturing dilution of 1:4 to promote optimal growth conditions for subsequent *in vitro* and *in vivo* experiments.

$\alpha_v\beta_6$ and $\alpha_v\beta_3$ expression by flow cytometry

To study the selective affinity of the peptide towards integrins, $\alpha_v\beta_6$ and $\alpha_v\beta_3$ were investigated. To analyze the expression of $\alpha_v\beta_6$ and $\alpha_v\beta_3$ in the several cell lines, 5×10^5 cells per tube were incubated at 4 °C for 1 hour in DMEM containing 0.1% BSA and 1 mM Mg²⁺. The primary antibody was used at a 1:50 dilution, followed by a 45-minute incubation with a secondary antibody at a 1:100 dilution.

For $\alpha_v\beta_6$ detection, the primary antibody was Anti-Integrin $\alpha_v\beta_6$ Mouse Monoclonal (MAB2077Z, Millipore, clone 6A2987), and the secondary antibody was Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Catalog # A-21131, Invitrogen).

For $\alpha_v\beta_3$ detection in BxPc3, Panc1, MIApaca2 and Capan1 cells, the same protocol was followed using PBS with 0.1% BSA as the buffer. The primary antibody for this analysis was the Human Integrin $\alpha_v\beta_3$ Alexa Fluor® 488-conjugated Antibody (Monoclonal Mouse IgG1, Clone #23C6, Catalog Number: FAB3050G, ReDSsystem, a Biotechne brand) with a secondary antibody conjugated to Alexa Fluor 647.

Flow cytometry analysis was conducted on a BD Celesta FACS instrument, providing data on integrin expression levels across the different cell lines.

$\alpha_v\beta_6$ and $\alpha_v\beta_3$ expression by Western Blot

For Western blot analysis, cell lysates were prepared by first collecting cell pellets. The old medium was removed, and cells were rinsed with cold PBS. They were then detached using a non-dissociating solution, inactivated with fresh medium, and centrifuged at 11000 RPM for 5 minutes at 4 °C. Approximately 2.5×10^6 were isolated, recentrifuged at 1100 RPM, and stored at -80 °C until protein extraction. U87MG and HT29 cell lines were used as control samples in the study, serving as positive and negative controls, respectively. U87MG cells are positive for β_3 integrin and negative for β_6 integrin, while HT29 cells are positive for β_6 integrin and negative for β_3 integrin. These controls were included to validate the specificity of the integrin-targeting treatments and to compare the effects across different integrin profiles.

For protein extraction, cell pellets were resuspended in a minimal volume of lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM $MgCl_2$, 1% Triton X-100, 10% glycerol) with 1 mM PMSF and protease inhibitors. A PBS-based solution with SDS and Triton X-100 was used to enhance lysis. The samples were vortexed for 30 minutes, sonicated, and then centrifuged at 13200 RPM for 20 minutes at 4 °C. Supernatants were collected for protein analysis. Throughout the procedure, samples were kept on ice, and lysates were stored at -80 °C. Protein concentration was determined using the BCA assay (Bicinchoninic acid assay).

A Western blot was conducted on BxPc3, Panc1, MIApaca2 cell lines, with U87MG and HT29 serving as positive and negative controls for the integrin subtypes $\alpha_v\beta_3$ and $\alpha_v\beta_6$. Primary antibodies used included Anti-Integrin $\alpha_v\beta_3$ (clone EM22703, ZooMAb® Rabbit Monoclonal ZRB1190) at a 1:500 dilution, and Anti-Integrin $\alpha_v\beta_6$ (clone EM05201, ZooMAb® Rabbit Monoclonal ZRB1104) at a 1:1000 dilution, with overnight incubation at 4 °C. The secondary antibody, an anti-Rabbit IgG, was diluted 1:15000 in 5% BSA in TBST and incubated for 1 hour. Protein transfer was performed on a PVDF membrane, which was pre-activated in methanol, to ensure efficient binding and signal detection. The data were then acquired at Chemidoc and analyzed using Image Lab software.

Formation and imaging of BxPc3 and MiaPaca2 spheroids

A total of 1×10^3 BxPc3 cells and 1×10^3 MIApaca-2 cells were seeded per well in a spheroid microplate (Corning, REF 4515, 96-well black plate with a clear, round, ultra-low attachment bottom) with complete RPMI medium. Spheroid formation was followed until day 7 from the seeding, and spheroid diameters were measured using a Spinning disk microscope in bright-field mode.

BxPc3 and MiaPaca-2 cells, which formed spheroids within one day after plating, were incubated with the photosensitizing drug for 2 hours. After a PBS wash, the spheroids were stained with DAPI (nuclear dye) and WGA (Wheat Germ Agglutinin to dye the plasma membrane) and then observed under a confocal microscope at the University of Turin laboratories.

Laser to conduct PDT

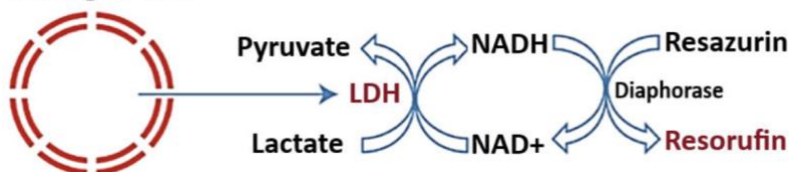
The FC-650/450 mW laser operates at a wavelength of $650 \text{ nm} \pm 5 \text{ nm}$ in continuous wave mode, delivering over 450 mW of power through a 400 μm fiber core with an SMA905 connector and 1 m fiber length. The laser has to maintain stable power output within a 5% RMS (Root Mean Square, power) variation over 4 hours and has a warm-up time of under 5 minutes. It operates within a

temperature range of 10–40 °C and supports modulation options from 1 Hz to 30 kHz. In laser therapy, power (measured in watts, W) is essential for effective tissue penetration, alongside wavelength. Higher power allows for deeper photon penetration and shortens treatment duration by delivering more photons efficiently, according to the formula $E = P/t$, where energy (E) is a function of power (P) and time (t). The laser was mounted in a protective enclosure that remains closed during use, with operators required to wear goggles that shield against red light. An adjustable arm holds the fiber in place, enabling precise control over power output, sample distance, and irradiation time for optimal treatment outcomes.

