

Exploring the therapeutic potential of a novel series of imidazothiadiazoles targeting focal adhesion kinase (FAK) for pancreatic cancer treatment:

Synthesis, mechanistic Insights and promising antitumor and safety profile

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ABSTRACT

Focal Adhesion Kinase (FAK) is a non-receptor protein tyrosine kinase that plays a crucial role in various oncogenic processes related to cell adhesion, migration, proliferation, and survival. The strategic targeting of FAK represents a burgeoning approach to address resistant tumors, such as pancreatic ductal adenocarcinoma (PDAC).

Herein, we report a new series of twenty imidazo[2,1-*b*][1,3,4]thiadiazole derivatives assayed for their antiproliferative activity against the National Cancer Institute (NCI-60) panel and a wide panel of PDAC models. Lead compound **10l** exhibited effective antiproliferative activity against immortalized (SUIT-2, CAPAN-1, PANC-1, PATU-T, BxPC-3), primary (PDAC-3) and gemcitabine-resistant clone (PANC-1-GR) PDAC cells, eliciting IC₅₀ values in the low micromolar range (1.04-3.44 μM), associated with a significant reduction in cell-migration and spheroid shrinkage *in vitro*. High-throughput kinase arrays revealed a significant inhibition of the FAK signalling network, associated to induction of cell cycle arrest in G2/M phase, suppression of tumor cell invasion and apoptosis induction. The low selectivity index/toxicity prompted studies using PDAC mouse xenografts, demonstrating significant inhibition of tumor growth and safety. In conclusion, compound **10l** displayed antitumor activity and safety in both *in vitro* and *in vivo* models, emerging as a highly promising lead for the development of FAK inhibitors in PDAC.

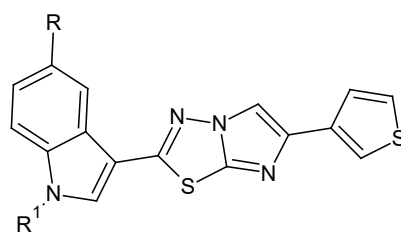
Keywords: PDAC; FAK inhibitors, anticancer agents, imidazo[2,1-*b*][1,3,4]thiadiazoles; PDAC xenograft mouse models

INTRODUCTION

Despite advancements in anticancer research, the persistently high global cancer mortality rate, currently at 10 million deaths per year, is expected to increase to 12 million by 2030 due to a projected rise in life expectancy [1]. Pancreatic ductal adenocarcinoma (PDAC), comprising more than 90% of pancreatic cancer cases, stands out as one of the deadliest tumors, with a 5-year overall survival rate of merely 13% [2]. It ranks as the seventh leading cause of cancer-related deaths globally, [3] but it is predicted to become the second cause of cancer-related deaths by 2030 due to rising incidence (1.1% annual growth) and to the very limited therapeutic options [4].

Currently, surgical resection remains the sole potential curative option for PDAC; however, this malignancy presents a considerable diagnostic challenge, often being diagnosed at advanced stages with local invasion or distant metastasis [5]. This late presentation is attributed to various factors, such as the non-specific symptoms associated with the disease and the proximity of vital blood vessels, which are susceptible to tumor invasion. Consequently, the vast majority of pancreatic tumors (around 80-85%) are deemed unresectable upon diagnosis [6]. The first-line treatment for locally-advanced or metastatic PDAC patients consists of cytotoxic chemotherapy, primarily FOLFIRINOX and gemcitabine-based regimens [7-9]. Indeed promising immunotherapeutic approaches that have shown success in various solid tumors, such as melanoma or renal cell carcinoma, are not applicable in the context of PDAC due to its nonimmunogenic, immune-suppressive, and therapy-resistant microenvironment [10]. Given this context, there is an urgent need for novel therapeutic approaches to effectively combat this malignancy [11].

Recently, we reported the synthesis and the remarkable antiproliferative activity against the PDAC cell lines SUIT-2, CAPAN-1 and PANC-1, of a novel series of imidazo[2,1-*b*][1,3,4]thiadiazole compounds of type **1** (Figure 1) [12].



1a-c

1a, R=R¹=H; **1b**, R=H, R¹=CH₃; **1c**, R=F, R¹=CH₃

Figure 1. Chemical structure of imidazo[2,1-*b*][1,3,4]thiadiazoles **1a-c**.

In particular, derivatives **1a-c**, bearing the imidazothiadiazole central core substituted in position 2 with an indole moiety and in position 6 with a thiophene ring, elicited GI₅₀ values ranging from sub-micromolar to micromolar levels (0.85-2.37 μM). A marked anti-migratory activity of compounds **1** was observed in several PDAC preclinical models and mechanistic studies, employing both kinase arrays and a specific ELISA assay, revealed that these compounds exert their action by inhibiting the phosphorylation of PTK2/FAK (protein tyrosine kinase 2/Focal adhesion kinase) [13].

By binding to the death domain of receptor-interacting protein (RIP), FAK plays a key role in regulating adhesion and cell migration dynamics, as well as promoting cell cycle progression [14-16]. Furthermore, FAK aids in cell survival under adverse growth conditions by enhancing the proteasomal degradation of the tumor suppressor p53 [17,18].

Prior research has demonstrated the anticancer properties of 1,3,4-thiadiazole derivatives containing the 1,4-benzodioxane scaffold, with their primary efficacy attributed to the inhibition of FAK [19]. Notably, the presence of the 1,4-benzodioxine moiety, widely used for its versatility when designing molecules with various biological activities, was identified as crucial for FAK inhibition in these compounds [20-22]. Analogously, the furan ring was

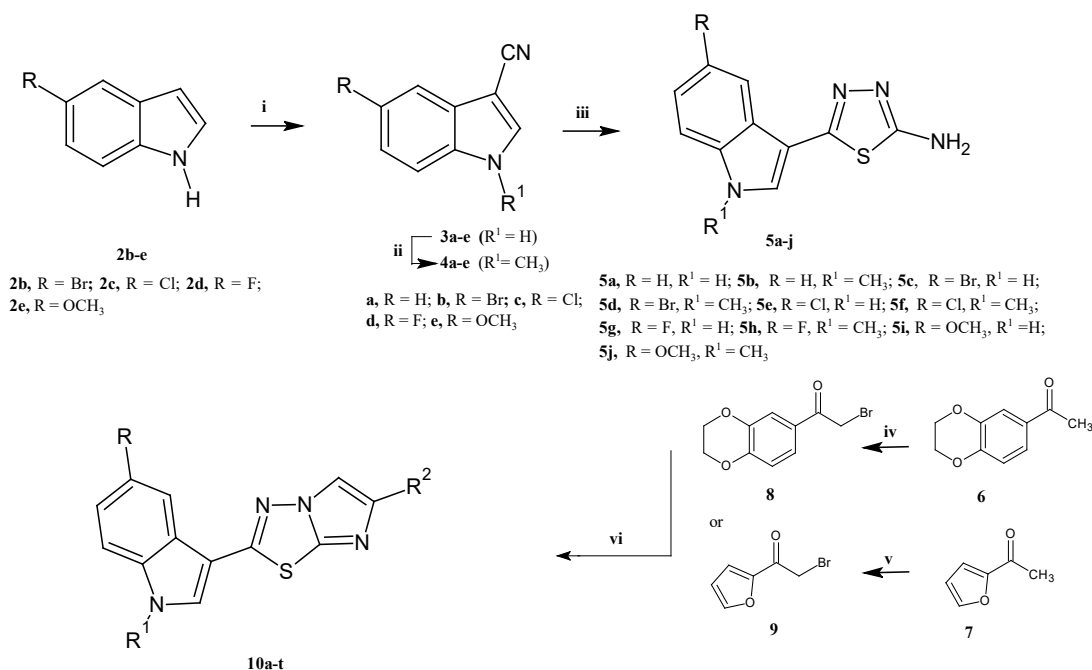
identified as promising scaffold for the development of novel potent dual inhibitors targeting both EGFR and FAK [23,24].

Building upon the encouraging results obtained for the compounds of type **1** together and on the promising anti-FAK properties reported for compounds bearing the 1,4-benzodioxine and the furan ring, as well as leveraging our knowledge in nitrogen heterocyclic systems with anticancer properties [25-30], we synthesized a new series of imidazothiadiazole compounds. In particular, the thiophene ring in position 6 was replaced with either a benzodioxane or a furan moiety, in order to obtain potent anticancer agents with an increased capacity to inhibit FAK.

Chemistry

Twenty new imidazothiadiazole derivatives **10a-t** were efficiently synthesized following the synthetic route described in Scheme 1. In particular, the carbonitriles **3b-e**, not commercially available, were prepared from appropriate 1H-indoles **2** with chlorosulfonyl isocyanate (CSI) in anhydrous acetonitrile under stirring at 0 °C as previously described [31].

The methylation of derivatives **3**, performed with dimethyl carbonate in anhydrous DMF under reflux at 130 °C, gave the corresponding 1-methyl-1H-indole-3-carbonitriles **4**. The thiadiazole intermediates **5a-j** were obtained in excellent yields by the reaction of derivatives **3a-e** or **4a-e** with thiosemicarbazide in trifluoroacetic acid (TFA) at 60 °C for 3 hours. Ultimately, by refluxing in anhydrous ethanol the thiadiazolamines **5** with the suitable α -bromoacetyl derivatives **8** or **9**, the desired compounds **10a-t** were obtained in good yields, ranging from 47% to 78% (Table 1).



Scheme 1. Synthesis of imidazo [2,1-*b*][1,3,4]thiadiazole derivatives **10a-t**.

Reagents and conditions: i) CH₃CN, CSI, 0 °C, 2 h, then DMF, 0 °C, 1.5 h (98-100%); ii) DMF, (CH₃O)₂CO, K₂CO₃, 130 °C, 3.5 h (98-100%); iii) trifluoroacetic acid, thiosemicarbazide, 60 °C, 3.5 h (98-100%); iv) pyridinium bromide-perbromide in anhydrous MeOH, anhydrous DCM, r.t., 24 h (70%); v) Br₂, ethyl ether, 24 h, (75%); vi) anhydrous ethanol, reflux, 24 h (47-83%).

The two α-bromoacetyl derivatives **8** or **9**, used in the final reaction, were obtained in good yields following two different procedures. In particular, the 2-bromo-1-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-ethanone **8** was obtained in good yield by treating a solution of 3',4'-(ethylenedioxy)-acetophenone **6** with pyridinium bromide-perbromide in methanol, whereas, the 2-bromo-1-furan-2-yl-ethanone **9** was prepared through the bromination of 2-acetylfuran **7** with bromine in diethyl ether.

Table 1. New imidazothiadiazole compounds **10a-t**.

Cpd	R	R ¹	R ²	Cpd	R	R ¹	R ²
10a	H	H	2,3-dihydro-benzo[1,4]dioxin-6-yl	10k	H	H	furan-2-yl
10b	H	CH ₃	2,3-dihydro-benzo[1,4]dioxin-6-yl	10l	H	CH ₃	furan-2-yl
10c	Br	H	2,3-dihydro-benzo[1,4]dioxin-6-yl	10m	Br	H	furan-2-yl
10d	Br	CH ₃	2,3-dihydro-benzo[1,4]dioxin-6-yl	10n	Br	CH ₃	furan-2-yl
10e	Cl	H	2,3-dihydro-benzo[1,4]dioxin-6-yl	10o	Cl	H	furan-2-yl
10f	Cl	CH ₃	2,3-dihydro-benzo[1,4]dioxin-6-yl	10p	Cl	CH ₃	furan-2-yl
10g	F	H	2,3-dihydro-benzo[1,4]dioxin-6-yl	10q	F	H	furan-2-yl
10h	F	CH ₃	2,3-dihydro-benzo[1,4]dioxin-6-yl	10r	F	CH ₃	furan-2-yl
10i	OCH ₃	H	2,3-dihydro-benzo[1,4]dioxin-6-yl	10s	OCH ₃	H	furan-2-yl
10j	OCH ₃	CH ₃	2,3-dihydro-benzo[1,4]dioxin-6-yl	10t	OCH ₃	CH ₃	furan-2-yl

Material and methods

Chemistry

The substances necessary to carry out the reported syntheses, both reagents and solvents, have been acquired from Merck, VWR International, Alfa Aesar and Acros Organics. Dichloromethane was anhydriified by using calcium hydride and stored over 4 Å molecular sieves. For the analytical thin-layer chromatography (TLC), silica gel 60 F254 plates (0.25 mm thickness) were used and the developed plates were observed under ultraviolet (UV) light. A Buchi-Tottoly capillary instrument was employed for the melting points evaluation. For recording IR spectra in bromoform, we used a Shimadzu FT / IR 8400S spectrophotometer and reported the peaks in wavenumber (cm⁻¹). ¹H and ¹³C NMR spectra were acquired at 200 and 50 MHz, respectively, through a Bruker Avance II series 200 MHz spectrometer. The purification of the compounds was carried out by using chromatography column with MERK silica gel 230-400 mesh ASTM or with Buchi Sepacore chromatography module (prepacked cartridge reference).

