Chapter

KRas4BG12C/D/PDE6δ Heterodimeric Molecular Complex: A Target Molecular Multicomplex for the Identification and Evaluation of Nontoxic Pharmacological Compounds for the Treatment of Pancreatic Cancer

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Abstract

The search for new targeted therapies to improve the quality of life of patients with pancreatic cancer has taken about 30 years. Compounds that can inhibit the K-Ras4B oncoprotein signaling pathway have been sought. Taking into account that the interaction of KRas4B with PDE6δ is essential for its transport and subsequent activation in the plasma membrane, our working group identified and evaluated in vitro and in vivo small organic molecules that could act as molecular staples to stabilize the KRas4B/PDE6δ heterodimeric complex. From this group of molecules, 38 compounds with high interaction energies on the structure of the crystallized molecular complex were selected, indicating that they efficiently stabilized the molecular complex. In vitro evaluation of compounds called D14, C22, and C19 showed significant specific effects on the cell viability of pancreatic cancer cells (and not on normal cells), thus inducing death by apoptosis and significantly inhibiting the activation of the pathways, signaling AKT and ERK. In addition to these experimental findings, we were also able to detect that compounds D14 and C22 showed significant tumor growth inhibitory activity in pancreatic cancer cell-induced subcutaneous xenograft models.

Keywords: KRas4B, PDE6, therapy

1. Introduction

About 95% of the pancreatic ductal adenocarcinoma (PDAC) originates in the exocrine pancreas, and 5% is generated in the endocrine pancreas. There are several precursors for the development of PDAC; among them, noninfiltrating lesions, called pancreatic intraepithelial neoplasia or PanIN [1], are remarkable. The follow-up of the PanIN toward an infiltrating lesion is given by the abnormal distribution inside the pancreas. These lesions can be located in the pancreatic parenchyma, which causes its infiltration. Currently, the development of a pancreatic adenocarcinoma is monitored by measuring the overexpression of EGFR, KRAS, MUC1, and MUC4 genes or the inactivation of INK4A, TP53, and BRCA2 genes, which are essential for proper cell functioning [1–4, 5–8].

The invasive ductal adenocarcinoma is the most common pancreatic neoplasm, as it occurs in 85% of the cases. Eighty percent of the patients with this type of neoplasm have an average survival of 3–6 months after the detection; that is why this adenocarcinoma has been proposed as one of the most deadly existing [1, 9–11]. The invasive micro ductal adenocarcinoma deforms the small pancreatic glands, infiltrates the stroma, and triggers a fibrous coating, where 98% of the cases present mutations in the KRas4B gene [2, 10]. One of the most important factors for the development, maintenance, and progression of this disease is the presence of mutations in the KRas4B oncoprotein, which is mutated in 99% of PDAC cases [12]. Kras4B is a small GTPase, which belongs to the RAS protein subfamily, and it has essential functions in the control and regulation of normal cell proliferation. Human tumors almost always express mutated KRas4B proteins, from 90 to 99% of cases; specific mutations of this protein occur in codons 12, 13, or 61, which leaves the KRas4B protein constitutively active [11]. The active state of KRas4B proteins contributes significantly to develop the malignant phenotype, such as the deregulation of tumor cell growth, the evasion of programmed cell death, invasion, and angiogenesis [13]. There are three genes that code for RAS proteins in the mammalian genome: HRas, NRas, and KRas; four isoforms are obtained by alternative splicing: H-Ras, N-Ras, K-Ras4A, and K-Ras4B [14].

RAS subfamily proteins are also members of a broad class of proteins known as CAAX proteins [15] like this because the C-terminal end sequence has the CAAX amino acids (C: cysteine, A: aliphatic amino acid, and X: any amino acid), and this sequence is modified post-translationally in order to confer Ras protein affinity for the plasma membrane (for its subsequent activation). This process is regulated by three enzymes that work sequentially: first, the farnesyltransferase enzyme participates in the prenylation of the CAAX sequence; second, a protein called Ras-converting enzyme (RCE1) cleaves the last two amino acids of the CAAX sequence; third, a methyltransferase (ICMT) allows adding a methyl group to the carboxyl of the cysteine terminal to finally generate the mature RTP GTPase [16]. Farnesylation in the 185 cysteine terminal allows Ras proteins to increase their affinity for cell membranes and for many farnesyl group binding proteins that are analogous to RhoGDI transporters, such as phosphodiesterase 6 delta subunit (PDE6δ), which has been described as an indispensable molecule in the traffic of some GTPases of the Ras family [14, 17]. After the findings about the presence of KRas4B and its importance in the formation, maintenance, and progression of the most deadly neoplasms such as the PDAC [18, 19], studies have been conducted to discover and develop pharmacological inhibitors against oncogenic KRas4B. The approaches include: (a) finding small molecules that interact with KRas4B directly in order to prevent its activation [18, 19]; (b) finding enzyme inhibitors responsible for the post-translational modifications in order to prevent the transport of KRas4B to the

plasma membrane; (c) finding compounds that inhibit the KRas4B downstream signaling pathway, as well as autophagy inhibitors and inhibitors of neoplastic cell metabolism [18, 19].

Despite the enormous prevalence of Kras4B mutations in pancreatic cancer, an efficient targeted treatment against aberrant signaling of this oncoprotein has not been found. It is known that pancreatic cancer cells with mutated Kras4B exhibit a phenomenon called "oncogene addiction", in which their survival becomes dependent on Kras4B signaling. Therefore, the inhibition of the Kras4B function promotes the inhibition of the viability of cancer cells; this eventually leads to cell death by apoptosis and the regression of the tumor [20, 21]. Therefore, it is necessary to find new strategies that allow us to inhibit the molecular mechanisms of activation and/or signaling of mutated Kras4B in pancreatic cancer. In this chapter, we describe new organic molecules that inhibit the dissociation of the heterodimeric molecular complex KRas4BG12C-D/PDE6δ; thus promoting that KRas4BG12C/D cannot bind to the plasma membrane, and consequently, it cannot be activated in pancreatic tumor cells.