Photodynamic therapy on BxPc3 spheroids

After a 2-hour dark incubation with the photosensitizer, the spheroids were irradiated with a 650 nm laser (model FC-650, 450 mW output power, fiber core diameter 400 μm) at a dose of 40 J/cm². The plate was then returned to the incubator for 24 hours. Following this incubation, the photodynamic effect was assessed using a Spinning Disk microscope in bright-field mode, and a fluorescence-based cytotoxicity assay was performed to evaluate treatment efficacy (CyQUANT™ LDH Cytotoxicity Assay – Fluorescence Kit. (Catalog Nos. C20302 and C20303). Pub. No. MAN0018656 Rev. A.0.

Damaged Cells



Scheme 4. The CyQUANT LDH Cytotoxicity Assay measures cytotoxicity based on lactate dehydrogenase (LDH) release. LDH is a cytosolic enzyme released into the culture medium when the cell membrane is damaged. In this assay, LDH catalyzes the conversion of lactate to pyruvate, resulting in the production of NADH. Subsequently, NADH reduces resazurin to resorufin, a highly fluorescent compound, which can be quantified by measuring fluorescence (excitation at 560 nm, emission at 590 nm).

The level of fluorescence directly correlates with the amount of LDH released, providing an indication of cytotoxicity.

The percentage of cytotoxicity was determined using the formula:

$$\% \text{ Cytotoxicity} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100$$

Spontaneous LDH activity is the baseline LDH release in untreated cells, and the Maximum LDH activity corresponds to total LDH release following complete cell lysis. Finally Compound-treated LDH activity represents the LDH released from cells treated with the test compound. This calculation yields the percentage of cells with membrane damage attributed to the treatment, providing a measure of treatment-induced cytotoxicity.

Animal model: defining the tumor growth curve

The study utilized 16 female NOD/SCID mice, aged 5 weeks upon arrival, supplied by Envigo, Italy. This choice of model was based on its suitability for xenograft studies of human pancreatic cancer.¹⁰⁹ The experimental design involved two groups of animals, each inoculated with BxPc3 cells to induce subcutaneous tumor. Each group is composed by 8 animals, with the first group receiving 1.5×10^6 cells and the second group receiving 3×10^6 cells. Prior to inoculation, cell viability was assessed using the Trypan Blue exclusion test with an automatic cell counter, ensuring only cell cultures with viability above 70% were used.

The BxPc3 cells were washed in serum-free medium, resuspended in 0.1 mL of serum-free medium, and injected into the right flank of 6-week-old NOD/SCID mice anesthetized with sevoflurane gas. Tumor development was monitored by measuring with caliper three times for week starting from the seventh day post-inoculation, and tumor volume was calculated using the formula $(L \times W^2)/2$.

Animals were sacrificed upon reaching one of the following endpoints: $\geq 20\%$ weight loss, tumor volume $\geq 1.5 \text{ cm}^3$, tumor ulceration, signs of severe or prolonged stress, tail necrosis, or 60 days post-tumor induction.

Efficacy PDT study on a subcutaneous pancreatic adenocarcinoma murine model

- **Experimental Design**
NOD/SCID mice were subcutaneously inoculated with BxPc3 cells and divided into four groups (n=8 per group): a high-dose PDT group, a low-dose PDT group, a drug control, and a laser control. Each tumor bearing animal received 1.5×10^6 BxPc3 cells, and tumor volumes were measured three times a week to track growth. Treatments began when tumor volumes reached 60-90 mm³, prior to the onset of exponential growth.
- **Photosensitizer administration**
Each animal received an intravenous dose of 10 nmol of Silicon Phthalocyanine-2c[FRGDIAFp(NMe)K] in a solution of PBS containing 10% DMSO. PDT treatment was conducted 4 hours after drug administration. Group 1 received laser irradiation at the dose of 116 J/cm², while Group 2 received irradiation at the dose of 56 J/cm². Groups 3 and 4 served as controls, receiving only the drug or laser treatment, respectively.
- **Laser treatment and tumor monitoring**
Mice were anesthetized with sevoflurane, and the tumor area was shaved for laser exposure. The tumor site was exposed to laser light. Post-irradiation, tumor progression was monitored for up to 60 days or until tumors reached 800 mm³. Tumor volumes were measured with caliper, and humane endpoints were applied as necessary.

Histological analysis

For *ex vivo* analysis, tumor tissues were excised and processed into frozen OCT or FFPE blocks. Sections of 10 μm (frozen) and 5 μm (FFPE) were prepared from anterior, middle, and posterior regions of the tumors and stained with Hematoxylin and Eosin (H&E). The staining of the vessels with CD105 was carried out.

Digital scans were conducted with an Aperio microscope, and image analysis was performed using Imagescope software (Leica).

Target expression in tumor tissues

Representative samples were then lysed, and Western blot analysis was conducted according to the previously described protocol to examine integrin expression in the tumor tissues.

4.3

Results

The solid-phase synthesis of the peptide achieved a 40% yield, resulting in the simultaneous presence of two peptide subspecies (Fig. 1). Analytical methods identified two peaks in the final product (at 4.51 min and 4.91 min), both corresponding to the same molecular weight of 1045.60 g/mol.

