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New Imidazo[2,1-*b*][1,3,4]Thiadiazole Derivatives Inhibit FAK Phosphorylation and Potentiate the Antiproliferative Effects of Gemcitabine Through Modulation of the Human Equilibrative Nucleoside Transporter-1 in Peritoneal Mesothelioma

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Abstract. *Background/Aim: A new class of imidazo[2,1-b][1,3,4]thiadiazole compounds have recently been evaluated as inhibitors of phosphorylation of focal adhesion kinase (FAK) in pancreatic cancer. FAK is overexpressed in mesothelioma and has recently emerged as an interesting target for the treatment of this disease. Materials and Methods: Ten imidazo[2,1-b][1,3,4]thiadiazole compounds characterized by indole bicycle and a thiophene ring, were evaluated for their cytotoxic activity in two primary cell cultures of peritoneal mesothelioma, MesoII and STO cells. Results: Compounds 1a and 1b showed promising antitumor activity with IC_{50} values in the range of 0.59 to 2.81 μM in both cell lines growing as monolayers or as spheroids. Their antiproliferative and antimigratory activity was associated with inhibition of phospho-FAK, as detected by a specific ELISA assay in STO cells. Interestingly, these compounds potentiated the antiproliferative activity of gemcitabine, and these results might be explained by the increase in the mRNA expression of the key gemcitabine transporter human equilibrative nucleoside transporter-1 (hENT-1). Conclusion: These promising results support further studies on new imidazo[2,1-b][1,3,4]thiadiazole compounds as well as on the role of both FAK and hENT-1 modulation in order to develop new drug combinations for peritoneal mesothelioma.*

Malignant mesothelioma refers to a rare but aggressive tumor derived from mesothelial cells. They form a monolayer that covers the body's serous cavities and whose main function is to provide a protective membrane for the lung (pleural), the intestine (peritoneum), the heart (pericardium) and the *tunica vaginalis*. The thorax and abdominal cavity are the primary sites for the development of cancer, with a rate of 80-90% and 10-15%, respectively (1).

Diffuse malignant peritoneal mesothelioma (DMPM) is difficult to diagnose, both clinically and histologically, and is characterized by a dismal prognosis. Most patients benefit from a multimodal treatment that includes the combination of surgery and chemotherapy. In particular, the standard of care consists in cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) (2-4). However, many patients still suffer from disease recurrence, and new therapeutic options to implement in the current surgical and HIPEC procedures are warranted.

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Though all mesotheliomas originate in serous membranes, the efficacy of conventional chemotherapy varies per location (5). Like many other solid tumors, mesotheliomas develop as a result of different molecular aberrations. To understand these events, research is directed towards: first, to identify new molecules with antitumor activity and second, to assess the activity of compounds already known for their mechanism of action and used for the treatment of various diseases alone or in combination with other drugs. Recently, we reported the antitumor activity of a new class of imidazo[2,1-*b*][1,3,4]thiadiazole compounds on pancreatic ductal adenocarcinoma, highlighting their ability to reduce FAK phosphorylation on tyrosine residue (Y-397) (6). Moreover, we previously observed good results with combinations of new drugs with the antimetabolite gemcitabine in preclinical models of DMPM (7). Encouraged by these findings as well as by studies supporting 'drug repositioning' in drug discovery (8), we studied the antitumor activity of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds (Figure 1) on two primary cultures of DMPM cells, growing as monolayers or spheroids and evaluated the potential mechanisms underlying the pharmacological interaction with gemcitabine.

Materials and Methods

Drugs and chemicals. The imidazo[2,1-*b*][1,3,4]thiadiazole compounds were synthesized, and dissolved in DMSO, as described previously (6). Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml) were from Gibco (Gaithersburg, MD, USA). All other chemicals were from Sigma (Zwijndrecht, the Netherlands). Gemcitabine was a gift from Eli-Lilly.

Cell cultures. Human DMPM primary cultures (STO and MesoII) were derived from patients who underwent surgery (9). The cells were maintained in F-12 for less than 20 passages, supplemented with 10% heat-inactivated-FBS, 1% penicillin/ streptomycin, and routinely tested for mycoplasma.