2. Targeted drugs for the inhibition of KRas4B

The direct inhibition of KRas4B has been a difficult task. Sites susceptible to pharmacological interaction have been identified by means of bioinformatic programs; therefore, compounds such as SCH-53239 and its analogue SCH-54292, which presented low affinity with respect to KRas4B, have been identified. These compounds were designed to interact with the Switch II of KRas4B, competing with the GDP. These compounds have in their chemical structure a hydroxylamine, which is essential for their cytotoxic activity. These compounds present a high level of toxicity in murine models, so they are in the improvement phase [22]. In 2012, several research groups reported a compound called DCAI [22, 23], which interacts with KRas4B at the site located between the α2 helix and β4 loop; this compound was able to inhibit the interaction of SOS1 with KRas4B with an IC_{50} of 340 μM, having an EC_{50} of 15.8 μM; therefore, it is so far one of the compounds considered for the treatment of PDAC [22, 23]. Also, different research teams have been working on 11,000 analogues of the DCAI compound in silico, based on the nuclear magnetic resonance (NMR). One of the analogues called VU0460009, showed an IC_{50} of 240 μM; although the concentration of the mean inhibition decreased, this compound did not have a considerable effect in murine models, so it was not possible to consider it as a candidate for treatment of PDAC [22, 23]. In order to find an organic compound that was capable of inhibiting the activation of KRas4B, studies were conducted to direct a specific molecule to the location site of the KRas4BG12C mutation, which is the most frequent in lung cancer [22]. One of the compounds studied was the so-called SCH-54292 [12], which is capable of binding to the α2 and α3 helices of KRas4B. This compound showed activity only in the cell lines that present KRas4BG12C, and with this finding, the researchers have intended to identify and study the analogues of SCH-54292 with the greatest effect on cancer cell lines [12]. Another group of researchers created a GDP analogue called SML-8-73-1, which could covalently bind to the cysteine of KRas4BG12C without taking into account the affinity of GDP with its binding site in KRas4B. These compounds did not show the expected effect on lung cancer cell lines, so they are in the improvement phase [22]. In recent years, several research groups have been trying to selectively inhibit mutated KRas activation and signaling. One way to prevent the activation of KRas and, therefore, its effector pathways, is through allosteric inhibition. Consequently, several research groups have developed

experimental models based on the *in silico* search for compounds that selectively bind and inhibit KRas. This strategy was carried out with an initial virtual coupling test of a library of compounds based on the reconstructed pocket structure of the switch I of RAS crystal, which resulted in an *in silico* coupling based on the pocket structure. The pocket consists of a hydrophilic part, which is composed of negatively charged residues such as Glu47, Asp48, and Asp 67, and a hydrophobic part, which consists of Leu66, Met77, and Tyr 81; on its surface, it is partially bordered by charged residues such as Lys15 and Asp67. These structural characteristics were used to establish a pharmacophore for the screening of charged residues, such as Asp67 (which corresponds to Asp57 of Ras), located in the lower center of the hydrophilic pocket in order to ensure the specificity of binding and energy. After the coupling analysis, compounds that *in vitro* effectively decreased the activation of Ras as well as its effectors were detected [21]. The development of small molecules that irreversibly bind to the oncogenic mutant KRasG12C allows the interruption of switch-1, and this alters the preference of native nucleotides in order to favor GDP over GTP and, consequently, this prevents its binding with the Raf effector. On the other hand, and using a similar strategy of *in silico* analysis and development of analogues with a favorable balance of ADME attributes (absorption, distribution, metabolism, and excretion), in vivo stability and specificity, in 2018, Janes and collaborators reported the design and characterization of switch-IIP inhibitors of KRasG12C with enhanced potency and pharmacological properties. Using tests based on liquid chromatography-tandem mass spectrometry (LC/MS-MS), compounds that covalently bound to Cys12 of K-Ras were measured directly and quantitatively. Pharmacological inhibition of KRasG12D with compound ARS-1620 suppressed the growth of cancer cells. ARS-1620 exhibited excellent oral bioavailability in mice and sufficient blood stability and, importantly, induces tumor regression through a specific mechanism of action.

Another strategy, proposed by Zeng and collaborators in 2017, is the possibility of designing compounds that incorporate elements of both the switch-IIP and the guanosine pharmacophores, or through the development of bivalent compounds that could recruit ligases to promote the degradation of RAS mediated by ubiquitination. The compounds were prepared with fluorophenyl and piperazinyl substituents and an electrophilic acrylamide warhead attached to the piperazine in order to effectively bind to KRasG12C. Subsequently, the 1_AM analogue was developed with an amino amide substituent and showed a more complete binding with KRas. In addition, 1_AM was compared with its serial head [1], and its properties were examined in H358 cells. The 1_AM inhibitor decreased levels of KRas bound to GTP by ~80% compared to the performance of inhibitor 1, and likewise the decrease of the ERK effector phosphorylated.

An interesting strategy, which has been recently addressed, is the blocking of the interaction of RAS with its effector Raf. A cyclic peptide called cyclosarin 9A5 blocked the RAS-RAF interaction. The amino acids present in cyclosarin such as nal, Fpa, Thr, norleucine (nle), and Trp are critical for binding with KRas. Cyclosarin 9A5 showed improved cell permeability and an affinity for KRas with an IC_{50} = 0.12 μM. Cyclosarin reduced the proliferation and induced cell death by apoptosis in tumor cells with mutated KRas [24].

Similarly, in 2019, MacCarthy and collaborators used a variety of computational approaches in order to describe four binding sites in K-Ras for allosteric ligands. The new inhibitors bind to the pocket p1 with submicromolar affinity and function primarily by directly inhibiting the interaction of KRas with its effector proteins. This potential inhibitor forms multiple favorable interactions with residues in the pocket p1 of nonmutated KRas (WT) and with residues of the mutants of K-RasG12D, G12C, and Q61H in the active state bound to GTP. In addition, the authors report that the inhibitor of KRas binding to its effectors decreases the

levels of phosphorylation of ERK and cRAF in BHK cells that express the mutant of KRasG12D and G12V, which suggests the inhibition of KRas signaling through the MAPK pathway. However, the problem of allosteric KRas inhibitors that prevent interaction with their effectors is that they do not exhibit selectivity toward a particular RAS isoform or KRas WT vs. mutated KRas, which raises the toxicity problem in cells and therefore in healthy organs when they are used in vivo.