Synthesis of 1H-indole-3-carbonitriles (**3b-e**)

Chlorosulfonyl isocyanate (CSI) (0.44 mL, 5.10 mmol) was added dropwise to a solution of the appropriate indole **2** (5.10 mmol) in anhydrous acetonitrile (4.5 mL). After two hours under stirring at 0°, anhydrous dimethylformamide (DMF) (2.8 mL, 36.39 mmol) was slowly added and the reaction mixture was kept at 0 °C for another 1.5 h. The solution was then poured into crushed ice and the solid product obtained was filtered and dried (yields 98-100%). Analytical and spectroscopic data for compounds **3b-e** are in agreement with those previously reported [12,31].

Synthesis of 1-methylindole-3-carbonitriles (**4a-e**)

A mixture of the proper 3-cyanoindole **3** (7.03 mmol), anhydrous DMF (10 mL), 3.61 mmol of K₂CO₃ and dimethyl carbonate (1.8 mL, 21.4 mmol) was heated under stirring at 130 °C for 3.5 h. After this time, the mixture was cooled at 0-5 °C, and water and ice (25 mL) was slowly added. The extraction of the resulting suspension with diethyl ether (3 x 10 mL), followed by washing the organic phase with brine and drying over Na₂SO₄, allowed to obtain the 3-cyano-1-methylindoles **4** in excellent yields (98-100%). Analytical and spectroscopic data are in accordance to those reported in literature [31].

Synthesis of 5-(1H-indol-3-yl)-1,3,4-thiadiazol-2-amines (**5a-j**)

The suitable indole-3-carbonitrile **3a-e**, or the methyl analogue **4a-e** (5 mmol), was treated with thiosemicarbazide (5 mmol) in trifluoroacetic acid (5 mL) under stirring at 60 °C for 3.5 h. After cooling, the mixture was poured into ice and treated with NaHCO₃ saturated solution up to pH 7. The solid was filtered off and washed with water, cyclohexane and diethyl ether to obtain the desired 5-(1H-indol-3-yl)-1,3,4-thiadiazol-2-amines **5a-j** in excellent yields.

Analytical and spectroscopic data for the derivatives **5a-j** are in accordance to those reported in literature [12].

Synthesis of 2-bromo-1-(2,3-dihydro-1,4-benzodioxin-6-yl)ethanone 8

To a solution of 1-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-ethanone **6** (1.68 mmol) in 3 mL of anhydrous DCM, pyridinium tribromide (3.53 mmol) solubilized in anhydrous methanol (2.8 mL) were slowly added at room temperature and the reaction mixture was stirred for 24 h. The resulting solid precipitate was filtered off and washed with cold ethanol to obtain the pure compound **8** in good yields (70%).

Analytical and spectroscopic data for the derivative **8** are in accordance to those reported in literature [32].

Synthesis of 2-bromo-1-(furan-3-yl)ethanone 9

To a solution of 2-acetylfuran **7** (20 mmol) in 30 mL of anhydrous diethyl ether, bromine (20 mmol) was added dropwise under stirring at the temperature of 0-5°C (ice bath). The reaction mixture was stirred at room temperature for 24h. After this time, 20 mL of water were added and the obtained solution was extracted with diethyl ether (3 x 10 mL), the combine organic layers were washed with a saturated aqueous NaHCO₃ and a solution of Na₂S₂O₃, dried over anhydrous Na₂SO₄. The solvent was evaporated under reduced pressure to obtain the crude product **9**, which was purified by column chromatography using DCM/ethyl acetate 9:1 as eluent (75%). Analytical and spectroscopic data for the derivative **9** are in accordance to those reported in literature [33].

General procedure for the synthesis of 3-[6-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1H-indole derivatives 10a-j and 3-(6-furan-2-yl-[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indole derivatives 10k-t

5-(1H-Indol-3-yl)-1,3,4-thiadiazol-2-amine **5a-f** (0.92 mmol) and the suitable α -bromoacetyl derivative **3** or **4** (0.92 mmol) were refluxed in 40 mL of anhydrous ethanol for 24 h. For the synthesis of derivatives **10k-t**, 0.13 mL of triethylamine were added to the mixture. After cooling at room temperature the solid was filtered off and washed with cold ethanol, obtaining the imidazothiadiazole **10** as pure compound, except for **10c,f,m-r** which were purified by silica gel column chromatography eluting by dichloromethane - ethyl acetate, 9:1.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1H-indole (10a)

Yield: 65 %; yellow solid; m.p.: 230 °C; IR (cm⁻¹): 3020 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ : 4.28 (4H, s, CH₂), 6.94 (1H, d, *J* = 8.2 Hz, Ar), 7.27-7.56 (5H, m, Ar), 8.15 (1H, d, *J* = 5.4 Hz, Ar), 8.40 (1H, s, Ar), 8.71 (1H, s, Ar), 12.21 (1H, s, NH). ¹³C NMR (50MHz, DMSO-*d*₆) δ : 64.6 (t), 64.7 (t), 106.6 (s), 106.9 (s), 113.1 (d), 113.8 (d), 115.1 (d), 117.9 (d), 118.3 (d), 119.7 (d), 120.8 (d), 122.1 (d), 123.8 (d), 124.2 (s), 126.1 (s), 126.7 (s), 137.2 (s), 142.9 (s), 143.7 (s), 144.1 (s). Anal. calculated for C₂₀H₁₄N₄O₂S (MW 374.42): C, 64.16; H, 3.77; N, 14.96%. Found: C, 64.32; H, 3.81; N, 15.01%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1-methyl-1H-indole (10b)

Yield: 67 %; light brown solid; m.p.: 230 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ : 3.90 (3H, s, CH₃), 4.29 (4H, s, CH₂), 6.94 (1H, d, *J* = 8.3 Hz, Ar), 7.31-7.38 (4H, m, Ar), 7.62 (1H, d, *J* = 7.4 Hz, Ar), 8.14 (1H, d, *J* = 6.6, Ar), 8.40 (1H, s, Ar), 8.69 (1H, s, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ : 33.6 (q), 64.4 (t), 65.1 (t), 105.6 (d), 105.9 (s), 106.7 (s), 110.2 (d), 111.5 (d), 112.1 (d), 121.0 (d), 122.3 (d), 123.7 (d), 124.5 (s), 126.2 (s), 127.9 (s), 133.5 (d), 137.6 (s), 137.7 (d), 142.4 (s), 149.7 (s), 157.4(s) Anal. calculated for C₂₁H₁₆N₄O₂S (MW 388.44): C, 64.93; H, 4.15; N, 14.42 %. Found: C, 65.22; H, 4.33; N, 14.70%.

5-Bromo-3-[6-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1H-indole (10c)

Yield: 55 %; light brown solid; m.p.: 327 °C; IR (cm⁻¹): 3142 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 4.27 (4H, s, CH₂), 6.89 (1H, d, *J* = 8.0 Hz, Ar), 7.32 (1H, s, Ar), 7.35 (1H, s, Ar), 7.42 (1H, d, *J* = 8.6 Hz, Ar), 7.51 (1H, d, *J* = 8.6 Hz, Ar), 8.32 (1H, s, Ar), 8.37 (1H, d, *J* = 1.3 Hz, Ar), 8.64 (1H, s, Ar), 12.21 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 64.6 (2xt), 106.7 (s), 110.3 (d), 113.6 (d), 114.5 (s), 115.1 (d), 117.7 (d), 118.1 (d), 123.1 (d), 125.9 (s), 126.7 (d), 128.1 (s), 131.1 (d), 135.9 (s), 143.0 (s), 143.2 (s), 143.9 (s), 145.1 (s), 156.8 (s). Anal. calculated for C₂₀H₁₃BrN₄O₂S (MW 453.32): C, 52.99; H, 2.89; N, 12.36%. Found: C, 53.25; H, 2.97; N, 12.60%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-5-fluoro-1-methyl-1H-indole (10d)

Yield: 58 %; yellow solid; m.p.: 257 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.88 (3H, s, CH₃), 4.28 (4H, s, CH₂), 6.88 (1H, d, *J* = 8.2 Hz, Ar), 7.33 (2H, d, *J* = 7.8 Hz, Ar), 7.47 (1H, d, *J* = 8.7 Hz, Ar), 7.59 (1H, d, *J* = 8.7 Hz, Ar), 8.31 (2H, d, *J* = 9.7 Hz, Ar), 8.58 (1H, s, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 33.8 (q), 64.3 (t), 65.2 (t), 102.8 (s), 105.6 (d), 113.7 (d), 114.9 (d), 115.8 (d), 118.3 (d), 122.6 (d), 123.4 (d), 124.4 (s), 126.2 (s), 134.5 (s), 136.5 (d), 143.9 (s), 146.8 (s), 148.0 (s), 150.1 (s), 156.0 (s), 159.8 (s). Anal. calculated for C₂₁H₁₅BrN₄O₂S (MW 467.34): C, 53.9; H, 3.24; N, 11.99%. Found: C, 53.81; H, 3.35; N, 12.23%.

5-Chloro-3-[6-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1H-indole (10e)

Yield: 75 %; yellow solid; m.p.: 340 °C; IR (cm⁻¹): 3136 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 4.28 (4H, s, CH₂), 6.91 (1H, d, *J* = 7.5 Hz, Ar), 7.43 – 7.27 (3H, m, Ar), 7.57 (1H, d, *J* = 7.6

Hz, Ar), 8.17 (1H, s, Ar), 8.40 (1H, s, Ar), 8.65 (1H, s, Ar), 12.30 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 64.6 (2xt), 106.8 (s), 110.2 (d), 113.6 (d), 114.6 (d), 117.7 (d), 118.1 (d), 120.0 (d), 123.7 (d), 125.3 (s), 126.5 (s), 128.0 (s), 131.2 (d), 135.7 (s), 143.1 (s), 143.2 (s), 143.9 (s), 145.1 (s), 156.8 (s). Anal. calculated for C₂₀H₁₃ClN₄O₂S (MW 408.86): C, 58.75; H, 3.20; N, 13.70%. Found: C, 58.91; H, 3.48; N, 13.93%.

5-Chloro-3-[6-(2,3-dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-1-methyl-1H-indole (10f)

Yield: 78%; brown solid; m.p.: 270 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.88 (3H, s, CH₃), 4.27 (4H, s, CH₂), 6.89 (1H, d, *J* = 8.1 Hz, Ar), 7.34 (3H, m, Ar), 7.64 (1H, d, *J* = 8.7 Hz, Ar), 8.13 (1H, s, Ar), 8.36 (1H, s, Ar), 8.59 (1H, s, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 33.8 (q), 64.4 (2xt), 105.7 (d), 110.3 (d), 113.3 (d), 113.6 (d), 117.7 (s), 118.1 (d), 120.1 (d), 123.7 (s), 125.4 (d), 126.9 (s), 127.9 (d), 134.6 (s), 136.3 (s), 142.9 (s), 143.2 (s), 143.9 (s), 145.1 (s), 156.4 (s). Anal. calculated for C₂₁H₁₅ClN₄O₂S (MW 422.89): C, 59.64; H, 3.58; N, 13.25%. Found: C, 59.81; H, 3.40; N, 13.50%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-5-fluoro-1H-indole (10g)

Yield: 70 %; beige solid; m.p.: 330 °C; IR (cm⁻¹): 3193 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 4.28 (4H, s, CH₂), 6.90 (1H, d, *J* = 8.0 Hz, Ar), 7.16 (1H, t, *J* = 8.5 Hz, Ar), 7.35 (2H, d, *J* = 8.5 Hz, Ar), 7.56 (1H, dd, *J* = 8.5, 4.2 Hz, Ar), 7.84 (1H, d, *J* = 9.8 Hz, Ar), 8.37 (1H, s, Ar), 8.59 (1H, s, Ar), 12.21 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 64.6 (2xt), 105.8 (d), 107.2 (s), 110.2 (d), 111.9 (d), 113.6 (d), 114.3 (d), 117.7 (d), 118.1 (d), 124.5 (s), 128.1 (s), 131.4 (d), 133.8 (s), 143.2 (s), 143.9 (s), 145.1 (s), 156.9 (s), 157.1 (s), 160.2 (s). Anal. calculated for C₂₀H₁₃FN₄O₂S (MW 392.41): C, 61.22; H, 3.34; N, 14.28%. Found: C, 61.48; H, 3.58; N, 14.07%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-5-fluoro-1-methyl-1H-indole (10h)