Inhibition of cell growth. The cell growth inhibitory effect of imidazo[2,1-*b*][1,3,4]thiadiazoles was evaluated by the Sulforhodamine-B (SRB) assay, as described previously (10). Cells were seeded into 96-well flat-bottom plates (5×10^3 cells in 100 μl/well). After 24 h, cells were treated with eight different concentrations of the compounds (from 0.3 to 40 μM) for 72 h. Thereafter, cells were fixed with 25 μl of 50% cold trichloroacetic acid and kept for at least 60 minutes at 4˚C. The plates were washed gently with deionized water, dried at room temperature (RT) overnight and stained with 50 μl of 0.4% SRB solution in 1% acetic acid for 15 minutes at RT. The excess of SRB was removed on dry tissues and the plates were washed with a 1% acetic acid solution and dried at RT overnight. Finally, the SRB was dissolved in 150 μl of tris(hydroxymethyl)aminomethane solution pH 8.8 (TRIS base), and the optical density (OD) was measured at wavelenghts of 490 nm and 540 nm. The cell growth inhibition was calculated as the percentage of the OD drug-treated cells *versus* the OD of vehicle-treated cells ("negative control") (corrected for OD before drug addiction, "day-0").

Half maximal inhibitory concentration (IC_{50}) values were calculated with GraphPad Prism 7 (GraphPad, San Diego, CA, USA). In the combination studies with gemcitabine, we used the most promising compounds at their IC_{50} concentrations and gemcitabine at its IC_{25} concentration.

Wound healing assay. The *in vitro* wound-healing assay was performed as previously described (11). MesoII and STO cells were seeded in 96-well flat-bottom plates at the density of 5×10^4 cells/well and the confluent layer was scratched with a pin-tool. Thereafter, the medium was replaced in the control wells with only medium or with medium containing the compounds of interest. Wound closure was monitored by phase-contrast microscopy using the Leica-DMI300B microscope and pictures were captured immediately after scratch (T=0), and after 4, 8 and 20 h. Results were analyzed with the Scratch-Assay 6.2 software (Digital Cell Imaging Labs, Keerbergen, Belgium).

Spheroids assay. MesoII and STO spheroids were created as reported previously (12). Cells were seeded at a density of 7×10^4 cells/well for MesoII and 5×10^4 cells/well for STO, in cell repellent U-bottom plates (Greiner, Kremsmünster, Austria). After three days the spheroids were treated with **1a** and **1b** at IC_{50} and 5× IC_{50} concentrations. Pictures were taken every two days after replacing the medium of the control wells or adding medium with compounds to the experimental wells, and the experiment lasted seventeen days. The reduction in size of spheroids was monitored by phase-contrast microscopy and pictures were analysed with ImageJ (NIH, Bethesda, MD, USA), as described previously (13).

Enzyme-linked immunosorbent assay (ELISA) to evaluate FAK. To investigate whether our imidazothiadiazole compounds were able to reduce FAK phosphorylation at tyrosine residue 397 (FAK [pY397]) in the DMPM, we performed a quantitative analysis using a specific ELISA, as described (6). This assay was carried out on lysates of cells treated with compounds **1a** and **1b** at 5× IC_{50}s concentrations for 2 h.

Quantitative-PCR. Total RNA was extracted and reverse transcribed from cells treated with **1a** and **1b** at 5× IC_{50} concentrations for 24 h. The resulting cDNA was amplified by quantitative-PCR with the ABIPRISM-7500 instrument (Applied Biosystems, Foster City, CA, USA) using specific hENT-1 and primers, as previously described (12).

Statistics. All experiments were performed in triplicate and repeated at least three times. Data were expressed as mean values±SEM and were analysed by Student's *t*-test or ANOVA followed by the Tukey's multiple comparison, setting the level of significance at $p<0.05$.