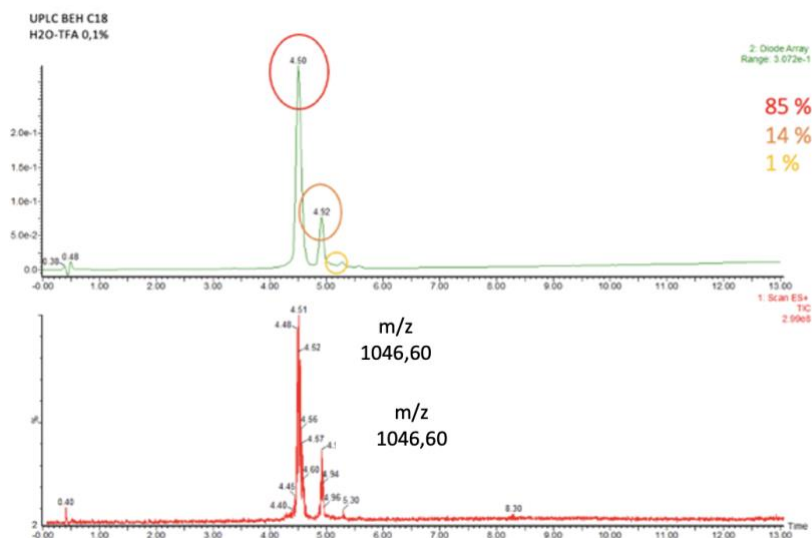


Figure 1. UPLC chromatograms of the purified cyclopeptide revealed by absorbance at 220 nm (top) and by total ion count (bottom). The mass-to-charge ratio (m/z) of the mono- and di-protonated ions are reported for each UPLC peaks.

The UPLC analysis showed no change in retention time after heating, with the main peaks representing 85% and 14% of the total area. This consistent peak distribution indicates that the two species called here A and B do not interconvert under the applied thermal conditions.

Successively, a two-dimensional NMR analysis was performed on the more abundant species A. The chemical shift assignment of cFRGDIAFp(NMe)K in DMSO- d_6 at 300 K (BBI, Broadband Inverse.) confirmed the presence of all expected peptide signals and showed that all observed NMR signals were consistent with those reported in the literature (Supporting Information of Reference 54) verifying the structural integrity and confirming the chemical identity of the compound indicated as "A".

The peptide demonstrated a high affinity for integrin $\alpha_v\beta_6$, with an IC_{50} value of 10 nM, stronger than its affinity for integrin $\alpha_v\beta_3$ (IC_{50} of 14.6 μ M, data not shown). This result confirms the selectivity of the peptide for the $\alpha_v\beta_6$ integrin target.

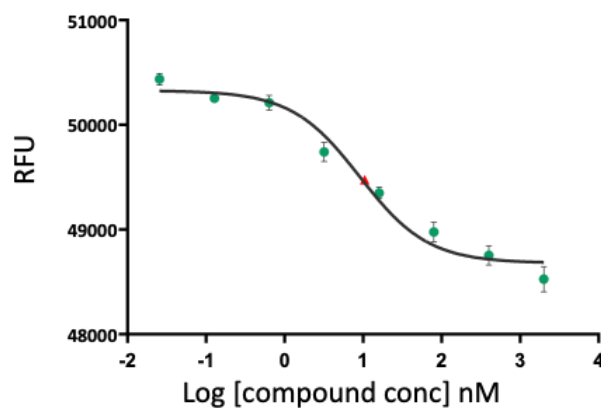


Figure 2. The affinity of the peptide towards $\alpha_v\beta_6$ (IC_{50} 10 nM) was found to be largely higher than that towards $\alpha_v\beta_3$ (IC_{50} 14.6 μ M) confirming the selectivity to the target.

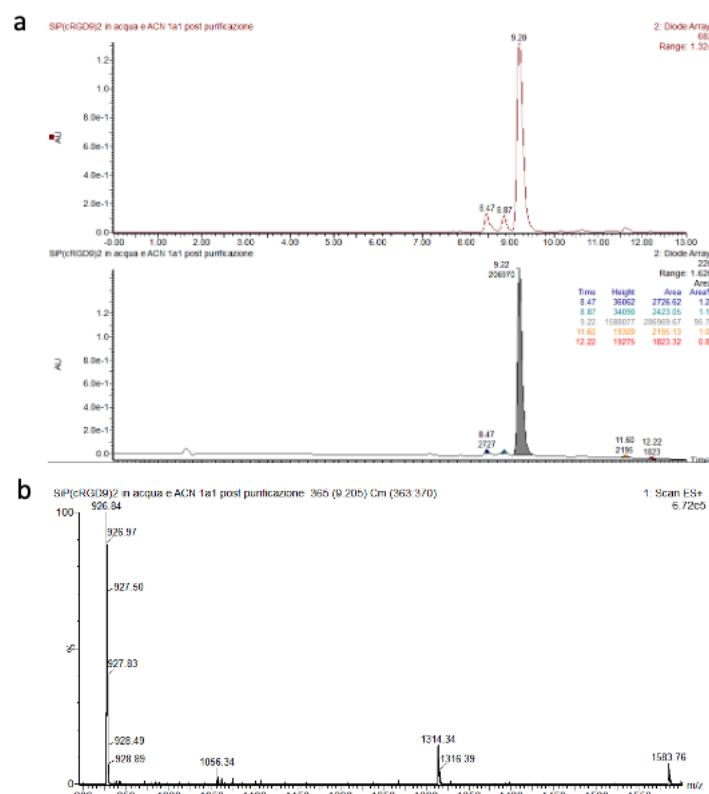


Figure 3. a) Chromatogram of SiPc-2c[FRGDLAfp(NMe)K] compound recorded at 682 nm and 220 nm (retention time of 9.22 min and purity of 95.7%); b) Mass spectrum (ESI+) of the peak at 9.21 min. *MS (ESI): m/z calculated for $C_{168}H_{212}N_{38}O_{28}Si$ $[M + H]^+ = 3168.87$, $[M + 2H]^{2+} = 1584.43$, found 1583.76, $[M + 3H]^{3+} = 1056.29$, found 1056.34*

Compound A (Fig. 3A) was conjugated to SiPc, and after purification, a pure product (blue powder, 3 mg, yield 12.6%) was obtained. The purity of the compound was 95.7 %.