Yield: 65%; beige solid; mp: 271 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.89 (3H, s, CH₃) 4.28 (4H, s, CH₂), 6.89 (1H, d, *J* = 8.0 Hz, Ar), 7.22 (1H, t, *J* = 8.5 Hz, Ar), 7.35 (2H, s, Ar), 7.63 (1H, dd, *J* = 8.1, 3.7 Hz, Ar), 7.82 (1H, d, *J* = 9.4 Hz, Ar), 8.35 (1H, s, Ar), 8.54 (1H, s, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 33.9 (q), 64.6 (2xt), 105.6 (d), 105.9 (s), 106.1 (d), 110.3 (d), 111.8 (d), 112.0 (d), 113.1 (d), 114.5 (s), 124.9 (s), 134.5(d), 134.9 (s), 137.7(d), 142.4 (s), 143.6 (s), 149.7 (s), 157.0 (s), 157.4 (s), 160.6 (s). Anal. calculated for C₂₁H₁₅FN₄O₂S (MW 406.43): C, 62.06; H, 3.72; N, 13.79%. Found: C, 62.32; H, 3.98; N, 13.85%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-5-methoxy-1H-indole (10i)

Yield: 67 %; off-white solid; m.p.: 265 °C; IR (cm⁻¹): 3197 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.85 (3H, d, *J* = 2.6 Hz, CH₃), 4.27 (4H, s, CH₂), 6.98 – 6.86 (2H, m, Ar), 7.35 (2H, dd, *J* = 7.7, 2.5 Hz, Ar), 7.44 (1H, dd, *J* = 8.8, 2.5 Hz, Ar), 7.63 (1H, s, Ar), 8.23 (1H, s, Ar), 8.59 (1H, d, *J* = 2.7 Hz, Ar), 11.98 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 55.9 (q), 64.6 (2xt), 102.8 (d), 106.8 (s), 110.2 (d), 113.5 (d), 113.6 (d), 113.8 (d), 117.7 (d), 118.1 (d), 124.9(s), 128.0 (s), 130.0 (d), 132.1 (s), 143.0 (s), 143.2 (s), 143.9 (s), 144.9 (s), 155.6 (s), 157.5 (s). Anal. calculated for C₂₁H₁₆N₄O₃S (MW 404.44): C, 62.36; H, 3.99; N, 13.85%. Found: C, 62.55; H, 3.71; N, 13.98%.

3-[6-(2,3-Dihydro-benzo[1,4]dioxin-6-yl)-imidazo[2,1-b][1,3,4]thiadiazol-2-yl]-5-methoxy-1-methyl-1H-indole (10j)

Yield: 47 %; light brown solid; m.p.: 238 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.86 (6H, s, CH₃), 4.27 (4H, s, CH₂), 6.88 (1H, d, *J* = 8.3 Hz, Ar), 6.99 (1H, dd, *J* = 8.9, 2.2 Hz, Ar), 7.35

(2H, d, $J = 7.9$ Hz, Ar), 7.51 (1H, d, $J = 8.9$ Hz, Ar), 7.62 (1H, d, $J = 2.1$ Hz, Ar), 8.22 (1H, s, Ar), 8.55 (1H, s, Ar). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 33.7 (q), 55.9 (q), 64.6 (2xt), 102.9 (d), 105.6 (s), 110.2 (d), 112.3 (d), 113.4 (2xd), 113.6 (d), 117.7 (d), 118.1 (d), 125.2 (s), 128.1 (s), 132.9 (s), 133.4 (s), 143.1 (s), 143.9 (s), 145.0 (s), 155.9 (s), 156.9 (s). Anal. calculated for $\text{C}_{22}\text{H}_{18}\text{N}_4\text{O}_3\text{S}$ (MW 418.47): C, 63.14; H, 4.34; N, 13.39%. Found: C, 63.31; H, 4.62; N, 13.53%.

3-(6-Furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indole (10k)

Yield: 47 %; white solid; m.p.: 218°C; IR (cm^{-1}) 3152 (NH); ^1H NMR (200 MHz, DMSO- d_6) δ : 6.59 (1H, d, $J = 1.3$ Hz, Ar), 6.71 (1H, d, $J = 3.4$ Hz, Ar), 7.34 – 7.25 (2H, m, Ar), 7.55 (1H, dd, $J = 6.8, 2.7$ Hz, Ar), 7.70 (1H, d, $J = 2.1$ Hz, Ar), 8.18 (1H, dd, $J = 4.9, 2.6$ Hz, Ar), 8.33 (1H, d, $J = 2.8$ Hz, Ar), 8.41 (1H, d, $J = 4.3$ Hz, Ar), 12.14 (1H, s, NH). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 105.6 (d), 106.9 (s), 110.22(d), 112.1 (d), 113.0 (d), 120.9 (d), 121.9 (d), 123.7 (d), 124.2 (s), 129.9 (d), 137.2 (s), 137.6 (s), 142.4 (d), 143.8 (s), 149.7 (s), 157.8 (s). Anal. calculated for $\text{C}_{16}\text{H}_{10}\text{N}_4\text{OS}$ (MW: 306.34): C, 62.73; H, 3.29; N, 18.29%. Found: C, 62.98; H, 3.43; N, 18.51%.

3-(6-Furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1-methyl-1H-indole (10l)

Yield: 56 %; yellow solid; m.p.: 188 °C; ^1H NMR (200 MHz, DMSO- d_6) δ : 3.90 (3H, s, CH_3), 6.59 (1H, s, Ar), 6.70 (1H, s, Ar), 7.44 – 7.26 (2H, m, Ar), 7.62 (1H, d, $J = 7.3$ Hz, Ar), 7.71 (1H, s, Ar), 8.18 (1H, d, $J = 6.3$ Hz, Ar), 8.34 (1H, s, Ar), 8.40 (1H, s, Ar). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 33.6 (q), 105.6 (d), 105.9 (s), 110.2 (d), 111.5 (d), 112.1 (d), 121.0 (d), 122.3 (d), 123.7 (d), 124.5 (s), 133.5 (d), 137.6 (s), 137.7 (s), 142.4 (d), 143.7 (s), 149.7 (s), 157.4 (s). Anal. calculated for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{OS}$ (MW: 320.07): C, 63.73; H, 3.78; N, 17.49%. Found: C, 63.91; H, 3.58; N, 17.67%.

5-Bromo-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indole (10m)

Yield: 55 %; brown solid; m.p.: 285 °C; IR (cm⁻¹) 3147 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 6.59 (1H, d, *J* = 1.4 Hz, Ar), 6.69 (1H, d, *J* = 3.0 Hz, Ar), 7.42 (1H, d, *J* = 7.6 Hz, Ar), 7.52 (1H, dd, *J* = 8.6, 3.2 Hz, Ar), 7.70 (1H, s, Ar), 8.32 (1H, s, Ar), 8.39 (1H, d, *J* = 3.0 Hz, Ar), 8.48 (1H, d, *J* = 3.6 Hz, Ar), 12.31 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 105.6 (d), 106.6 (s), 110.4 (d), 112.1 (d), 114.6 (s), 115.1 (d), 123.1 (d), 125.9 (s), 126.3 (d), 131.3 (d), 135.9 (s), 137.7 (s), 142.4 (d), 143.7 (s), 149.7 (s), 157.3 (s). Anal. calculated for C₁₆H₉BrN₄OS (MW: 385.24): C, 49.88; H, 2.35; N, 14.54%. Found: C, 49.95; H, 2.61; N, 14.33%.

5-Bromo-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1-methyl-1H-indole (10n)

Yield: 48 %; pale yellow solid; m.p.: 245 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.89 (3H, s, CH₃), 6.58 (1H, dd, *J* = 3.0, 1.6 Hz, Ar), 6.69 (1H, d, *J* = 3.1 Hz, Ar), 7.48 (1H, dd, *J* = 8.7, 1.5 Hz, Ar), 7.60 (1H, d, *J* = 8.8 Hz, Ar), 7.69 (1H, s, Ar), 8.30 (1H, s, Ar), 8.37 (1H, s, Ar), 8.44 (1H, s, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 33.8 (q), 105.5 (d), 105.7 (s), 110.4 (d), 112.0 (d), 113.7 (s), 115.0 (d), 123.1 (d), 126.0 (s), 126.3 (d), 134.7 (d), 136.5 (d), 137.6 (s), 142.4 (s), 143.5 (s), 149.7 (s), 156.9 (s). Anal. calculated for C₁₇H₁₁BrN₄OS (MW: 399.26): C, 51.14; H, 2.78; N, 14.03%. Found: C, 51.45; H, 2.92; N, 14.15%.

5-Chloro-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indole (10o)

Yield: 52 %; yellow solid; m.p.: 248°C; IR (cm⁻¹) 3150 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 6.58 (1H, dd, *J* = 3.2, 1.7 Hz, Ar), 6.69 (1H, d, *J* = 3.1 Hz, Ar), 7.31 (1H, dd, *J* = 8.6, 2.0 Hz, Ar), 7.57 (1H, d, *J* = 8.7 Hz, Ar), 7.70 (1H, s, Ar), 8.17 (1H, d, *J* = 1.7 Hz, Ar), 8.40 (1H, d, *J* = 3.0 Hz, Ar), 8.46 (1H, d, *J* = 3.5 Hz, Ar), 12.31 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 105.7 (d), 106.8 (s), 107.8 (d), 110.4 (d), 114.6 (s), 114.7 (d), 120.1 (d), 123.8 (d), 125.3 (s), 126.3 (s), 126.6 (s), 131.5 (d), 135.7 (s), 137.7 (s), 143.7 (d), 149.7 (s). Anal. calculated for

C₁₆H₉ClN₄OS (MW: 340.79): C, 56.39; H, 2.66; N, 16.44%. Found: C, 56.51; H, 2.86; N, 16.63%.

5-Chloro-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1-methyl-1H-indole (10p)

Yield: 55 %; white solid; m.p.: 253 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.90 (3H, s, CH₃), 6.58 (1H, dd, *J* = 3.0, 1.7 Hz, Ar), 6.69 (1H, d, *J* = 3.0 Hz, Ar), 7.49 (1H, dd, *J* = 8.7, 1.5 Hz, Ar), 7.65 – 7.56 (1H, m, Ar), 7.68 (1H, d, *J* = 3.4 Hz, Ar), 8.31 (1H, s, Ar), 8.37 (1H, d, *J* = 4.9 Hz, Ar), 8.42 (1H, d, *J* = 3.5 Hz, Ar). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 33.8 (q), 105.5 (d), 105.7 (s), 110.4 (d), 112.0 (d), 113.3 (s), 115.0 (d), 120.1 (d), 126.0 (s), 127.0 (d), 134.7 (d), 136.3 (d), 137.7 (s), 142.4 (s), 143.6 (s), 149.7 (s), 156.8 (s). Anal. Calculated for C₁₇H₁₁ClN₄OS (MW: 354.81): C, 57.55; H, 3.12; N, 15.79%. Found: C, 57.84; H, 3.31; N, 15.91%.

5-Fluoro-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indole (10q)

Yield: 50 %; off-white solid; m.p.: 265 °C; IR (cm⁻¹) 3153 (NH); ¹H NMR (200 MHz, DMSO-*d*₆) δ: 6.58 (1H, d, *J* = 1.3 Hz, Ar), 6.69 (1H, d, *J* = 2.9 Hz, Ar), 7.17 – 7.11 (1H, m, Ar), 7.58 – 7.53 (1H, m, Ar), 7.70 (1H, s, Ar), 7.86 (1H, dd, *J* = 9.5, 1.7 Hz, Ar), 8.39 (1H, d, *J* = 2.8 Hz, Ar), 8.41 (1H, s, Ar), 12.22 (1H, s, NH). ¹³C NMR (50 MHz, DMSO-*d*₆) δ: 105.6 (d), 105.9 (d), 107.1 (s), 110.3 (d), 111.8 (d), 112.1 (d), 114.4 (d), 124.5 (s), 124.7 (s), 131.6 (d), 133.8 (s), 137.6 (s), 142.4 (d), 149.7 (s), 157.0 (s), 157.4 (s), 160.3 (s), 166.9 (s). Anal. Calculated for C₁₆H₉FN₄OS (MW: 324.33): C, 59.25; H, 2.80; N, 17.27%. Found: C, 59.43; H, 2.93; N, 17.41%.