Another approach to inhibit the interaction of KRas with its effectors is the search for macromolecules that selectively bind to KRas at an allosteric lobe site that encompasses histidine 95 residue at the interface between the helix α3/loop 7/helix α4. Designed ankyrine repeat proteins (DARPins) K13 and K219 inhibit the interactions with the effectors and the nucleotide exchange of KRas. Similarly, K13 and K19 induce selective inhibition of the RAF/MEK/ERK signaling pathway in cells with the Kras4BG13D mutation but not in cells with other mutated isoforms such as HRasG12V and/or RAS WT. This suggests that K13 and K19 selectively inhibit the function of mutated KRas without affecting cancer cells with RAS WT; however, its toxicity in healthy cells has not been proven [25].

3. Targeted drugs for PDE6δ inhibition

Post-translational modifications are important for the recognition and transport of KRas4B to the plasma membrane; therefore, the study of molecules responsible for recognizing these post-translational modifications may be an important therapeutic target against PDAC [26]. One of the proteins responsible for recognizing the post-translational modification is phosphodiesterase 6δ (PDE6δ), which recognizes the farnesylation or geranyl-geranylation present in the cysteine of the CAAX motif of Kras4B protein [27]. A group of German researchers identified and evaluated the compound called Deltarasin, which interacts with PDE6δ, with a K_d of 38 nM, prevents the recognition of the post-translational modification present in KRas4B, arresting KRas4B in the cytosol, and consequently preventing its activation and tumor progression. This compound was named the first generation of PDE6δ inhibitors [26]. However, its evaluation in noncancerous pancreatic duct cell lines showed high cytotoxicity; this affected considerably the cell viability at low concentrations [28]. In 2016, the analogue of the compound Deltarasin (second generation of PDE6δ inhibitors) was reported, and it was called deltazinone. This analogue presented constant dissociation of K_d 38 nM to K_d 4 nM, thus showing to be a compound with better interaction energy than the analogue of first generation. Deltazinone showed cytotoxic effects on pancreatic cancer cell lines at a concentration of 24 μM, but it took about 8 h to have an anti-proliferative effect on pancreatic cancer cell lines. Conversely, Deltarasin, at a concentration of 5 μM showed the same effect in 1 hour. Considering these data, the first generation of PDE6δ inhibitors have a better performance than the ones from the second generation [29, 30]. In 2017, the third generation of specific PDE6δ inhibitors, which were called Deltasonamides, was reported. This new generation shows more interaction energy than the ones from the first generation and greater cytotoxic effects on pancreatic cancer cell lines at concentrations from 1 to 12 μM [31].

In early 2019, drugs based on triazoles arose. These compounds can be considered as the fourth generation of PDE6δ inhibitors since they used the structure of Deltarasin to be able to find the functional group with the highest interaction energy with PDE6δ. This fourth generation is still in *in vitro* studies in order to evaluate its cytotoxic effect [32]. At the beginning of 2020, the fifth generation of PDE6δ inhibitors, called Deltaflexin, arose; although they are analogues of Deltarasin, they do not have the same cytotoxic effects showed by the first generation of PDE6δ inhibitors [33].

4. Drugs capable of stabilizing the KRas4B/PDE6δ protein complex

The search for new targeted therapies aimed at trying to improve the quality of life of patients with pancreatic cancer has taken about 30 years; along this time, researchers have looked for compounds that can inhibit the signaling pathway of the KRas4B oncoprotein. One of the most important mechanisms for the activation of KRas4B is the transport from the cytosol toward the plasma membrane by the PDE6δ, which recognize the farnesyl group of KRas4B present at carboxyl terminal (**Figure 1a**). It was believed that KRas4B/PDE6δ was transported as a dimer, and it is now known that it forms a cluster of 6–12 proteins or 3–6 dimers (**Figure 1b**). Because of this, our work group looked for a plate of the heterodimer using the crystal of the heterodimeric complex of the cluster of 6 proteins (**Figure 1c**) in order to identify small organic molecules capable of stabilizing the interaction of the molecular complex KRas4B/PDE6δ with the purpose of avoiding the activation of KRas4B as well as its signaling pathway dependent of this oncoprotein. An exhaustive search was carried out in public chemical libraries of organic compounds