Mass spectrometry analysis confirmed the identity of the compound SiPc-2c[FRGDLAfp(NMe)K] (Fig. 3B).

The absorbance spectrum of the photosensitizer was measured in two different solutions: 5% DMSO/PBS and 5% DMSO/serum. In the serum solution, the spectrum remained stable over a 24-hour period, consistently maintaining its characteristic absorbance profile. This stability in serum suggests that the photosensitizer retains its optical properties under conditions that mimic physiological environments.

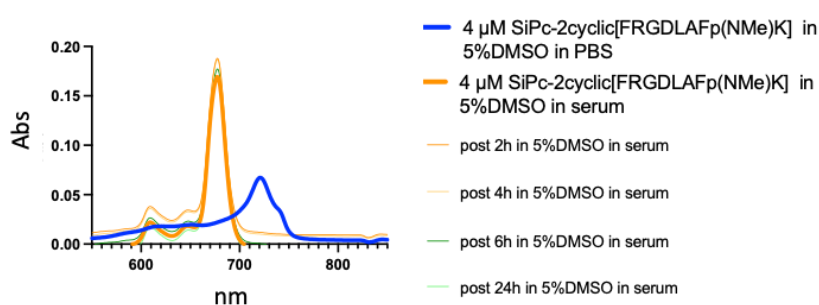


Figure 4. Absorbance spectrum of the photosensitizer in 5% DMSO/PBS and 5% DMSO/serum. The spectrum in the serum solution remains stable over 24 hours, consistently maintaining its characteristic absorbance profile.

The flow cytometry analysis confirmed high expression of the transmembrane protein $\alpha_v\beta_6$ in the BxPc3 and Capan1 cell lines (Figure 5a and b). In contrast, integrin $\alpha_v\beta_6$ was notably absent in the MIApaca2 and PANC1 cell lines, which served as negative controls. This differential expression underscores the relevance of $\alpha_v\beta_6$ as a biomarker in pancreatic carcinoma, identifying BxPc3 and Capan1 as suitable models for targeted therapeutic research and reinforcing the specificity of $\alpha_v\beta_6$ -associated pathways in tumor development.

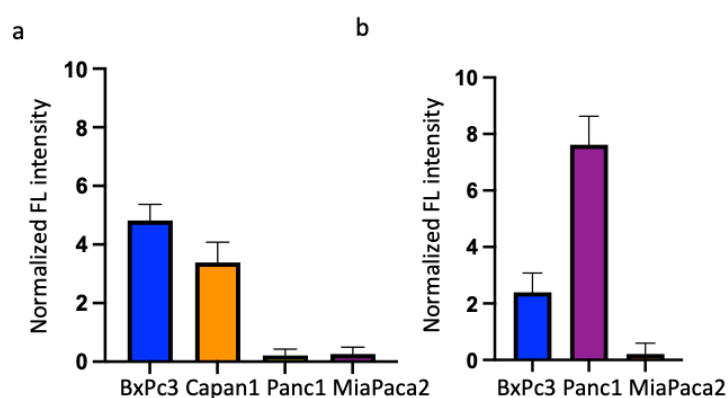


Figure 5. Normalized fluorescence measurements in pancreatic adenocarcinoma cells confirm β_6 expression in BxPc3 and Capan1 (a) and β_3 expression in Panc1, with moderate levels observed in BxPc3.

Protein quantification was performed using the BCA assay to ensure equal protein loading for subsequent Western blot analyses. Based on the calibration curve generated with BSA, the protein

concentrations ($\mu\text{g}/\mu\text{L}$) for each cell line were determined as follows: U87MG (4.5), HT29 (3.1), BxPc3 (2.6), PANC1 (3.3), and MIAPaca2 (1.4). These values allowed for normalization across samples, ensuring reliable comparative analysis. These values were used to normalize protein amounts in subsequent WB experiments, ensuring consistent and comparable results across different samples.

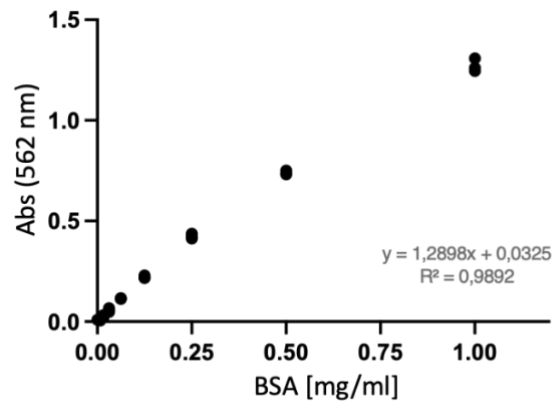


Figure 6. Calibration curve obtained using BSA as a standard for protein quantification by BCA assay. This curve was used to determine the protein concentrations of different cell lines, ensuring accurate and standardized protein loading for Western blot analyses.

The Western Blot experiment confirmed high expression of the integrin $\alpha_v\beta_6$ in the BxPC3 cell line and low levels of integrin $\alpha_v\beta_3$, which were instead present in the Panc1 cell line (Figure 7 a and b). This allowed the identification of BxPC3 as a suitable cell line for subsequent photodynamic therapy experiments.