5-Fluoro-3-(6-furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1-methyl-1H-indole (10r)

Yield: 65%; off-white solid; m.p.: 246 °C; ¹H NMR (200 MHz, DMSO-*d*₆) δ: 3.90 (3H, s, CH₃), 6.58 (1H, s, Ar), 6.69 (1H, d, *J* = 3.0 Hz, Ar), 7.22 (1H, td, *J* = 9.1, 2.0 Hz, Ar), 7.64 (1H, dd,

$J = 9.0, 4.4$ Hz, Ar), 7.69 (1H, s, Ar), 7.85 (1H, dd, $J = 9.7, 2.0$ Hz, Ar), 8.37 (2H, s, Ar). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 33.9 (q), 105.6 (d), 105.8 (s), 106.1 (d), 110.3 (d), 111.8 (d), 112.1 (d), 113.0 (d), 124.7 (s), 134.5 (s), 134.9 (d), 137.7 (s), 142.4 (d), 149.7 (s), 157.0 (s), 157.4 (s), 160.5 (s). Anal. Calculated for $\text{C}_{17}\text{H}_{11}\text{FN}_4\text{OS}$ (MW: 338.36): C, 60.34; H, 3.28; N, 16.56%. Found: C, 60.52; H, 3.35; N, 16.72%.

3-(6-Furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-5-methoxy-1H-indole(10s)

Yield: 50 %; yellow solid; m.p.: 252 °C; IR (cm^{-1}) 3147 (NH); ^1H NMR (200 MHz, DMSO- d_6) δ : 3.85 (3H, s, CH_3), 6.57-6.59 (1H, m, Ar), 6.69 (1H, d, $J = 3.2$ Hz, Ar), 6.94 (1H, dd, $J = 8.8, 2.4$ Hz, Ar), 7.44 (1H, d, $J = 8.8$ Hz, Ar), 7.64 (1H, d, $J = 2.3$ Hz, Ar), 7.69 (1H, s, Ar), 8.24 (1H, d, $J = 3.0$ Hz, Ar), 8.40 (1H, s, Ar), 11.99 (1H, s, NH). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 55.9 (q), 103.0 (d), 105.6 (d), 106.8 (s), 110.3 (d), 112.0 (d), 113.5 (d), 113.8 (d), 124.9 (s), 130.2 (d), 132.2 (s), 137.6 (s), 142.3 (d), 143.7 (s), 149.8 (s), 155.7 (s), 157.9 (s). Anal. calculated for $\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}_2\text{S}$ (MW: 336.37): C, 60.70; H, 3.60; N, 16.66%. Found: C, 60.95; H, 3.79; N, 16.83%.

3-(6-Furan-2-yl-imidazo[2,1-b][1,3,4]thiadiazol-2-yl)-5-methoxy-1-methyl-1H-indole (10t)

Yield: 54 % ; brown solid; m.p.: 167 °C; ^1H NMR (200 MHz, DMSO- d_6) δ : 3.86 (6H, s, CH_3), 6.59 (1H, s, Ar), 6.69 (1H, s, Ar), 7.00 (1H, d, $J = 8.8$ Hz, Ar), 7.53 (1H, d, $J = 9.0$ Hz, Ar), 7.64 (1H, s, Ar), 7.70 (1H, s, Ar), 8.26 (1H, s, Ar), 8.39 (1H, s, Ar). ^{13}C NMR (50 MHz, DMSO- d_6) δ : 33.7 (q), 56.0 (q), 102.1 (s), 103.2 (d), 105.6 (d), 110.3 (d), 112.0 (d), 112.4 (d), 113.3 (d), 125.2 (s), 132.9 (s), 133.6 (d), 137.5 (s), 141.6 (s), 142.4 (d), 149.7 (s), 155.9 (s), 157.5 (s). Anal. calculated for $\text{C}_{18}\text{H}_{14}\text{N}_4\text{O}_2\text{S}$ (MW: 350.39): C, 61.70; H, 4.03; N, 15.99%. Found: C, 61.93; H, 4.21; N, 15.74%.

Biology

Drugs and chemicals

For the biological assays the newly synthesized compounds **10** were dissolved in DMSO. The medium, foetal bovine serum (FBS), penicillin (50 IU mL⁻¹) and streptomycin (50 mg mL⁻¹) were purchased from Gibco (Gaithersburg, MD, USA), whereas the other chemicals were acquired from Sigma (Zwijndrecht, the Netherlands).

Cell culture

The cells were cultured in RPMI-1640 (Roswell Park Memorial Institute 1640) enriched with 10% heat-inactivated newborn calf serum (NBCS), 1% penicillin/streptomycin, or in DMEM (Dulbecco's Modified Eagle's Medium), added with 10% heat-inactivated NBCS. They were maintained in a humidified environment containing 5% CO₂ and 95% air at 37 °C and harvested using trypsin-EDTA.

Cell growth inhibition

The cytotoxic activity of the imidazothiadiazole derivatives **10** against differentiated pancreatic cancer cells was assessed using the sulforhodamine B (SRB) chemosensitivity assay. Triplicate wells of 96-well flat-bottom plates were seeded with cells in volumes of 100 μ L, with different cell densities for each cell line (3 x 10³ cells/well for SUIT-2, PANC-1, PANC-1 GR, PATU-T, and PDAC-3; 4 x 10³ cells/well for BxPC-3; 5 x 10³ cells/well for CAPAN-1; and 8 x 10³ cells/well for the normal fibroblasts Hs27, used to evaluate the selectivity index (SI)). After incubating for 24 hours at 37 °C, cells were treated with various concentrations of the compounds (100 μ L) for 72 hours at 37 °C, 5% CO₂, and 95% humidity.

Upon completion of the incubation period, the cells were treated with 25 μ L of 50% cold trichloroacetic acid for fixation and maintained at 4 °C for a minimum of 60 minutes.

Subsequently, the plates were emptied, gently rinsed with deionized water, dried overnight at room temperature (RT), and stained with 50 μ L of 0.4% SRB solution in 1% acetic acid for 15 minutes at RT. After removing excess SRB stain, the plates were washed with a 1% acetic acid solution and allowed to air-dry overnight at RT. The SRB staining was dissolved in 150 μ L of tris(hydroxymethyl)aminomethane solution pH = 8.8 (TRIS base), and the absorbance, at wavelengths of 490 nm and 540 nm, was evaluated. Cell growth inhibition was determined as the percentage of drug treated cells compared to vehicle-treated cells (“untreated cells or control”) OD (adjusted for OD before drug exposure, "day-0").

The percentage of cell growth was calculated by comparing the average optical density of the growth in control wells and of the sample wells, through the following equation:

$$\% \text{ Cell Growth} = [(\text{mean OD}_{\text{compound}} - \text{mean OD}_{\text{day zero plate}}) / \text{mean (OD}_{\text{control cells}} - \text{mean OD}_{\text{day zero plate}})] \times 100$$

The outcomes achieved were calibrated using the day zero plate (wells harboring cells grown for just 24 hours) and standardized by the control cells (wells containing untreated cells) to obtain the viable cells rate. The 50% inhibitory concentration of cell growth (IC₅₀) was computed through non-linear least squares curve fitting (GraphPad PRISM version 9, Intuitive Software for Science, San Diego, CA). In the NCI procedure, the IC₅₀ is referred to as the GI₅₀, signifying the concentration at which 50% growth inhibition is attained. Data was expressed as mean values $\pm$ SEM.

Initially, the cells were exposed to three different screening concentrations (0.1, 1 and 10 μ M) of each compound **10** for a period of 72 hours. Subsequently, the SRB assay was executed to ascertain the inhibition of cell proliferation induced by the most active compounds. To determine the IC₅₀ values for each PDAC cell model, cells were treated with eight escalating

concentrations (ranging from 0.3 μ M to 40 μ M) of compounds **10f**, **10k**, **10l**, and **10q** for 72 hours.

Wound-healing assay

The *in vitro* scratch wound-healing assay was conducted following a previously established method [34]. BxPC-3, PATU-T, SUIT-2, CAPAN-1, PANC-1, PANC-1 GR, and PDAC-3 cells were seeded in 96-well flat-bottom plates at a density of $3\text{--}5 \times 10^4$ cells/well in a volume of 100 μ L. After 24 hours of pre-incubation at 37 °C, 5% CO₂, and 95% humidity, scratches of consistent width were created on the cell monolayers using a specialized multi-needle tool. Following the removal of detached cells by washing with phosphate-buffered saline, the control wells received only medium, while the experimental wells were supplemented with the compounds of interest. The wound confluence was monitored at specific time points (T = 0, 4, 8, 20, and 24 hours after treatment) using phase-contrast microscopy integrated with the Leica DMI300B migration station and Universal Grab 6.3 software from Digital Cell Imaging Labs (Keerbergen, Belgium). Images were captured, and the wound closure was analyzed using the Scratch Assay 6.2 software from Digital Cell Imaging Labs.

PamChip® kinase activity profiling

A PamChip array comprising 144 kinase peptide substrates was employed to assess the alteration in tyrosine kinase activity upon the application of **10l** compound. This study was conducted using SUIT-2 and PDAC-3 cells in biological triplicates, involving three untreated samples and three samples treated with **10l**, as outlined previously [35].

The cells were cultured in 25 cm² flasks until they attained 80% confluence, maintaining a temperature of 37 °C and a CO₂ concentration of 5%. Following this, both cell lines were exposed to 5 times the IC₅₀ concentration of **10l**, while the control cells underwent medium replacement with fresh medium. The treatment duration was 2 hours, after which the cells were

detached, and 10^6 cells/mL were harvested from each replicate in 1.5 mL tubes. To lyse the cells, 100 μ L of M-PER lysis buffer per 10^6 cells was utilized, comprising M-PER Mammalian Extraction Buffer (Thermo Scientific, Rockford, IL, USA), Halt protease inhibitor cocktail (Complete Mini EDTA-free Protease Inhibitor Cocktail, Roche #11836170001) diluted 1:100, and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher #78420) also diluted 1:100. The lysis process took at least 15 minutes at 4 °C. The lysates were then collected in 1.5 mL tubes and centrifuged at 16,000g for 15 minutes at 4 °C. The resulting supernatants were gathered and stored at -80 °C until further use. The protein concentration of the samples was estimated employing the Bio-Rad protein assay, which is based on the Bradford method (BioRad, Hercules, CA). The PamChips were prepared in order to reach a concentration of 10 μ g protein/array. They were then added to the MasterMix (PamGene reagent kit 32116) which included PK buffer 10x, BSA solution 100x, PTK additive 10x, 1 M DTT solution, Complete Mini EDTA-free Protease Inhibitor Cocktail, Halt Phosphatase Inhibitor Cocktail 400x (Thermo Fisher), PY-20-FITC (fluorescent labeled antibody), and 4 mM ATP solution. The samples were added to the MasterMix promptly before loading onto the chip. Prior to sample loading, the PamStation®12 instrument performed a blocking step using 25 μ L of 2% BSA on each array, followed by three wash steps with PK buffer. Subsequently, 40 μ L of each sample mixture was loaded in duplicate onto the arrays. Throughout the 30 °C incubation, the sample mixture underwent pumping up and down through the array once every minute for a total of 60 cycles. Fluorescent imaging of each array was repeated using a 12-bit CCD camera, which monitored fluorescence intensities in real time.

The intensity of each spot at the end point was evaluated through an open-source software (ScanAnalyze) and then normalized to account for local background noise. As the negative control yielded a negative value, all intensities were shifted minimally to ensure the negative control value was set to zero. For the differential analysis, a Student t-test was conducted in R

(version 3.6.1), and the resulting p-values were corrected for false discovery rate (FDR). Peptides reaching a cutoff of $FDR < 0.01$ were considered statistically significant. Visualization of differentially phosphorylated protein was performed in Cytoscape (version 3.5.0).

FAK ELISA assay

The quantification of pFAK (phosphorylated focal adhesion kinase) at tyrosine residue 397 was carried out using an Enzyme-Linked Immunosorbent Assay (ELISA). The specific ELISA kit employed was the Invitrogen™ FAK[pY397] ELISA Kit (Cat. # KHO0441), following the manufacturer's instructions. Supernatants from SUIT-2 and PDAC-3 cells were collected 24 hours after treatment with imidazothiadiazole compounds **10k** and **10l** at a concentration corresponding to 5 times the IC_{50} values. The absorbance of the samples was measured at 450 nm. Additionally, we conducted a parallel ELISA test using the well-known FAK inhibitor defactinib at 5 times IC_{50} , which effectively inhibited FAK phosphorylation, as described previously [36].

Cell cycle assay

Cell cycle stage was analyzed by flow cytometry. Cells were seeded in 6 wells plate (2.5×10^5 cells/well). After an overnight incubation at 37 °C, the cells were treated with the compound **10l** at 5 times IC_{50} , and incubated for 24 hours. After treatment, the cells were harvested by trypsinization (0.3 mL/well of trypsin-EDTA), incubated until the cells were detached; cells were resuspended with 1.7 mL of medium and collected into FACS tubes. The samples were then centrifuged in order to get cells pellet (5 min at 1200 rpm). Finally, these pellets were fixed in ice-cold 70% ethanol, washed twice with phosphate-buffered saline (20 mM sodium phosphate pH 7.4, 150 mM NaCl) and incubated for 30 min at 37 °C with 50 µl of RNase (100 µg/mL) followed by incubation with 200 µl of propidium iodide solution (PI, 50 µg/mL). The

cycle analysis was performed on the FACS (Fluorescence Activated Cell Sorting) Calibur instrument, to evaluate the effects on the cell cycle distribution and cell viability.