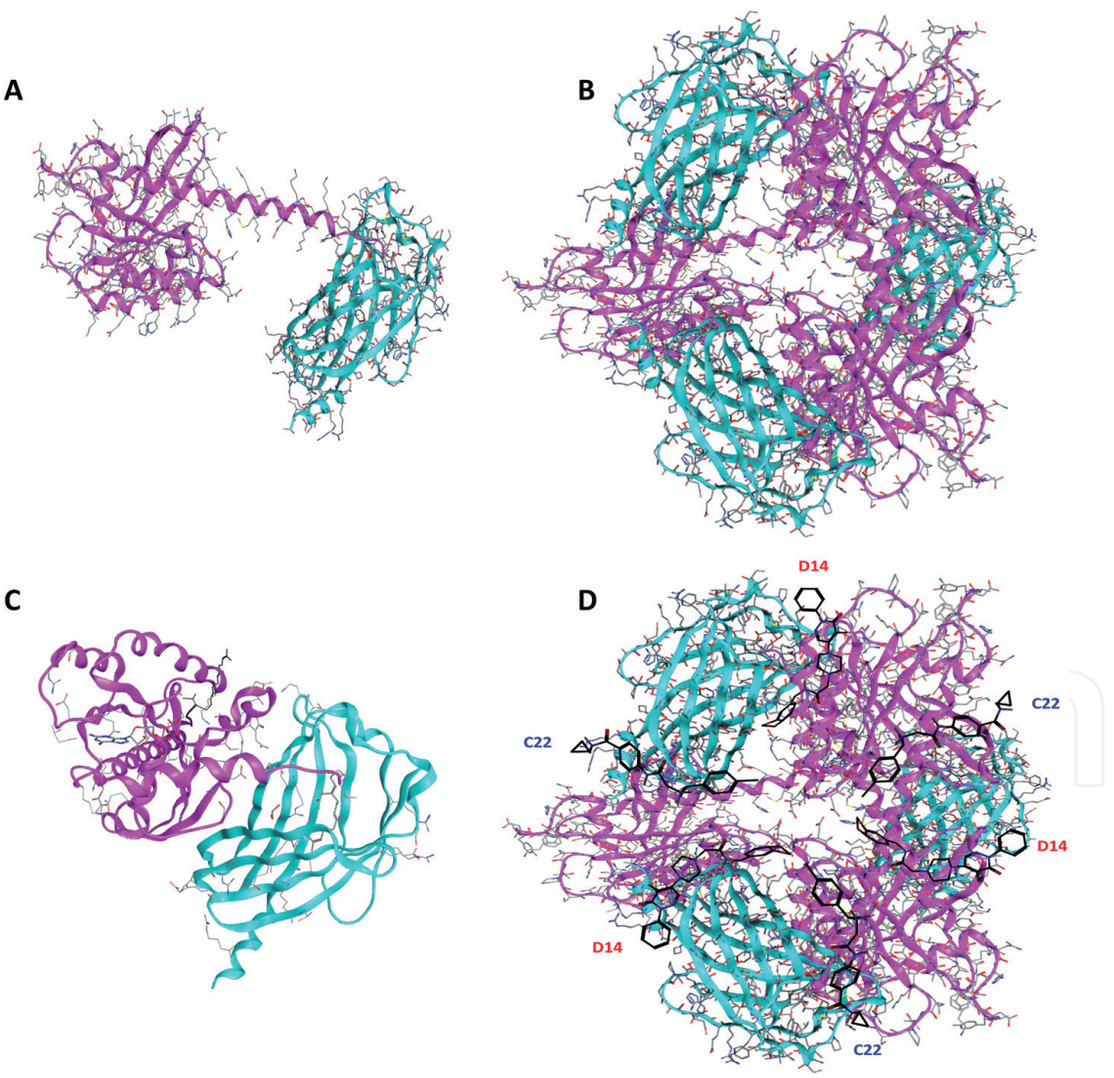

Figure 1.
Types of interactions between KRas4B/PDE6δ heterodimeric complex crystallized. (A) Interaction between K-Ras4B (pink) and PDE6δ (aqua) proteins. (B) Cluster formation among K-Ras4B/PDE6δ in multiheterodimeric molecular complexes crystallized. (C) Template of K-Ras4B/PDE6δ heterodimeric complex in a cluster used to docking and drug identification. (D) Molecular docking of D14 (N-[(2H-1,3-benzodioxol-5-yl)methyl]-2-[4-(5-chloro-6-oxo-1-phenyl-1,6-dihydropyridazin-4-yl)piperazin-1-yl]acetamide) and C22 (3-(2-{[1-(4 chlorophenyl)ethyl]amino}acetamido)-N-cyclopropylbenzamide) compounds and using a cluster of the heterodimeric K-Ras4B/PDE6δ molecular complexes.

with pharmacological potential, which could stabilize the KRas4B/PDE6δ complex. The identification of the compounds that had an *in silico* interaction with the complex, in addition these compounds was selected considering that they complied with the Lipinsky rule, which states that (1) the compounds should not have more than five hydrogen bridge donors; (2) they must not contain more than 10 hydrogen bridge acceptors; (3) they must have a molecular weight of less than 500 g/mol; (4) the compounds must have an octanol/water partition coefficient of less than five (log P < 5). Compounds identified as D14 and C22 showed different *in silico* interaction energies on the KRas4B/PDE6δ and K-Ras4BG12C/PDE6δ heterocomplex crystals; these interaction energies ranged from −143 to −162 ΔG [28].

An *in silico* analysis on the prediction of absorption using the ADME software made it possible to identify that compounds D14 and C22 have good absorption at the intestinal level and have low uptake by the permeability glycoprotein proteins that belong to the ABC transporter family. Their values are very low compared with the absorption of Gemcitabine and Deltarasin, which indicates that compounds D14 and C22 have a low chemoresistance when they are used as a treatment for pancreatic cancer cells (**Figure 2a**). Additionally, it was possible to observe a metabolism of compounds D14 and C22 by cytochromes P450 (CYP450), which indicated rapid liver metabolism and low toxicity since these compounds are coated by these enzymes (**Figure 2b**). Furthermore, it was observed that these compounds may have low toxicity compared to that obtained with the treatment of choice for pancreatic cancer such as Gemcitabine and with the PDE6δ Deltarasin inhibitor (**Figure 2c**).

Once identified that compounds D14 and C22 do not have toxic effects, we assessed the presence of KRas4B and PDE6d in pancreatic cancer cell lines by immunofluorescence using different cell lines with KRas4B (BxPC3), KRas4BG12D (PANC-1), and KRas4BG12C (MIA PaCa-2); we observed a greater presence of these proteins in the KRas PANC-1 and MIA PaCa-2-dependent cell lines (**Figure 3**). Having identified the cell lines with the highest presence of KRas4B and PDE6d, we treated them with a concentration of 200 μM of compounds D14 and C22 comparing their effect with hTERT-HPNE, which is a noncancerous cell line, and with 5 μM of Deltarasin (**Figure 4**). The results showed that compound D14 had a greater cytotoxic effect on the PANC-1 and MIA PaCa-2 cell lines, while compound C22 had a greater cytotoxic effect on the MIA PaCa-2 cell line. The comparison of these results with the effect obtained from Deltarasin, where the normal hTERT-HPNE cell line of pancreas was affected, suggested that our compounds do not have cytotoxicity in noncancerous cell lines.