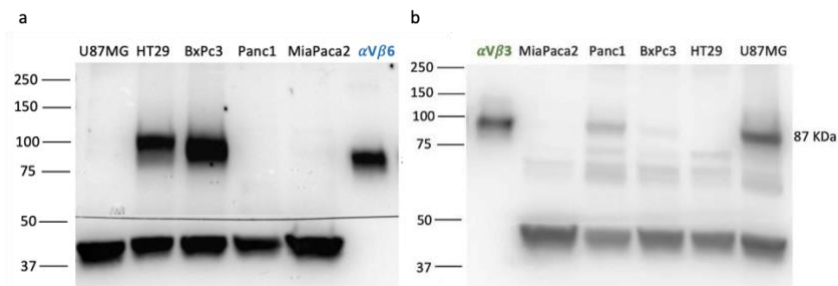


Figure 7. Western blot results showing β_6 and β_3 integrin expression levels normalized to protein concentration ($\text{pg } \beta/\mu\text{g}$ protein) across pancreatic adenocarcinoma cell lines, respectively (a) and (b). BxPc3 expresses both β_3 ($17 \text{ pg}/\mu\text{g}$) and β_6 ($1411 \text{ pg}/\mu\text{g}$), Panc1 expresses β_3 ($113 \text{ pg}/\mu\text{g}$), while MiaPaca2 shows no detectable levels of these integrins.

To gain further insights from the cellular model, spheroids were prepared using BxPC3 and MIAPaca2 cell lines, representing high and low expression levels of integrin $\alpha_v\beta_6$, respectively (Figure 8a). The spheroid diameter was measured over time, and they were visualized after staining with a photosensitizer, DAPI for nuclei, and WGA for the cell membrane (Figure 8 b and c shows BxPc3 spheroids, MIAPaca spheroids are not shown). It's important to note that, due to limited light penetration in confocal microscopy, the image captures only a semi-superficial layer of the spheroid.

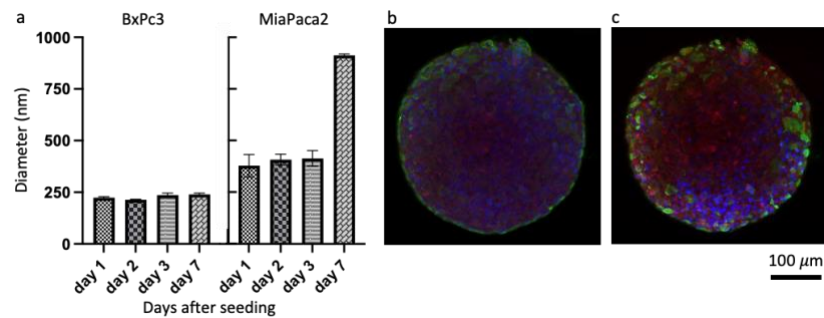


Figure 8. (a) BxPc3 spheroids form stable, viable 3D structures, while MIAPaca2 cells disaggregate by day 7. (b) Untreated spheroid; (c) drug-treated spheroid with drug in red, nuclei in blue, and membranes in green.

The photodynamic therapy experiment conducted on spheroids composed of BxPC3 cells revealed disintegration and partial cell death in the spheroids subjected to the full treatment, as observed under the microscope (Figure 9 a-d). This effect was not detected in the control conditions. A cytotoxicity rate of 30% was measured as a result of the treatment (Figure 9e).

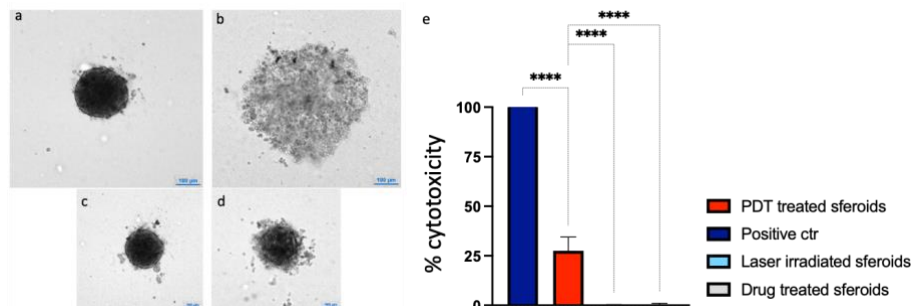


Figure 9. (a-d) Bright-field images of BxPc3 spheroids under various conditions: (a) untreated, (b) photodynamic therapy (drug and laser combined), (c) drug only, and (d) laser only. (e) Cytotoxicity percentage after photodynamic treatment, including control groups and a positive control with induced cell lysis for maximum response. Photodynamic treatment results in 30% cytotoxicity, with a statistically significant difference (****, $p < 0.0001$) compared to all other conditions.

Based on the data obtained from the three-dimensional model, the study progressed to establish an *in vivo* xenograft model using the BxPC3 cell line in NOD/SCID mice. This experiment aimed to investigate tumor model development and determine the optimal cell concentration for inoculation to achieve a reliable model for subsequent photodynamic therapy studies.

Considering Figure 10 a-c, the group of 8 animals inoculated with 1.5×10^6 cells per flank exhibited more consistent and homogeneous tumor growth compared to the group inoculated with 3×10^6 cells. As shown in Figure 10d, the animals in each group were treated according to the protocol when tumor volumes ranged between 60 and 90 mm³, ensuring that differences in tumor growth were not influenced by initial tumor volume at the time of treatment.