Evaluation of apoptosis

The evaluation of caspase-3 activity was chosen as a reliable method to measure apoptosis induction due to its well-established role as a key executioner caspase in the apoptotic pathway. To this goal we used a specific spectrofluorimetric activity assay (Human Active Caspase-3 Immunoassay Quantikine ELISA, Catalog Number KM300, R&D Systems, Inc., Minneapolis, MN). In summary, cells were seeded in 6-well plates at a density of 2×10^5 cells/mL and treated with **101** or defactinib at $5 \times \text{IC}_{50}$ for 24 hours. Following drug exposure, cell extracts were prepared, diluted, and combined with the provided reagents following the manufacturers' instructions. The absorbance was measured at 450 nm with a background subtraction at 540 nm. The relative caspase activity was determined by comparing the measurements to a standard curve generated using human recombinant caspase-3, as described previously [37].

Spheroids

PDAC-3 spheroids were grown in VWR®96-wells F-bottom plates (VWR, Cat No. 7342327) coated with 1.5% w/v agarose. Cells were seeded at the density of 2×10^4 cells per well and the plate was centrifuged at 1200 rpm, for 5 min at RT to facilitate cell aggregation, finally cells were incubated at 37°C, 5% CO₂ for 72 h in order to allow spheroids formation.

After 72 hours, before the treatment a picture of the plate was taken with an automated phase-contrast microscope DMI300B (Leica Microsystems, Eindhoven, Netherlands), and the subsequent pictures were taken every two days. Prior to treatment, automated phase-contrast microscope was used to capture an initial picture of the plate. Subsequent pictures were taken every two or three days.

After 72-hour incubation, the culture medium was replaced with drug-free medium for the control, whereas each testing group was treated with compounds of interest (at least 8 wells per condition). Compound **101** and defactinib were used at 5 times the IC₅₀, Gemcitabine was used as an additional “positive control” at 150 nM, as previously reported [12]. The treatment and change of medium was repeated after four days, to ensure the availability of nutrients.

Pictures were analysed with ImageJ Software (U.S. National Institute of Health, Bethesda, Maryland, USA) to determine the area of the spheroids treated and compare it to the area of the untreated spheroids, as described previously [38].

Confocal experiment of spheroids

PDAC-3 spheroids were obtained as previously described. Spheroids were exposed to 150 nM gemcitabine, 3 and 15 μM of **101** after 3 days from seeding, and the treatment was refreshed every 3 days. Untreated spheroids served as controls. Viability and cell death were measured by live fluorescence confocal imaging. After the first treatment, spheroids were stained with a staining solution containing PBS 1X, FBS 1X, 1 μM Calcein AM, 0.32 μM Hoechst33342 and 3 μM of propidium iodide (PI) for 3 hours. Staining was repeated on day 8 (from seeding). Washing steps were avoided to preserve the integrity of the spheroids. For confocal imaging, z-stacks were acquired with a Nikon Eclipse Ti2 confocal microscope with a temperature and CO₂ controlled incubator, using a 10x Plan dry objective. 4 images for each stack (~11 stacks, steps of 20 μm) were stitched with NIS Elements 5.02.02 software. At least 3 spheroids per condition were imaged, on the day of the first treatment, and every 2 (on day 3 and 5) days in the first week and every 3 days in the second week (day8 and 11). Composite images were obtained by doing a z-projection with ImageJ. Scale bar is 400 μm.

***In vivo* experiment using subcutaneous mice.**

In vivo experiments were performed on mice purchased from Charles River Laboratories (Calco, Italy). The working protocol was approved by the local committees on animal experimentation of the VU University Medical Center (VUmc, Amsterdam, The Netherlands) and of the University of Pisa (Pisa, Italy), according to the 2010/63/EU European Community Council Directive for laboratory animal care.

In vivo experiments were performed on subcutaneous primary PDAC models. Subcutaneous tumors were obtained by cell suspension injection of 1×10^6 primary PDAC-3 cells [39]. Mice were treated with compound **101**, solubilised in Polyethylene glycol 400 (PEG400, Sigma-Aldrich, St. Louis, MO), at 25 mg/kg, 3 days/week for 2 weeks (formulation concentration: 25 mg/mL in PEG400, 100 μ L of i.p. injection for a 25g mouse). When tumor volume reached an average size of 100 mm³ (day 5), the animals were randomly distributed into four groups ($n=5$ tumours per treatment group) as follows: (1) untreated mice, (2) mice treated with gemcitabine alone at 100 mg/kg, 2 days (days 1 and 4) for 2 weeks (formulation concentration: 25 mg/mL in PBS, 100 μ L of i.p. injection for a 25g mouse), (3) mice treated with **101** solubilised in PEG400, at 25 mg/kg, 3 days (days 1, 3 and 5) for 2 weeks (formulation concentration: 12.5 mg/mL in PEG400). The concentration of **101** was selected on the basis of previous experiments.

Tumor xenografts were measured as described previously [40]. Lidocaine was used as local anaesthetic on the skin of the mice. Mice were sacrificed before primary tumours and metastases can cause severe symptoms, via cervical dislocation.

Statistics

The *in vitro* experiments were performed in triplicates and repeated at least twice. The results reported in the figures are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical analyses were carried out by two-way ANOVA followed by Bonferroni's post-test (to adjust for multiple comparisons), using GraphPad-Prism version 9 (Intuitive Software for

Science, San Diego, CA). All analyses were two-sided, and statistical significance was set at $P < 0.05$. *In vivo* statistical analysis was conducted with ANOVA analysis with Bonferroni correction for post hoc analysis to evaluate differences in tumor size in the different groups of animals. $P < 0.05$ was considered significant. The results were expressed as mean values $\pm$ standard deviation (SD).

Results and Discussion

Antiproliferative activity

The newly synthesized imidazo [2,1-*b*][1,3,4]thiadiazoles **10a-t** were tested by the NCI (Bethesda, MD) for the evaluation of their antitumor activity. They were initially prescreened, according to the NCI protocol, at one-dose (10 μ M) on the full panel of 60 human cancer cell lines, derived from 9 cancer cell types and grouped into disease subpanels, including leukemia, non-small cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancers.

Among the tested compounds, the furan analogues demonstrated superior potency compared to the corresponding benzodioxane derivatives. In particular compounds **10k**, **10l**, **10n**, and **10r**, meeting the criteria set by the NCI, underwent further screening across the full panel at five concentrations, ranging from 10^{-4} to 10^{-8} M, in order to determine their half maximal inhibitory concentrations (GI_{50}). All four compounds exhibited significant antiproliferative activity against the tested cell lines, with GI_{50} values in the nanomolar or low micromolar range (Table 2). Compound **10l** displayed the highest potency, eliciting nanomolar GI_{50} values against fifteen different cancer cells. Notably, it exhibited GI_{50} values in the range from 0.45 to 3.09 μ M and from 0.52 to 3.64 μ M against the leukemia and colon cancer subpanels, respectively.

Table 2. Antiproliferative activity^a of compounds **10k**, **10l**, **10n** and **10r** expressed as GI_{50} ^b and TGI ^c.

Panel/Cell line	10k		10l		10n		10r	
	GI ₅₀ (μM)	TGI (μM)	GI ₅₀ (μM)	TGI (μM)	GI ₅₀ (μM)	TGI (μM)	GI ₅₀ (μM)	TGI (μM)
Leukemia								
CCRF-CEM	2.96	29.6	3.01	>100	33.5	>100	4.19	36.2
HL-60(TB)	2.10	89.8	0.92	>100	>100	>100	2.81	10.0
K-562	0.56	>100	0.45	>100	17.6	>100	2.18	17.8
MOLT-4	3.02	>100	1.90	>100	21.4	88.1	3.38	13.5
RPMI-8226	3.19	>100	3.09	>100	4.66	83.0	4.06	73.7
SR	0.69	>100	0.45	>100	35.6	>100	2.85	16.6
Non-Small Cell Lung Cancer								
A549/ATCC	4.09	>100	3.56	>100	58.9	40.3	7.75	45.2
EKVX	1.76	>100	1.56	>100	10.6	32.9	5.15	40.2
HOP-62	4.62	>100	3.19	>100	8.06	49.3	7.91	41.3
HOP-92	6.39	63.2	14.7	98.7	2.09	10.6	4.45	22.7
NCI-H226	24.0	>100	22.3	>100	n.t.	n.t.	n.t.	n.t.
NCI-H23	2.82	39.8	3.24	>100	7.83	27.1	7.45	39.4
NCI-H322M	3.46	>100	6.45	>100	6.97	52.9	10.9	49.7
NCI-H460	3.71	>100	2.42	>100	7.04	22.7	3.72	16.1
NCI-H522	1.97	18.2	0.72	17.7	17.0	48.1	5.17	35.2
Colon Cancer								
COLO 205	2.36	11.2	1.22	6.28	39.0	>100	6.67	39.2
HCC-2998	4.14	41.2	3.64	25.8	17.5	78.3	5.41	19.2
HCT-116	2.42	27.8	0.71	>100	2.74	8.65	4.39	16.2
HCT-15	1.55	>100	0.52	37.2	4.09	24.1	3.20	16.7
HT29	2.79	69.6	0.67	>100	15.2	64.1	4.44	20.2
KM12	3.07	25.7	1.71	>100	12.7	48.1	2.99	10.3
SW-620	2.46	>100	0.72	>100	17.2	41.3	4.31	49.3
CNS Cancer								
SF-268	9.10	>100	22.5	>100	6.76	30.3	10.3	42.8
SF-295	3.00	27.1	0.92	15.4	16.8	58.4	6.14	21.1
SF-539	2.82	12.9	1.66	4.98	4.72	20.4	5.92	19.9
SNB-19	6.68	>100	4.88	>100	12.6	27.1	11.0	30.4
SNB-75	1.42	8.39	0.48	6.29	21.1	61.5	8.45	34.5
U251	3.64	>100	3.69	>100	5.61	66.1	6.52	29.8
Melanoma								
LOX IMVI	2.98	33.5	2.40	28.7	3.65	14.4	4.88	17.0
MALME-3M	2.41	>100	1.12	27.0	7.64	29.8	4.44	20.1
M14	1.81	>100	0.93	>100	5.53	37.1	4.13	19.4
MDA-MB-435	0.39	1.79	0.23	0.60	7.58	67.8	1.85	4.02
SK-MEL-2	2.23		1.18	84.6	70.3	>100	4.41	21.3
SK-MEL-28	5.17	>100	3.86	51.7	11.9	27.9	9.81	32.2
SK-MEL-5	2.75		2.60	14.8	13.6	>100	3.57	14.7
UACC-257	4.99	>100	21.8	>100	83.6	>100	16.9	49.3
UACC-62	3.52	>100	0.97	33.5	10.3	46.0	8.01	37.6
Ovarian Cancer								
IGROV1	2.81	>100	1.62	34.2	11.8	60.8	8.44	36.7

OVCAR-3	2.84	>100	2.09	>100	11.3	36.4	4.04	17.2
OVCAR-4	16.1	>100	12.7	>100	2.34	6.06	15.0	61.0
OVCAR-5	4.07	>100	3.77	56.0	5.12	26.5	11.1	28.1
OVCAR-8	4.17	>100	4.12	>100	6.28	34.0	11.7	70.5
NCI/ADR-RES	1.26	4.40	0.51	4.60	3.07	10.7	3.12	15.2
SK-OV-3	4.47	>100	3.92	>100	51.0	>100	10.8	43.0
Renal Cancer								
786-0	2.06	>100	24.4	>100	2.73	6.05	15.4	31.9
A498	97.2	>100	35.0	>100	27.2	75.1	15.2	40.8
ACHN	6.06	>100	5.47	48.2	1.95	4.71	7.14	22.2
CAKI-1	2.82	>100	1.55	72.9	3.46	21.0	3.03	16.4
RXF 393	3.87	76.6	3.47	43.3	4.67	17.6	8.29	22.8
SN12C	3.77	>100	3.93	>100	16.6	94.8	15.2	46.0
TK-10	8.62	>100	15.7	>100	20.4	55.0	20.7	43.5
UO-31	2.60	>100	1.65	>100	1.72	9.08	2.71	20.3
Prostate Cancer								
PC-3	3.76	>100	4.01	>100	4.56	23.1	5.85	39.0
DU-145	4.25	>100	4.33	>100	7.04	>100	11.8	40.2
Breast Cancer								
MCF7	0.52	10.0	0.29	19.3	0.39	15.2	0.59	18.3
MDA-MB30.2-231/ATCC	2.10	15.6	1.59	27.4	1.79	11.3	2.71	17.6
HS 578T	2.20	21.0	3.80	>100	3.88	47.2	4.14	30.2
BT-549	2.96	>100	4.40	53.0	18.1	99.0	15.2	50.0
T-47D	n.t.	n.t.	n.t.	n.t.	2.42	>100	3.33	83.2
MDA-MB-468	3.38	>100	2.82	>100	3.57	18.3	2.60	9.88

^a Data obtained from the NCI in vitro disease-oriented human tumor cell line screen. ^b GI₅₀: concentration that inhibit 50% net cell growth. ^c TGI total growth inhibition.