Taking into account the results described above, we carried out Ras activation assays using the MIA PaCa-2 cell line. We obtained a dose response curve during 60 min, measuring Ras-GTP uptake by means of G-Lisa assays. Compounds D14 and C22 significantly decreased Ras activation over time; we obtained a 50% decrease in Ras activation at 60 min after treatment with the compounds (**Figure 5a**). As mentioned earlier during this chapter, the constitutive activation of KRas4B is essential for the development, progression, and maintenance of pancreatic cancer, and therefore, we performed subcutaneous xenograft tests by grafting 5 million cells of the MIA PaCa-2 cell line and administered via intraperitoneal 10 and 20 mg/kg of weight of compounds D14 and C22 for 15 days. The result was a 50% decrease in tumor growth in tumors treated with 20 mg/kg of weight of the two compounds, compared to the vehicle used as a control (**Figure 5b** and **c**).

Pancreatic ductal adenocarcinoma (PDAC) remains one of the leading causes of death by cancer, in addition to being one of the most aggressive types of cancer. The pancreatic cancer stem cell population (PCSCs) has been linked to this aggressiveness and poor prognosis. The cancer stem cell model proposes that tumor initiation,

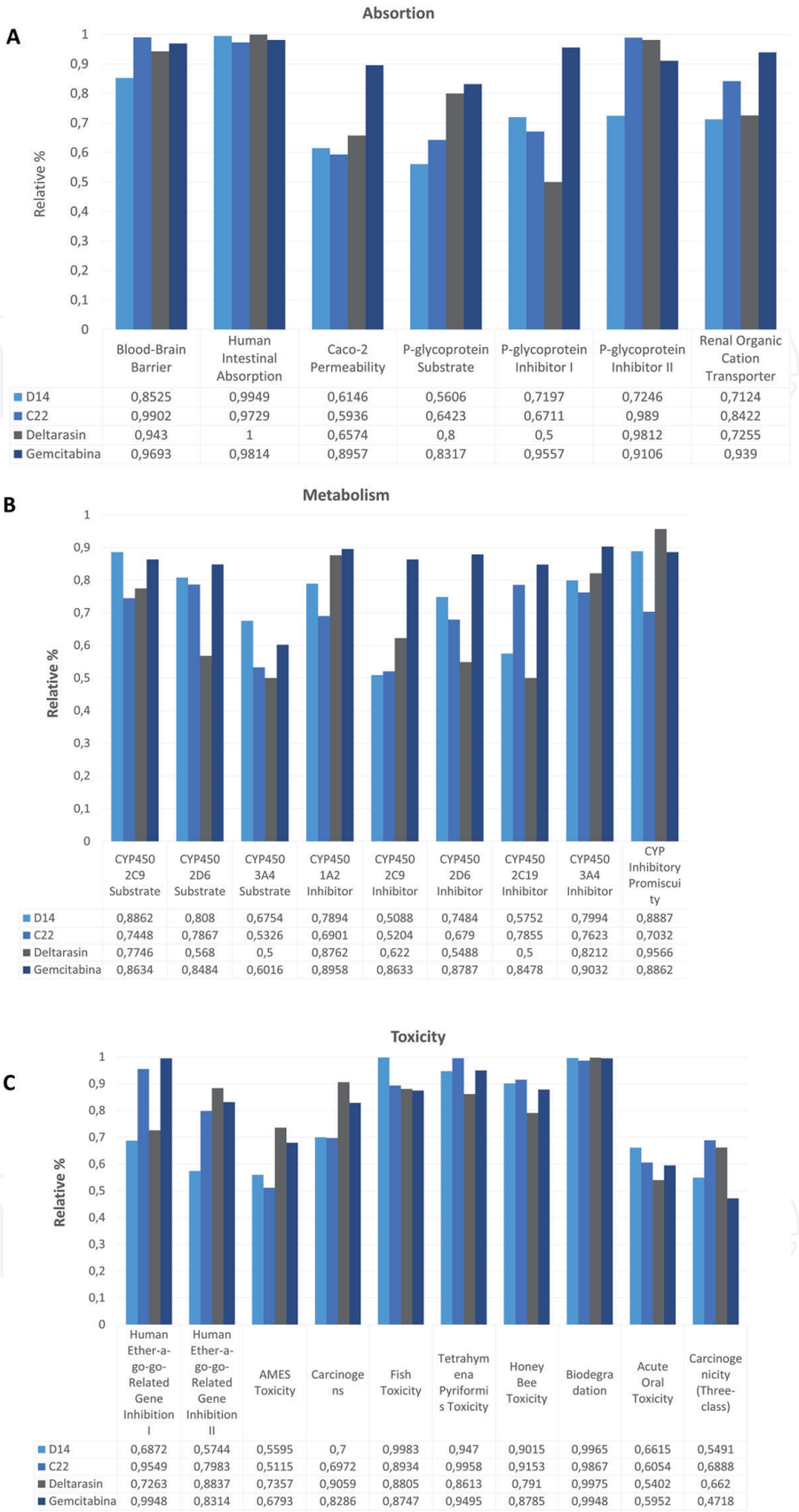

Figure 2.
Prediction of ADME processes of compounds D14 and C22. (A) Absorption of compounds D14 and C22 in epithelial barriers and their uptake by permeability glycoprotein proteins. (B) Metabolization of compounds D14 and C22 by means of cytochrome P450. (C) Toxicity of compounds D14 and C22.

maintenance, and growth are directed by the population of stem cancer cells (CSC) [34, 35], which have been identified in several types of cancer, *e.g.* breast, brain, head and neck, colon, and pancreas [36, 37]. CSCs are defined as those tumor cells