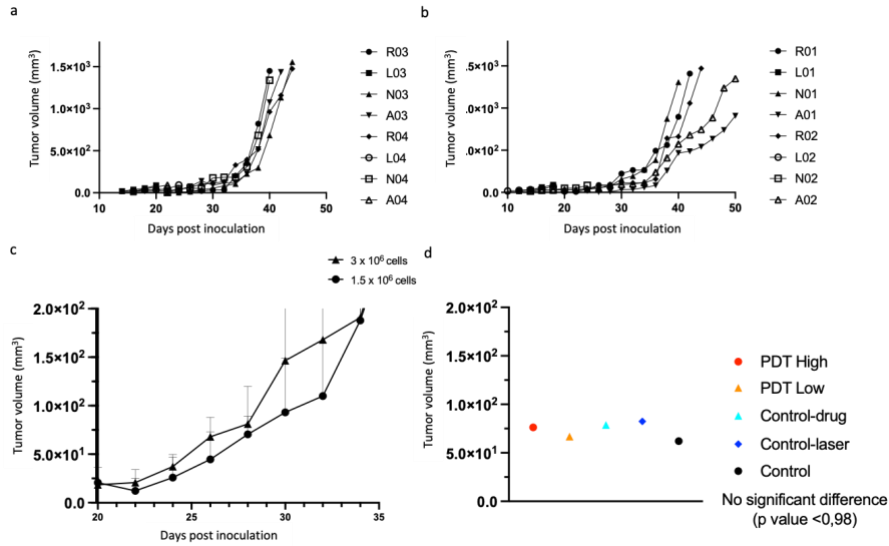


Figure 10. Tumor growth curves for BxPc3 cell inoculation in NOD/SCID mice with individual data points for each animal per group: (a) 1.5×10^6 cells per flank and (b) 3×10^6 cells per flank. (c) In all samples, tumor growth was observed to be linear up to day 32 post-inoculation, remaining linear rather than exponential within the 60–90 mm³ volume range. Tumors derived from the inoculation of 1.5×10^6 cells (●) and 3×10^6 cells (▲) followed a similar growth trend, with no significant deviation from linearity within the observed timeframe. (d) All groups received treatment within this specified linear growth phase.

In Figure 11a, a slowdown in tumor volume growth is observed in the data shown in red, corresponding to animals treated with high-dose PDT (116 J/cm²), compared to all other groups, including control animals and those treated with low-dose PDT. When analyzing individual animal data with the exponential growth model as shown in Figure 11 ($y = y_0 e^{Kx}$), the high-dose PDT treatment group showed a marked reduction in tumor growth rate compared to other groups. This finding (Figure 11 b and c) is reflected in the parameters K (the exponential growth rate of tumor volume) and τ (the characteristic growth time of the tumor).

The growth rate K , measured in days⁻¹ indicates how quickly the tumor volume expands. Higher K values mean faster growth, as more tumor volume is added each day.

The characteristic growth time τ , in days, represents the time it takes for the tumor volume to increase by a factor of e (about 2.718). A smaller τ , suggests faster growth, while a larger τ , indicates slower tumor progression.

In the high-dose PDT group, the K value is lower, and the τ value is higher, indicating a slower tumor growth rate compared to other groups. This pattern suggests that high-dose PDT effectively slows tumor progression by extending the time needed for the tumor to grow by a factor of e . The reduction in growth rate highlights the significant therapeutic impact of high dose PDT in tumor expansion.

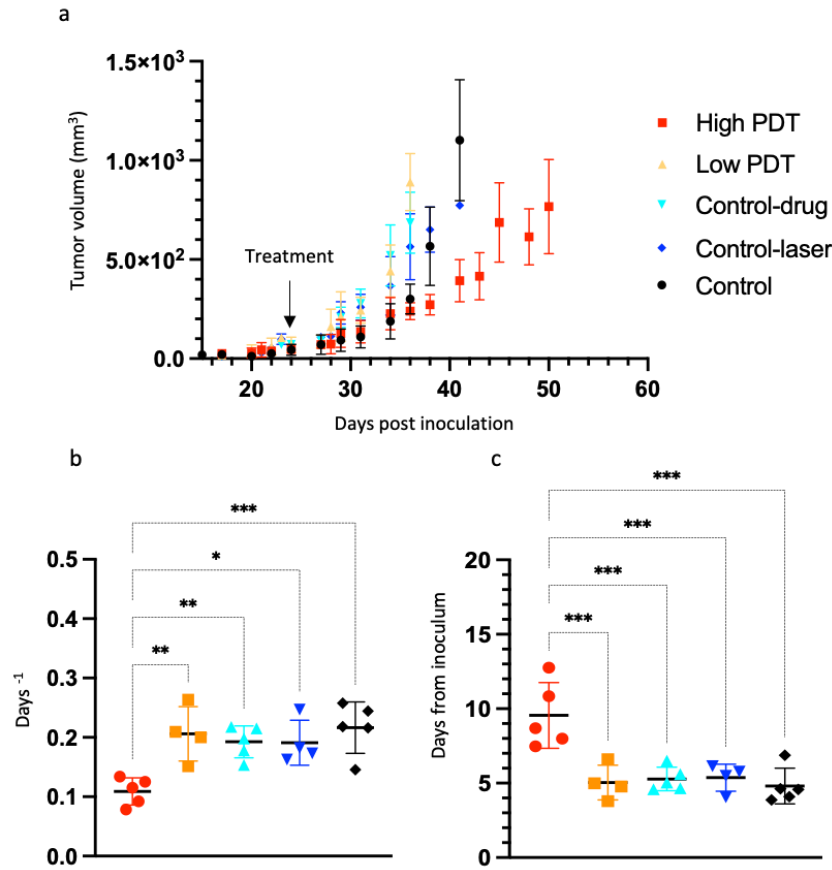


Figure 11. (a) Growth curves for each group, with the treatment day marked (when tumor size reached the target range, around days 24-26). (b) and (c) show the kappa and tau parameters, respectively, as indicators of tumor growth rate across the different groups. Significant differences are observed compared to the high-dose PDT-treated group. Statistical significance is indicated as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Ex vivo analyses of tumor tissues stained with hematoxylin and eosin revealed similar results across all groups, showing a tumor morphology characteristic of pancreatic adenocarcinoma with a predominant stromal component, as expected (Figure 12 a and b). Additionally, initial formation of ductal structures was observed, despite the xenograft nature of the model.