To broaden the investigation of the antiproliferative effects of compounds **10a-t** to cancer cells not covered in the NCI panel and considering the encouraging activity reported for imidazothiadiazole derivatives in previous studies targeting pancreatic cancer, we conducted additional experiments to evaluate the cytotoxicity of these compounds on a panel of cells representative of the subtypes of PDAC. Among the pancreatic cancer cells used in the study BxPC-3, CAPAN-1, and SUIT-2 can be categorized as epithelial subtypes, while PANC-1 and PATU-T are representative of the mesenchymal subtype. Additional experiments were performed on a gemcitabine-resistant clone of PANC-1 (PANC-1 GR [41]) and on the primary cell culture PDAC-3 (obtained from a surgically resected patient, as described previously [42]).

A preliminary screening was conducted using three concentrations (0.1, 1, and 10 μ M). Subsequently, the most promising compounds were subjected to cytotoxicity testing at various concentrations (ranging from 312 nM to 40 μ M) to determine their IC₅₀ values. Notably, compounds **10f**, **10k**, **10l**, and **10q** demonstrated significant antiproliferative activity across all the preclinical models. Particularly, the imidazothiadiazoles **10k** and **10l** exhibited IC₅₀ values ranging from 1.04 to 6.90 μ M, as shown in Table 3.

Table 3. Antiproliferative activity of compounds (Cpd) **10f**, **10k**, **10l** and **10q** on pancreatic cancer cells.

IC ₅₀ ^a (μ M)±SEM ^b							
Cpd	CAPAN-1	PANC-1	PATU-T	SUIT-2	BxPC-3	PDAC-3	PANC-1 GR
10f	4.78±0.01	7.40±0.13	8.43±0.08	10.8±0.12	16.62±0.26	>40	15.49±2.9
10k	2.64±0.06	3.16±0.007	3.73±0.12	2.19±0.17	2.67±0.15	5.54±0.33	6.90±0.05
10l	1.54±0.04	1.57±0.08	1.56±0.007	1.70±0.14	1.04±0.08	2.98±0.4	3.44±0.26
10q	6.38±0.1	8.65±0.03	12.68±0.7	6.6±0.1	8.83±0.64	12.94±2.7	15.92±2.6

^a The values are means ± SEM of three separate experiments.

^b SEM: Standard Error of the Mean.

Finally, we conducted additional experiments to assess the *in vitro* cytotoxicity of the new compound **10l** against normal fibroblasts (Hs27). The outcomes of these experiments enabled us to calculate the selectivity index (SI, calculated as IC₅₀ in non-tumor cell line/IC₅₀ in the tumor cells PDAC-3), resulting in values of 8.1 for compound **10l**, 5.6 for gemcitabine and 2.6 for defactinib, respectively (Table 4). Consequently, our compounds demonstrated a selectivity index similar to gemcitabine and significantly superior to defactinib, supporting their high cancer-selective profile and prompting further *in vivo* studies.

Table 4. Antiproliferative activity of compound **10l** and of reference compounds (gemcitabine and defactinib) on PDAC-3 tumor cells and Hs27 normal fibroblast cells, reported as 50% inhibitory concentration of cell growth (IC₅₀) mean values $\pm$ standard error of the mean (SEM) values of three separate experiments.

Cells	10l	Gemcitabine	Defactinib
PDAC-3	2.9 $\pm$ 0.4	0.03 $\pm$ 0.01	0.44 $\pm$ 0.06
Hs27	23.6 $\pm$ 2.5	0.17 $\pm$ 0.03	1.17 $\pm$ 0.27
SI	8.1	5.6	2.6

Abbreviations. SI, Selectivity index (IC₅₀ non-tumor cell line/IC₅₀ tumor cell line)

Inhibition of cell migration

Since the high metastatic potential of PDAC is considered one of the main causes of the poor outcome and the high aggressiveness of this disease, the ability of the compounds **10k** and **10l** in inhibiting cell migration of PDAC cells was investigated by scratch wound-healing assays. These experiments were performed on all preclinical models that were previously studied, including BxPC-3, PATU-T, SUIT-2, CAPAN-1, PANC-1, PANC-1 GR, and PDAC-3 cells. Briefly, 3 x 10⁴ to 5 x 10⁴ cells/well were seeded into 96-well flat-bottom plates in a volume of 100 μ L and incubated for 24 h to create a monolayer. The scratches were created in the center of the wells by scraping with a specific tool equipped with needles. The cells were then treated with the compounds at 5 times the IC₅₀ concentrations. The selected concentrations were determined through preliminary experiments, which showed no necrotic effects after 24 hours of exposure. The wound closure was monitored by phase-contrast microscopy and the pictures were captured immediately after scratch (T = 0), and at 8-, 20- and 24-hours post-treatment.

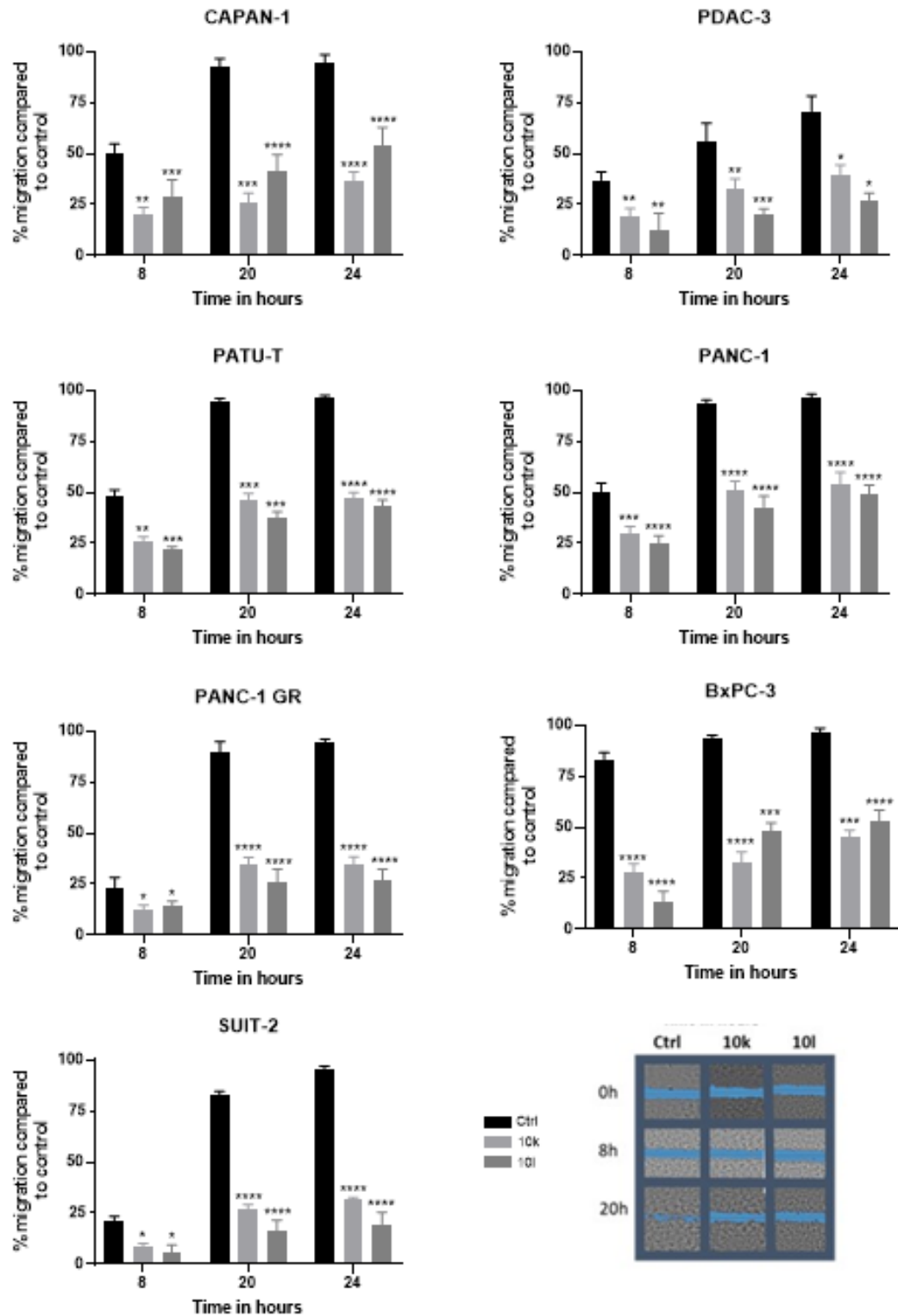


Figure 2. Percentage of migration monitored over time (0, 4, 8, 20 and 24 h) of PANC-1, BxPC-3, PATU-T, CAPAN-1, PDAC-3, PANC-1 GR and SUI-2 and cells untreated (control, Ctrl) and treated with the compounds 10k and 10l. The P values were calculated with Student's t-

test comparing untreated cells with cells treated at concentrations of 5 times IC_{50} . **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns= not significant.

As depicted in Figure 2, compounds **10k** and **10l** exhibited potent inhibition of cell migration, demonstrating consistent activity across all investigated PDAC cells. After 8 hours of exposure, the treated cells already displayed a wider scratch area compared to the control group. Remarkably, after 24 hours, a significant two-fold reduction in migration was observed in all PDAC cell lines treated with compound **10l**. Statistical analyses confirmed the significance of these differences when compared to their respective control groups (i.e., untreated cells) in all cell lines.

Specifically, when compared to the untreated cells set at 100%, the migration percentages for cells treated with compound **10k** and **10l** were 31% and 19%, respectively, in SUIT-2 cells, 34% and 27%, respectively, in the resistant clone PANC-1 GR, and 39% and 27%, respectively, in PDAC-3 primary cells.

The observed effects on cell migration are consistent with our prior studies on a series of imidazo [2,1-*b*][1,3,4]thiadiazole derivatives, which demonstrated their ability of modulate key regulators of epithelial-to-mesenchymal transition (EMT), including E-cadherin and vimentin, and inhibition of metalloproteinase-2/-9 [12].

Profiling of inhibition of kinase activity

In order to understand the mechanism of action underlying the above-mentioned promising antiproliferative and antimigratory effects against PDAC cells, we conducted a comprehensive analysis using the Pamgene tyrosine kinase peptide substrate array (PamChip).

This high-throughput assay involves four identical arrays, each comprising 144 peptide sequences immobilized on a porous ceramic membrane. These sequences contain one or more phosphorylation sites, which are derived from literature or computational predictions. The

amount of phosphorylated protein by tyrosine kinases from our samples was detected by using specific fluorescently labelled anti-phospho antibodies.

SUIT-2 and PDAC-3 cells, representing the most responsive and resistant cell models, were treated with compound **10I** at a concentration of 5 times the IC₅₀ for a duration of 2 hours. Subsequently, both treated and untreated cell lysates were passed through the porous array in the presence of ATP to enable the phosphorylation of peptides by the protein kinases. The assay mixture in both arrays included a fluorescein isothiocyanate (FITC)-labeled antibody, allowing for the quantification of peptide phosphorylation during the incubation period. To capture the real-time reaction kinetics, images measuring the fluorescent signal intensity were taken using a charge-coupled device camera. These kinetic images were captured at 6-second intervals throughout the entire duration of the program.

In SUIT-2 cells, compound **10I** demonstrated significant inhibition of the phosphorylation of 49 protein kinase peptide substrates, with inhibition percentages ranging from 27% to 66% compared to the untreated control. Similarly, in primary PDAC-3 cells, compound **10I** exhibited inhibitory effects on the phosphorylation of 53 different peptide substrates, with inhibition percentages ranging from 26% to 71% compared to the control. Figure 3 illustrates the kinases that showed inhibition percentages exceeding 50% in both cell lines. Remarkably, compound **10I** demonstrated the most remarkable activity against FAK1 and FAK2 in both SUIT-2 and PDAC-3 cell lines, with inhibition percentages of 66% and 71%, respectively.