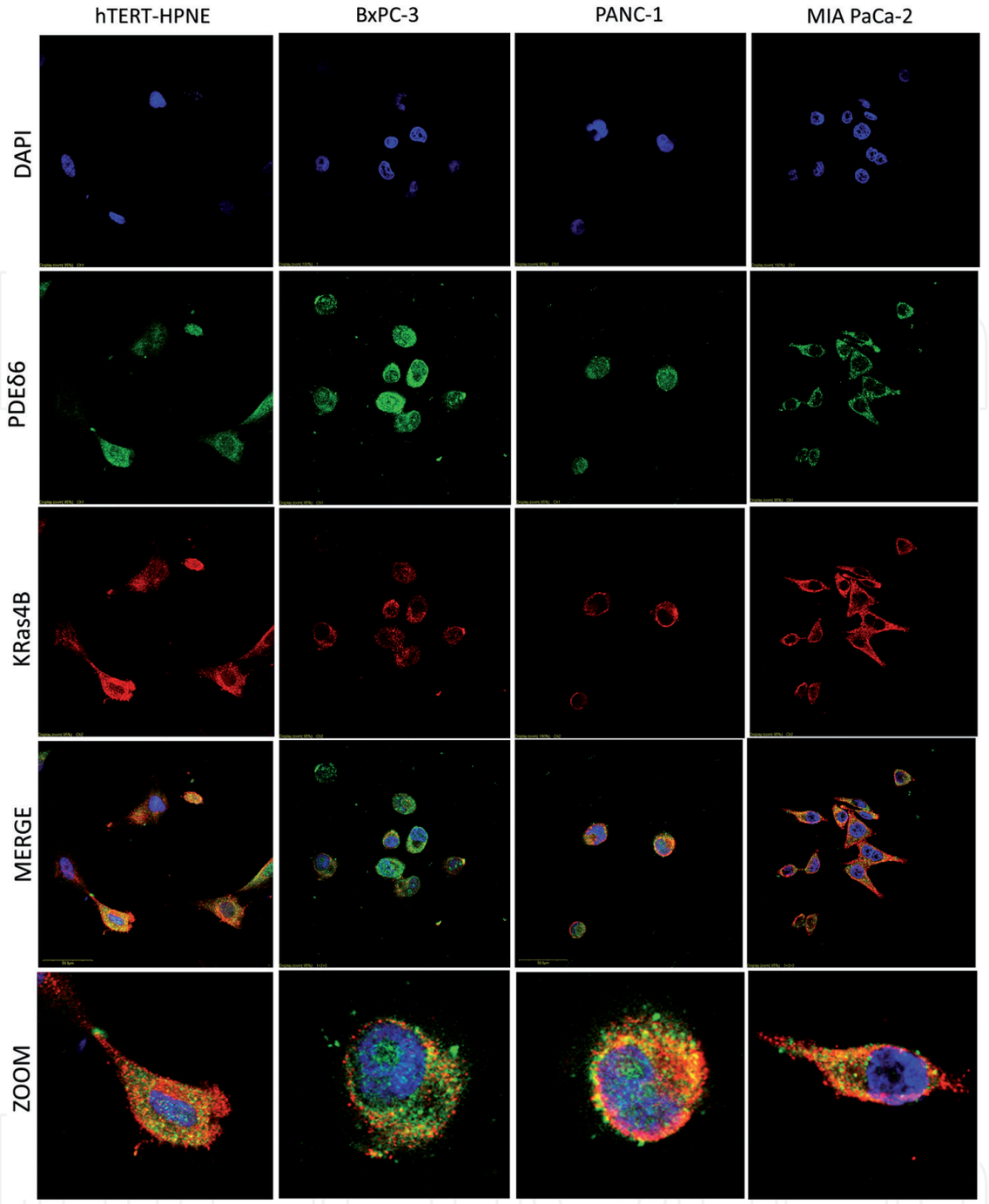

Figure 3.
KRas4B and PDE6δ are present in pancreatic cancer cell lines with different mutations in K-Ras. Images taken by confocal microscopy.

capable of self-renewal and production of heterogeneous lineages that comprise tumor volume [38]. In addition, several studies have reported evidence of the contribution of CSCs in resistance to conventional therapy, which causes metastasis and tumor recurrence [36, 39]. Different immunphenotypes have been reported for the identification of pancreatic cancer stem cells (PCSCs) [36, 37]. Due to the high fatality of PDAC, the importance of CSCs, and the participation of oncogenic KRas4B, we decided to evaluate the effect on the tumorigenicity of compounds D14 and C22 in CSC of PDAC; in this sense, cancer steam cells from BxPC3, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines, as well as in the hTERT-HPNE non-cancerous cell line, were growing in nonadherent conditions, forming spheroids or pancreatospheres and selected with the immunophenotypes positive to CD44, CD24, and ESA markers, which indicates an enrichment of PCSC (**Figure 6**). These