Figure 12c shows the vascularization observed in the samples, which appears to be widely present. This was demonstrated through endothelial structure staining, clearly visible in red.

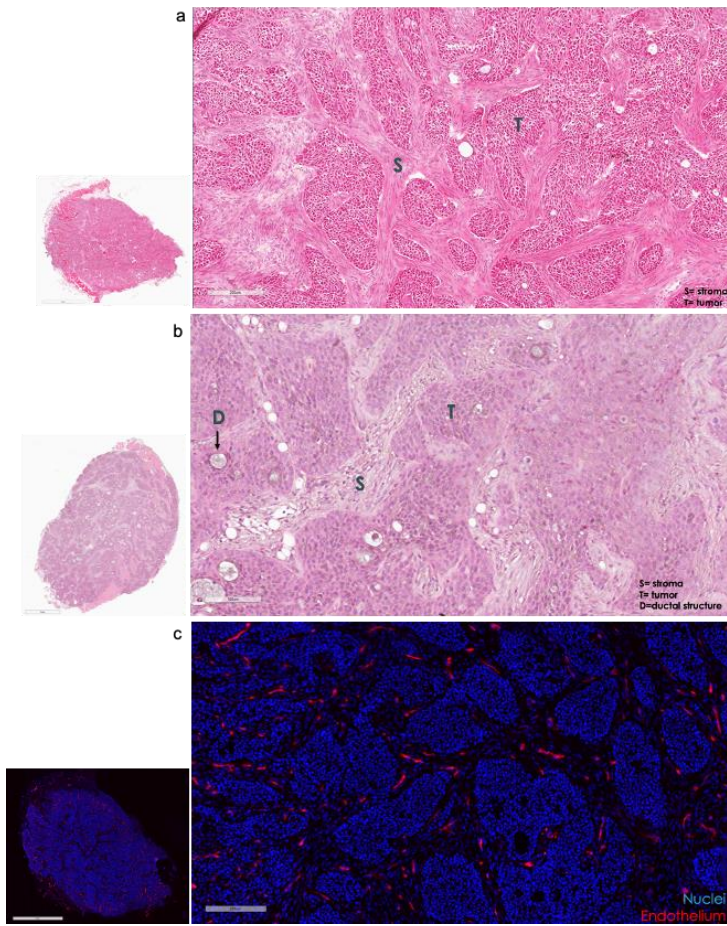


Figure 12. Histological view of BxPC3 xenografts: (a, b) H&E-stained sections showing tumor structure (scale bar: 200 μ m). (c) Blood vessels highlighted by immunofluorescence staining, using an anti-CD105 antibody to mark endothelial cells (scale bar: 200 μ m). Analysis shows that blood vessels occupy $1.9 \pm 0.45\%$ of the tumor area.

Western Blot analysis was performed on tumor tissues to detect the expression of integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$, aiming to better characterize the model and understand the involvement of these integrins in the activity of the photosensitizer. Tumor tissues from animals in the different groups were compared with a pellet of cells to assess the expression levels on both cells and tissue, respectively β_3 , β_5 , and β_6 (Figure 13 a-c). The presence of β_3 and β_5 integrins in the analyzed tumor tissues prevents a definitive determination of whether the photosensitizer, specifically the peptide designed to target β_6 , binds exclusively to this integrin subtype. Furthermore, tumor digestion from animal models also incorporates stromal components, where integrins are highly expressed in the extracellular matrix.

This complicates the evaluation of binding specificity and raises further questions regarding the selectivity of the treatment for tumors with high integrin expression.

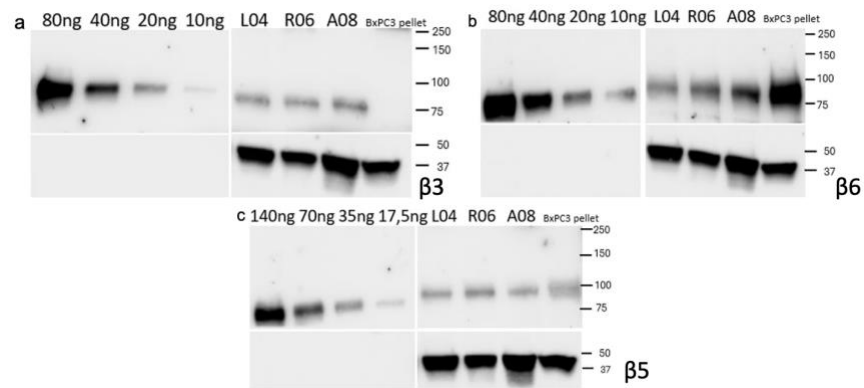


Figure 13. Analysis confirmed integrins β_3 (a), β_6 (b), and β_5 (c) were present in all tumor samples examined. Quantification showed average concentrations of β_3 at 56 pg per μg of protein (± 25), β_6 at 135 pg per μg of protein (± 55), and β_5 at 174 pg per μg of protein (± 30).