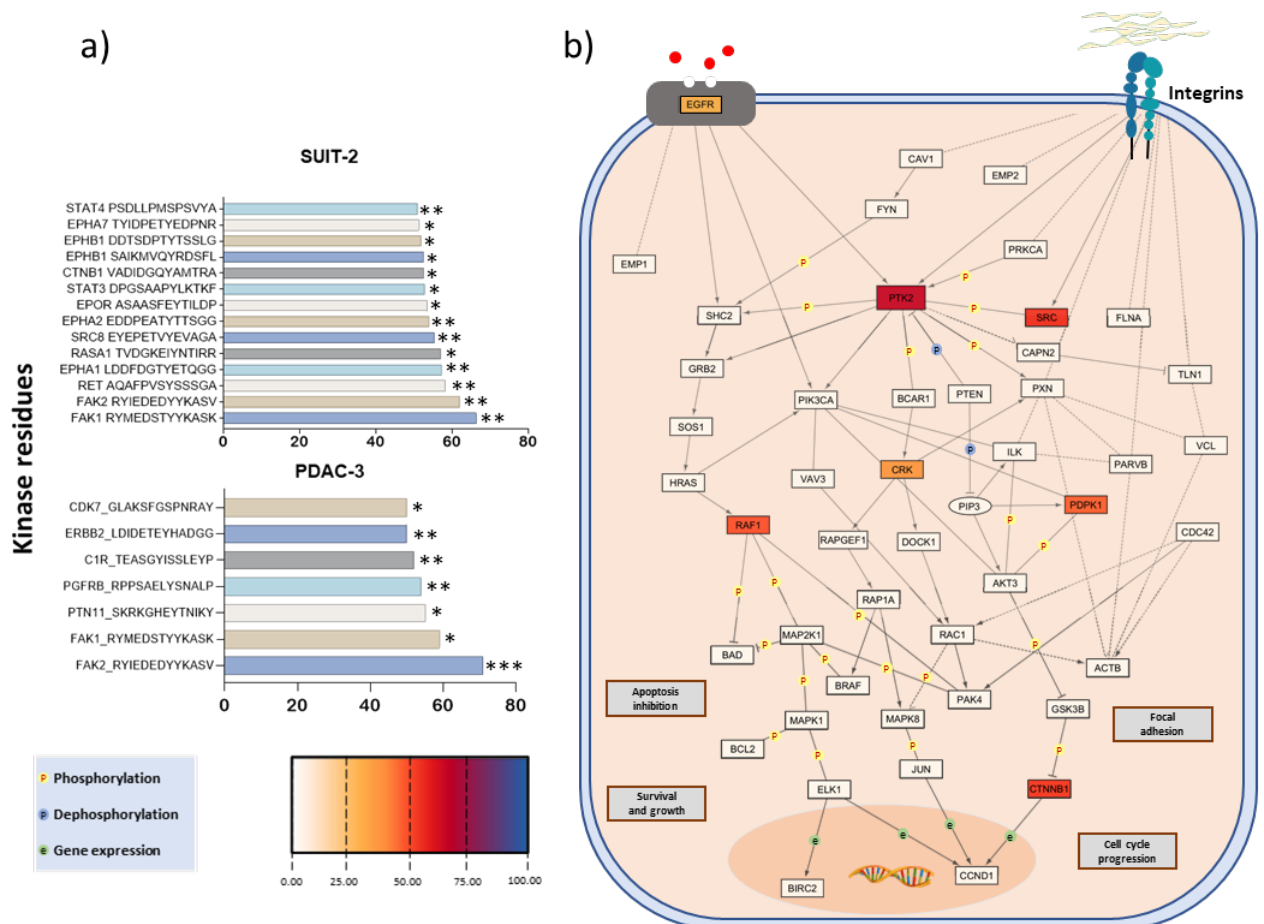


Figure 3. a) Barplot of kinase inhibition in untreated cells compared to cells treated with 5 times IC_{50} of **101**. P values were calculated with Student's t-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. b) FAK interaction network obtained with Cytoscape/KEGGscape; the color legend indicates the significance level of each inhibited protein by **101**.

It is worth mentioning that FAK exhibits known interactions with several kinases inhibited by the compound, including RAF, RAS1, EPHA1, EPHA2, SRC, KDR, and CTNNB1, as depicted in Figure 5. For instance, FAK activation can trigger RAS1 activation, facilitating downstream signaling via the MAPK pathway and influencing cellular processes such as cell growth and survival [43]. Moreover, FAK can form complexes with RAF, particularly in integrin-mediated signaling, leading to the activation of the RAF-MEK-ERK cascade, which impacts cell proliferation, survival, and migration [44]. FAK has also demonstrated interactions with Eph receptor family members, such as EPHA1, EPHA2, EPHA7, and EPHB1, modulating cell

adhesion and migration and suggesting potential shared downstream signaling pathways [45]. Furthermore, FAK and SRC can establish a reciprocal activation complex through phosphorylation, which plays a critical role in various cellular processes, including cell migration, invasion, and proliferation [46]. In addition, FAK can regulate the phosphorylation and activation of STAT3, contributing to cell survival, proliferation, and migration by activating STAT3-dependent gene expression, as previously described in MIA-PaCa-2 PDAC cancer cells [47]. Lastly, FAK and JAK1 can interact and mutually regulate each other's activity, as assessed in PDAC preclinical models. In addition, FAK phosphorylation can activate JAK1, initiating downstream signaling events involved in immune response and cytokine signaling [48].

Hence, the obtained results indicate that the inhibition of FAK phosphorylation serves as a mechanism of action for the new imidazothiadiazole compounds. To validate this mechanism, a specific ELISA assay was employed, and the details of this assay are described in the following section.

Inhibition of FAK as assessed by ELISA

Considering the intriguing biological activity observed for the new compounds and taking into account the FAK inhibitory potential demonstrated in our previously synthesized series of imidazo [2,1-*b*][1,3,4]thiadiazole derivatives [12], we conducted an analysis using an Enzyme-Linked Immunosorbent Assay (ELISA) to investigate the FAK inhibitory activity of the most active compounds of our new series. Figure 4 illustrates the ability of compounds **10l** and **10k** to inhibit FAK phosphorylation at tyrosine residue 397 (FAK [pY397]), which is crucial for the kinase activity of this protein. Comparatively, both compounds exhibited stronger inhibitory activity than the well-known FAK inhibitor defactinib, used as a reference compound. Specifically, **10l** and **10k** reduced FAK phosphorylation to less than 30% compared to the levels observed in the untreated (control) SUIT-2 and PDAC-3 cells.

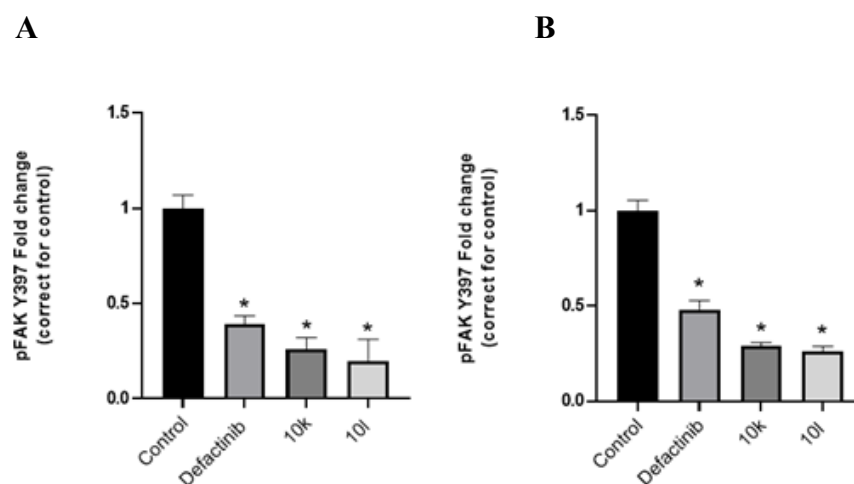


Figure 4. The impact of compounds **10k** and **10l** on the modulation of phosphorylated-FAK at tyrosine residue 397 (pFAK Y397) was assessed in SUIT-2 (**A**) and PDAC-3 (**B**) cells. After treating the cells with compounds **10k**, **10l** at 5 times the IC₅₀ concentration for 24 hours, the amount of pFAK was measured in cell lysates. Defactinib, at 5 times IC₅₀, was used as a reference compound. The statistical analysis was performed using Student's t-test, and *p<0.05 denotes significance.

These results are extremely important because FAK is a protein that plays a crucial role in promoting tumorigenesis and cancer progression in several tumors, including PDAC (Kanteti et al. 2016). Additionally, the translocation of FAK in the nucleus induces the inhibition of p53 activity and its downstream gene transcription [50]. As a result, it confers resistance to various anti-cancer treatments, such as chemotherapy and radiation therapy, resulting in treatment failure. Against this background, inhibiting FAK activity can potentially impede tumor growth and metastasis by disrupting these protumorigenic features.

Effects on cell-cycle modulation

Because of the key role of FAK in cell cycle regulation by promoting cell proliferation and progression through the G1 phase, we assessed the impact of the most cytotoxic compound

(compound **10l**) on cell cycle dynamics of SUIT-2 and PDAC-3 cells after 24-hour exposure. Cell cycle progression was analyzed by cytofluorimetry, using propidium iodide (3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6phenyl- diiodide, PI) staining solution.

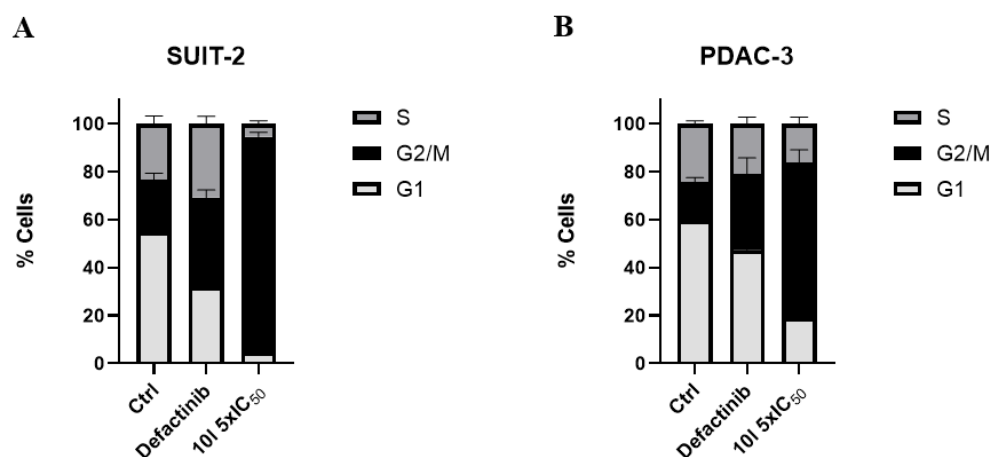


Figure 5. Effects of imidazothiadiazole compound **10l** on cell cycle modulation. SUIT-2 (A) and PDAC-3 (B) cells were treated for 24 hours. Stacked bar graphs show the mean percentage of cells at various stages of cell cycle, G1 (yellow), S (grey), and G2/M (red) phase, in untreated control and after treatment with the compound. Error bars report standard deviations of three separate experiments. Defactinib, at 5-times IC₅₀, was used as a reference compound.

In both cell lines, the compound **10l** demonstrated potent activity in arresting cell cycle in phase G2/M. Specifically, after 24 hours of treatment, more than 70 and 90% of total SUIT-2 cells and PDAC-3 cells were observed to be blocked in the G2/M phase, respectively (Figure 5). These findings align with the mechanism of action involving FAK inhibition, which is known to induce cell cycle arrest at the G2/M phase and a significant reduction in the S phase [13].

Effects on apoptosis induction

FAK can influence apoptosis signaling pathways, with its overexpression often associated with enhanced cell survival and resistance to apoptosis. Thus, induction of apoptotic response after

exposure to compound **10I** was evaluated by the analysis of the stimulation of caspase-3 in both SUIT-2 and PDAC-3 cells (Figure 6).

The immunoassay conducted in the present study revealed significantly higher levels of active caspase-3 in SUIT-2 cells that were treated with **10I** and defactinib, compared to the untreated cells. Similar results were observed in PDAC-3 cells, although the levels of active caspase-3 were slightly lower (0.34 ng/mL in untreated cells, 0.75 ng/mL in defactinib-treated cells, and 0.87 ng/mL in **10I**-treated cells). In both PDAC models, the induction of apoptosis was directly proportional to the increasing concentrations of **10I**. Of note, we observed that compound **10I** at 2 μ M induces high levels of active Caspase-3 protein expression, achieving a comparable result to defactinib, which was used at 5 times its IC₅₀.

The maximum effect was observed in SUIT-2 cells treated with **10I**, resulting in a concentration of active caspase-3 of 1.75 ng/mL.