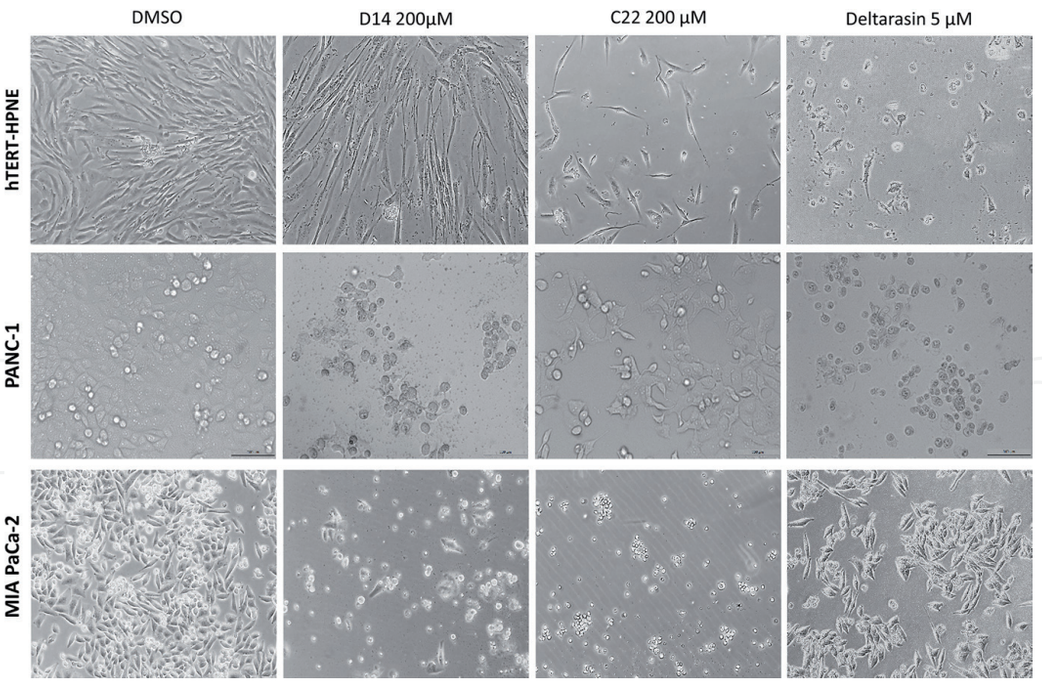

Figure 4.
Morphological visualization of hTERT-HPNE, PANC-1, and MIA PaCa-2 cell lines treated with compounds D14 and C22 at 200 μM compared with the effect of Deltarasin.

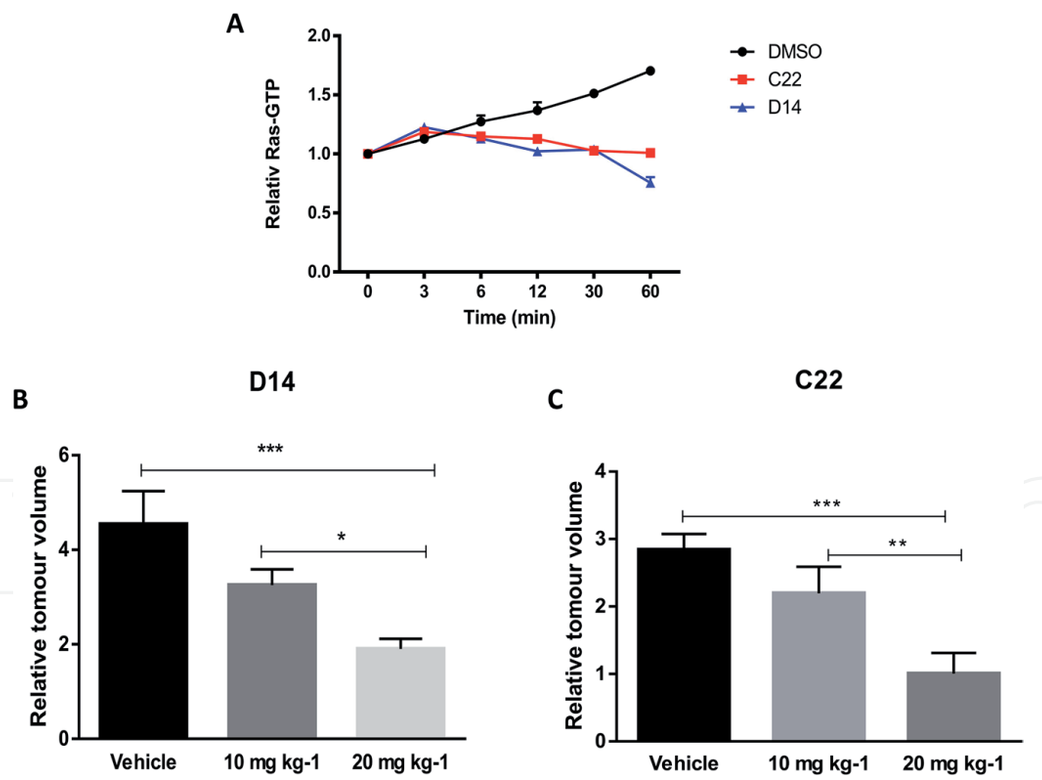

Figure 5.
Compounds D14 and C22 decrease the activation of Ras in the MIA PaCa-2 cell line promoting the decrease of tumor growth. (A) Ras activation decreases by more than 50% in the MIA PaCa-2 cell line treated with D14 and C22. (B) and (C) Compounds D14 and C22 decrease tumor growth in subcutaneous xenograft models, using 20 mg/kg and 10 mg/kg intraperitoneally for 15 days.

were treated with 49.65, 99.3, and 148.9 μM of compound D14, and with 494 nM of Gemcitabine. It was found that the treatment with compound D14 was able to break up the pancreatospheres formed by BxPC3 and MIA PaCa-2 more efficiently than the first-line treatment with Gemcitabine (**Figure** 7).