4.4

Discussion and conclusions

The design of the molecule used in this study was motivated by the need to develop a selective and specific probe, while also enhancing the solubility of a promising photosensitizer. Additionally, the compound was designed to exhibit high selectivity for the integrin target $\alpha_v\beta_6$, which is known to be a promising target for pancreatic adenocarcinoma treatment. This design was achieved by synthesizing a cyclic nonapeptide containing the RGD sequence, which was then conjugated to a silicon phthalocyanine, improving its water solubility and enhancing its stability, optical properties, and serum compatibility.¹¹⁰ The conjugation of both axial position of silicon phthalocyanine with cyclic peptides, above and below the plane, helped overcome aggregation issues typical of hydrophobic photosensitizers, making it a more viable candidate for therapeutic applications.¹¹¹ A critical consideration in the design of this molecule was the multimerization approach, which involves the assembly of multiple functional units, and while beneficial for improving affinity and stability, may also increase the likelihood of interactions with other integrin subtypes. This could challenge the target specificity of the molecule, and it can potentially account for some of the observed effects in the study related to the multiple interactions with integrins.¹¹²

Regarding the expression of the target integrins, the pancreatic cancer cell lines, specifically BxPc3 cell line, confirmed the expected levels of integrin β_3 and β_6 . The drug was internalized by the BxPc3 spheroids, which highly express $\alpha_v\beta_6$, although it could not be conclusively determined that the photosensitizer exclusively entered through this specific receptor.¹¹³

Despite this uncertainty, the use of BxPC3 pancreatic cancer spheroids served to more closely mimic *in vivo* conditions, such as extracellular matrix density, spatial organization, and oxygen gradients, characteristic of solid tumors like pancreatic adenocarcinoma.

While *in vitro* studies on spheroids showed significant photodynamic activity with approximately 30% cytotoxicity, this effect translated to moderate results *in vivo*. The spheroids, with diameters over 300 μm before treatment, remained viable and did not form necrotic cores, suggesting adequate oxygenation to initiate PDT.

Qualitative assessments confirmed the internalization of the photosensitizer and induced cytotoxicity, although imaging limitations prevented precise localization confirmation. This highlights the challenges in drug delivery, particularly in fibrotic tumors like pancreatic adenocarcinoma, which may limit the penetration of therapeutic agents.¹¹⁴ *In vivo*, the chosen model demonstrated a moderate response to phototherapy.

However, the involvement of integrin subtypes β_5 and β_3 could not be excluded, as stromal components contributed to the increased expression of integrin $\alpha_v\beta_5$. Therefore, further competition experiments *in vitro* and *in vivo* are necessary to better understand the full range of integrin interactions contributing to the observed effects.

The *in vivo* photodynamic therapy study showed that high-dose PDT delayed tumor progression, with the drug reaching the tumor site, being activated, and exerting its effect in the well-oxygenated tumor microenvironment. Tumor masses became softer 3-5 days post-treatment, confirming the photodynamic reaction, although histological analysis did not reveal necrosis, indicating that the PDT effect on tumor integrity was not strong enough to provoke extensive tissue breakdown and inflammatory responses typically seen with PDT.¹¹⁵

The high binding affinity of the PS for $\alpha_v\beta_6$, combined with the RGD sequence in the peptide, which allows binding to other integrins, likely contributed to its greater uptake at the tumor site despite the dense, stromal nature of the tumor, which poses a challenge to drug penetration.¹¹⁶

In addition to these considerations, the future need emerges to create different setups for photodynamic therapy dosing, starting from the high dose used in this study and increasing it, or by evaluating repeated treatments over time. The advantage of repeated treatments would be to gradually target the tumor mass, making it progressively less intact and thus more responsive to the therapy.¹¹⁷ In the use of the laser, ensuring homogeneous and stable illumination of the field is a crucial step in the optimization of the laser dose, as it minimizes treatment variability.

To enhance the efficacy of high-dose PDT, it is critical to optimize the interplay between dose, tumor oxygenation, stromal components, and laser precision to achieve more targeted and effective therapeutic outcomes. Furthermore, the choice of preclinical models significantly impacts the translational relevance of therapeutic evaluations. Conventional cell lines, maintained in two-dimensional cultures, often exhibit artificially high target expression, which can lead to an overestimation of treatment efficacy. In contrast, patient-derived xenograft (PDX) models better preserve the genetic heterogeneity, tumor-stroma interactions, and microenvironmental complexity observed in clinical tumors. These differences underscore the limitations of cell line-based studies in accurately predicting therapeutic responses in patients. The use of more physiologically relevant models, such as PDX or organoid systems, is therefore essential to improve the reliability of preclinical findings and enhance the success of targeted therapies in clinical settings.

This study demonstrated the significant potential of the photosensitizer on spheroids, suggesting promising avenues for further development within a more refined and optimized experimental framework.

5 General discussion and conclusions

The results of these two studies on photodynamic therapy (PDT) with integrin-targeted photosensitizers underscore both the promise and the challenges of this approach in preclinical cancer models. The photosensitizers demonstrated effective internalization into tumor cells, driven by high affinity for integrins, and the design significantly improved solubility, stability, and cellular binding.

One notable observation from these studies is the impact of multivalency on integrin interactions. While the photosensitizers were designed with a primary focus on targeting $\alpha_v\beta_3$ or $\alpha_v\beta_6$ integrin, the RGD peptide-phthalocyanine conjugates likely enhanced binding across a broader range of integrins. This broader integrin engagement may have improved the overall cellular uptake and distribution of the photosensitizers within the tumor microenvironment. Although the multivalent approach may have reduced target specificity, the additional binding interactions likely contributed to the observed therapeutic effects, particularly in complex tumor settings characterized by heterogeneity in integrin expression.

Despite these advancements, the *in vivo* outcomes highlighted the need for further optimization. The dense stromal composition of certain tumors like pancreatic adenocarcinoma, coupled with variations in oxygenation and integrin expression, likely influenced the efficacy of PDT. These findings suggest that while multivalency enhances affinity and internalization, it does not guarantee optimal therapeutic outcomes without addressing other factors such as tumor architecture and oxygen availability.

In conclusion, these studies reinforce the therapeutic potential of integrin-targeted PDT and highlight the importance of multivalency as a strategy to enhance photosensitizer performance. The ability to engage multiple integrin subtypes may have contributed to the observed efficacy, particularly in challenging tumor models. Future research should focus on refining the balance between target specificity and binding affinity, alongside addressing tumor-specific characteristics such as stromal density and oxygenation profiles. Such advancements will be critical for optimizing PDT as a precise and effective treatment modality in oncology.

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