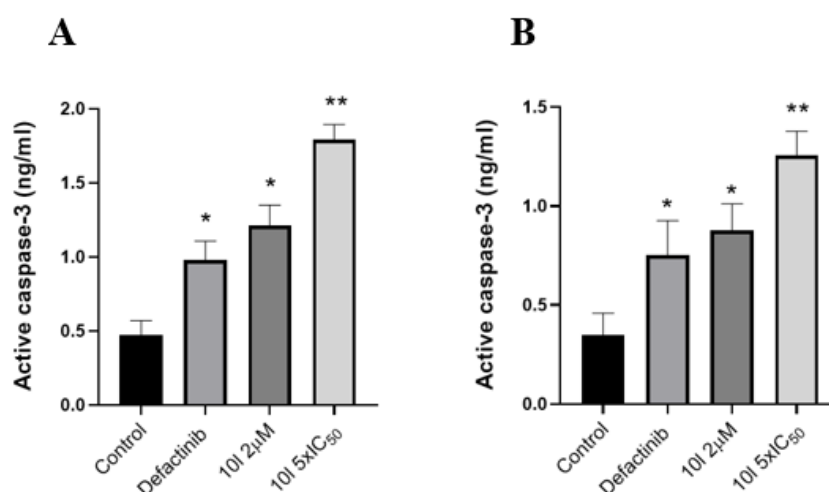


Figure 6. Induction of apoptosis as assessed by evaluation of the levels of active caspase-3 in SUIT-2 cells (A) and PDAC-3 cells (B) treated with **10I**, or defactinib, used at 5 times IC₅₀, for 24 hours, compared to control/untreated cells. Measurements were performed in triplicate, and data are presented as means $\pm$ SD. *p < 0.01 versus control; **p < 0.001 versus control.

Effects on spheroids

FAK plays a role in promoting the attachment of cancer cells to the extracellular matrix, thereby facilitating their invasive potential and aiding in the progression of invasive diseases such as PDAC. Additionally, FAK has been implicated in the formation and maintenance of spheroid structures in PDAC cells, which are associated with increased resistance to therapy and tumor progression [51]. Thus, we conducted further evaluations on three dimensional (3D) spheroids of PDAC-3 cells. These primary cultures successfully formed spheroids that closely resemble the *in vivo* aggregation of tumor cells, consistent with findings from our previous studies [12]. After three days of growth, spheroids were treated with compound **10I** at IC₅₀ concentration and concentration 5 times the IC₅₀. Additionally, spheroids treated with defactinib and gemcitabine were used as positive control at concentration 5 times IC₅₀. As summarized in Figure 7A, both treatment concentrations of compound **10I** inhibit tumor cell proliferation and reduction of the spheroid volume after 11 days. This decrease is plotted as the fold-change between treated and control spheroids and was statistically significant (p-value < 0.0001). A representative picture was captured on Day 1, and subsequent treatments were repeated every four days (Day 5 and Day 8) with corresponding images taken immediately before each treatment (Figure 7B). Moreover, to provides a comprehensive overview of the cell responses to therapy, viability and cell death were analysed by live fluorescence confocal imaging in our 3D models (Figure 7C). Indeed, employing confocal fluorescence microscopy with a correct staining strategy enabled us to visual examination of the inner core of spheroids [52]. After proving antiproliferative activity on 2D cell culture, compound **10I** confirmed its promising activity on 3D PDAC models which better represent the complex tumor structure.

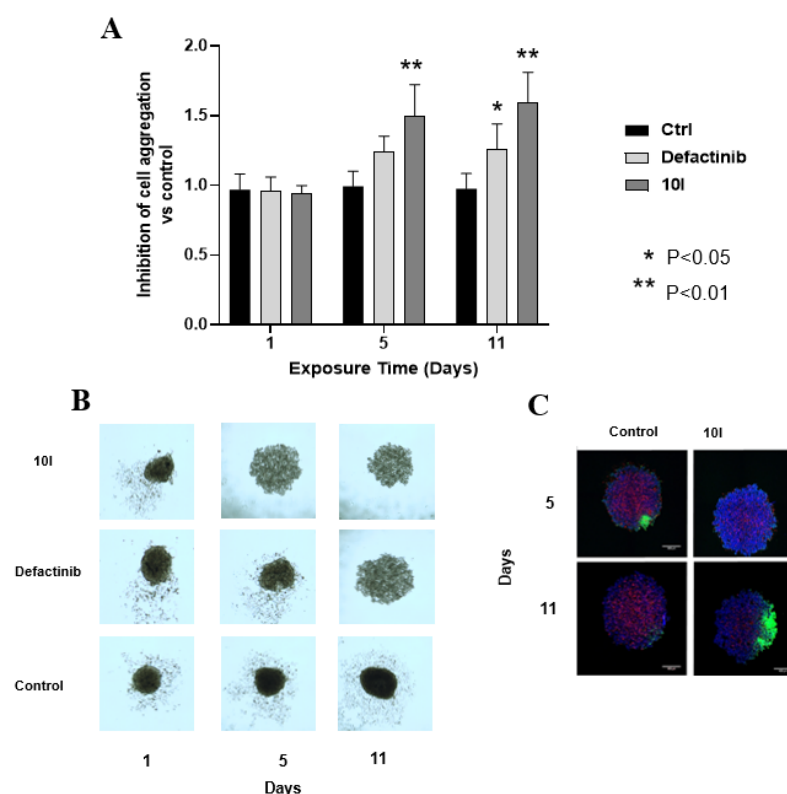


Figure 7. Size reduction of PDAC-3 spheroids treated with compounds **10I** at 5 times the IC_{50} (i.e. $15\mu M$); A) Inhibition of cell aggregation, corrected for control, of the spheroids, at Day 1, Day 5, Day 11; B) Representative pictures of PDAC-3 spheroids exposed to **10I** and gemcitabine, taken at day 1 of treatment, Day 5, and Day 11 with an automated phase-contrast microscope. C) Representative pictures of PDAC-3 spheroids exposed to **10I**, compared to control, taken at day 5 of treatment, and Day 11 with a live fluorescence confocal microscope. p-values were determined by Two-way ANOVA followed by Tukey's multiple comparisons test, **** = $p < 0.0001$, ** = $p < 0.01$. The values were obtained considering the mean values of the areas of at least 8 different spheroids, in two different separated experiments.

Effects on *in vivo* models

To confirm our findings from *in vitro* PDAC models, we further analysed our most promising compound **10I** on *in vivo* model. Therefore, compound **10I** was tested on a subcutaneous

xenograft model, obtained via subcutaneous injection of 1×10^6 primary PDAC-3 cells (Figure 8a).

After 5 days from tumor implantation, when tumor volume threshold (100 mm^3) was reached, cohorts of mice were randomised into different treatment groups (N=5 each). Three experimental groups were treated with 1) **10I** (25 mg/kg) three times for two week or 2) gemcitabine (100 mg/kg) or 3) two times per week for two weeks.

Remarkably, compound **10I** demonstrated a significant effect ($p < 0.05$) on mice already after two weeks from the initial treatment, leading to a reduction in tumor volume compared to the untreated cohort (Figure 8b). After three weeks, all experimental groups exhibited a substantial reduction in tumor volume (Figure 8c,d). Animals treated with **10I** displayed a significant 17.7% reduction in tumor mass compared to the untreated/control group ($354.8 \text{ vs. } 431.4 \text{ mm}^3$, $p < 0.05$). The gemcitabine group showed a similar effect with a 13.2% reduction in tumor mass compared to untreated/control animals ($374.4 \text{ vs. } 431.4 \text{ mm}^3$, $p < 0.05$).

Of note, **10I** was well tolerated; no toxic deaths or signs of toxicity were observed, and the body weight of animals given **10I** was similar to that of the untreated group, as reported in Figure 9. To summarize, our *in vitro* evidence has been confirmed in an *in vivo* model where the administration of compound **10I** demonstrated comparable inhibition of tumor growth to that observed with gemcitabine, which is commonly used in the clinical treatment of PDAC.

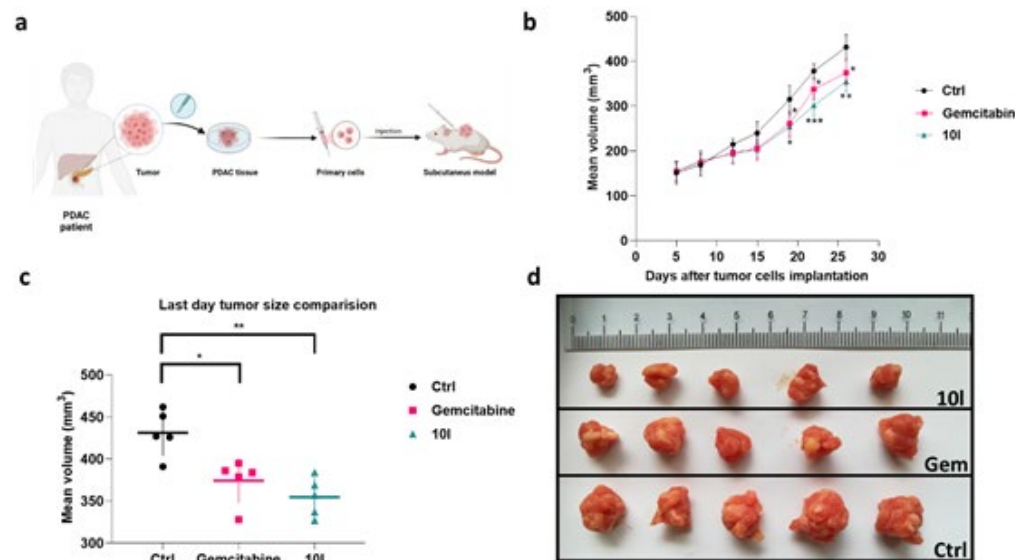


Figure 8. *In vivo* tumor growth results. a) Schematic illustration of tumor inoculation to the obtain subcutaneous model. b) Tumor growth curves show a significant difference between control and treated groups (gemcitabine and **10I**) from day 19th (mean $\pm$ SD). c) Significant tumors volume reduction after 2 weeks of treatments d) representative tumors' images from mice subcutaneously injected with PDAC-3 cells.

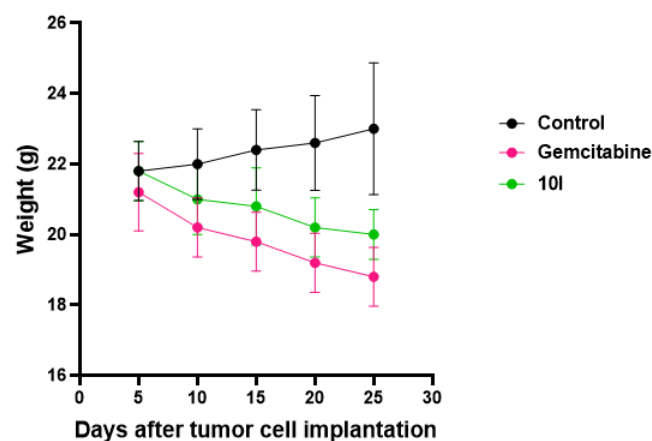


Figure 9. Body weight of mice monitored during treatment.

Conclusions

FAK is an important emerging target in PDAC because it enhances cancer cell survival by inhibiting apoptosis and prompting cell proliferation signals, while promoting cell migration, immune evasion, and tissue remodeling. In this study, we reported the synthesis and the ability to inhibit cell proliferation and migration in PDAC cells of a novel series of imidazo[2,1-*b*][1,3,4]thiadiazole compounds. Four of the new derivatives, **10f**, **10k**, **10l**, and **10q**, exhibited potent antiproliferative activity against PDAC cell lines showing IC₅₀ values ranging from 1.04 to 16.62 μM. High-throughput kinase arrays, carried out for the most promising compound **10l**, unveiled a noteworthy suppression of FAK phosphorylation in SUIT-2 and PDAC-3 cells, suggesting it as the main mechanism of action. This hypothesis was subsequently confirmed by a specific ELISA assay. The *in vitro* results related to the effects of these compounds on cell migration, as well as their ability to block the cell cycle at the G2/M phase, are congruent with a mechanism involving FAK inhibition. Noteworthy, the significant antiproliferative activity of compound **10l** was corroborated in 3D spheroids of PDAC-3 cells and in a xenograft *in vivo* model, in which a significant reduction of tumor volume was observed after two weeks of treatment. Additionally, this compound did not cause any signs of toxicity and no reduction in mice body weight. This aligns with its low selectivity index value *in vitro*, prompting forthcoming *in vivo* studies to validate this safety profile, which is crucial for the strategic planning of future clinical trials.

Altogether, the obtained results revealed the promising anticancer properties of a new series of FAK inhibitors with imidazothiadiazole structure laying the foundation for the development of new therapeutic approaches in the treatment of PDAC disease.

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