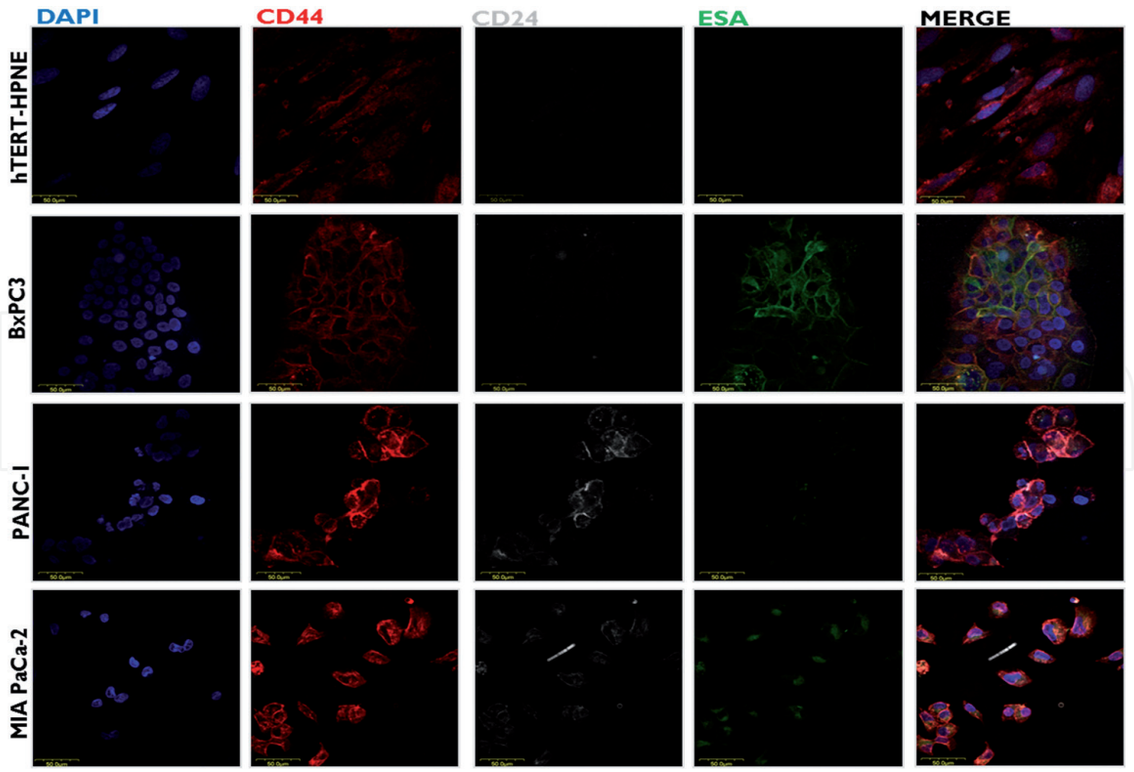

Figure 6.
CD44, CD24, and ESA immunophenotype in 3D cultures of BxPC3, PANC-1, and MIA PaCa-2. The expression of these markers is crucial for the identification of the cancerous trunk population.

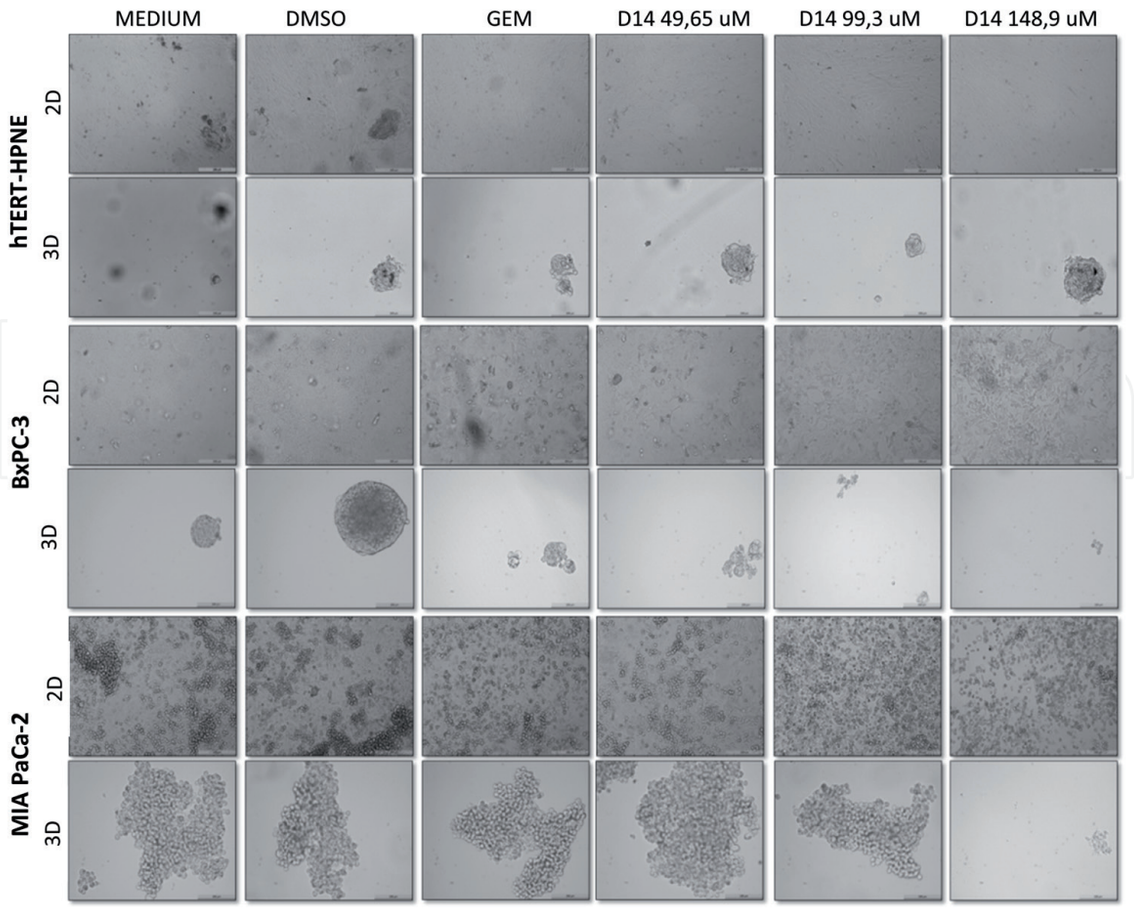

Figure 7.
Morphological visualization of the effect of compound D14 on the viability of BxPC3 and MIA PaCa-2 in 2D and 3D; the result is better than with Gemcitabine, which is the first-line treatment for PDAC.

5. Conclusion

The search for compounds that can stabilize the KRas4B/PDE6δ heterodimeric complex has provided a great pattern in the search for new and less toxic pharmacological alternatives for the treatment of pancreatic cancer and with fewer collateral effects due to their high specificity. Compounds D14 and C22 have shown great specific cytotoxic effects against pancreatic cancer cell lines as well as decreased tumor growth and, even better, in the possible reduction of the PCSC population. However, it is necessary to carry out additional experiments in order to identify the specific mechanisms of action of the cross-linking and stabilizing compounds of this protein multicomplex.

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