Results

Antiproliferative activity. The effect of ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds was evaluated on MesoII and STO cells, by the SRB assay. Only four out of the ten compounds (**1a, b, g** and **h**), showed more than 50% inhibition of growth at 10 μM and were explored in more

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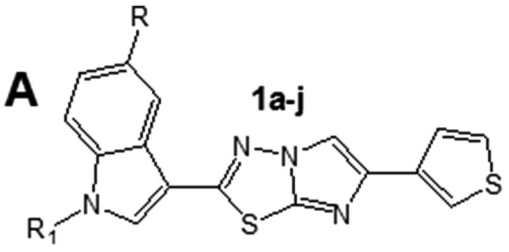

Compounds	R	R^1	IC_{50} values [μM] reported as Means ± SEM of 3 separate experiments: MesoII	STO
1a	H	H	2.81 ± 0.39	0.81 ± 0.35
1b	H	CH_3	2.58 ± 0.12	0.59 ± 0.11
1c	Br	CH_3	>10	>10
1d	Cl	H	>10	>10
1e	Cl	CH_3	>10	>10
1f	F	CH_3	>10	>10
1g	Br	H	5.9 ± 1.53	3.65 ± 1.03
1h	F	H	3.4 ± 0.29	1.14 ± 0.11
1i	OCH_3	H	>10	>10
1j	OCH_3	CH_3	>10	>10

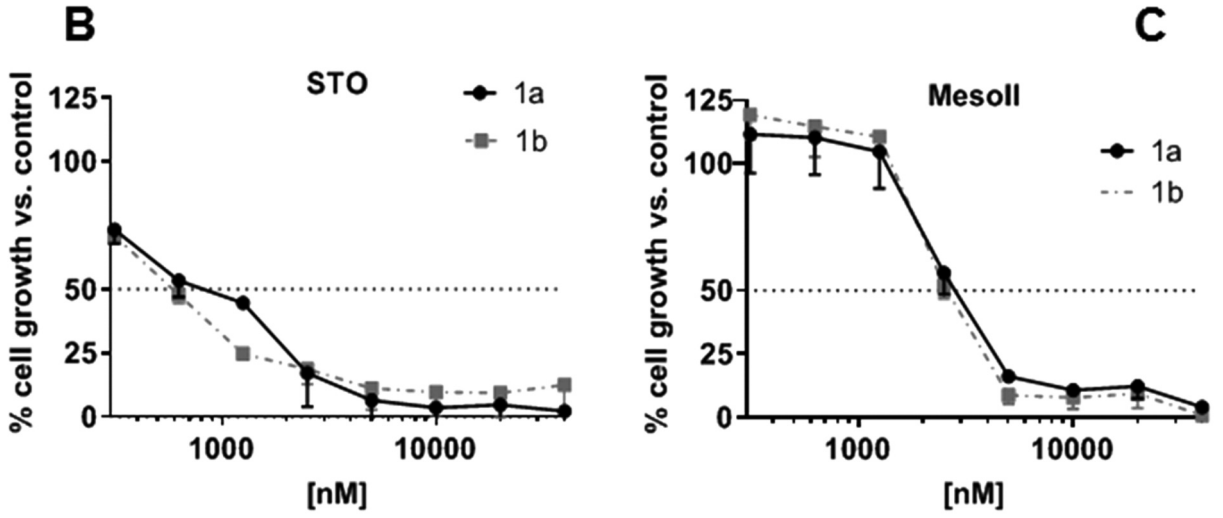

Figure 1. *Antiproliferative activity of compounds 1a-j against diffuse malignant peritoneal mesothelioma cells, MesoII and STO. (A) Chemical backbone structure of compounds 1a-j with the list of the chemical structure of the R and R1 substituents for each compound and the IC_{50} values in μM of each compound against the DMPM cell lines. (B, C) Representative growth curves of STO (B) and MesoII (C) cells after 72 h of exposure to 1a or 1b. Points, mean values obtained from one representative experiment; bars, SEM.*

detail. Figure 1A summarizes their IC_{50} values, ranging from 0.59 to 5.9 μM, with the lowest IC_{50} in STO cells (Figure 1B), while in MesoII cells these compounds were less effective (Figure 1C). The results obtained with the compounds **1a** and **1b**, prompted us to investigate their cytotoxic activity on three-dimensional (3D) models.

Volume reduction of MesoII- and STO-derived tumor spheres. Earlier studies reported that the drug activity found in the two-dimensional monolayers is different from that in 3D cell cultures (14), as the 3D model offers a more realistic representation of the tumor microenvironment, including the physical and mechanical properties, oxygen,

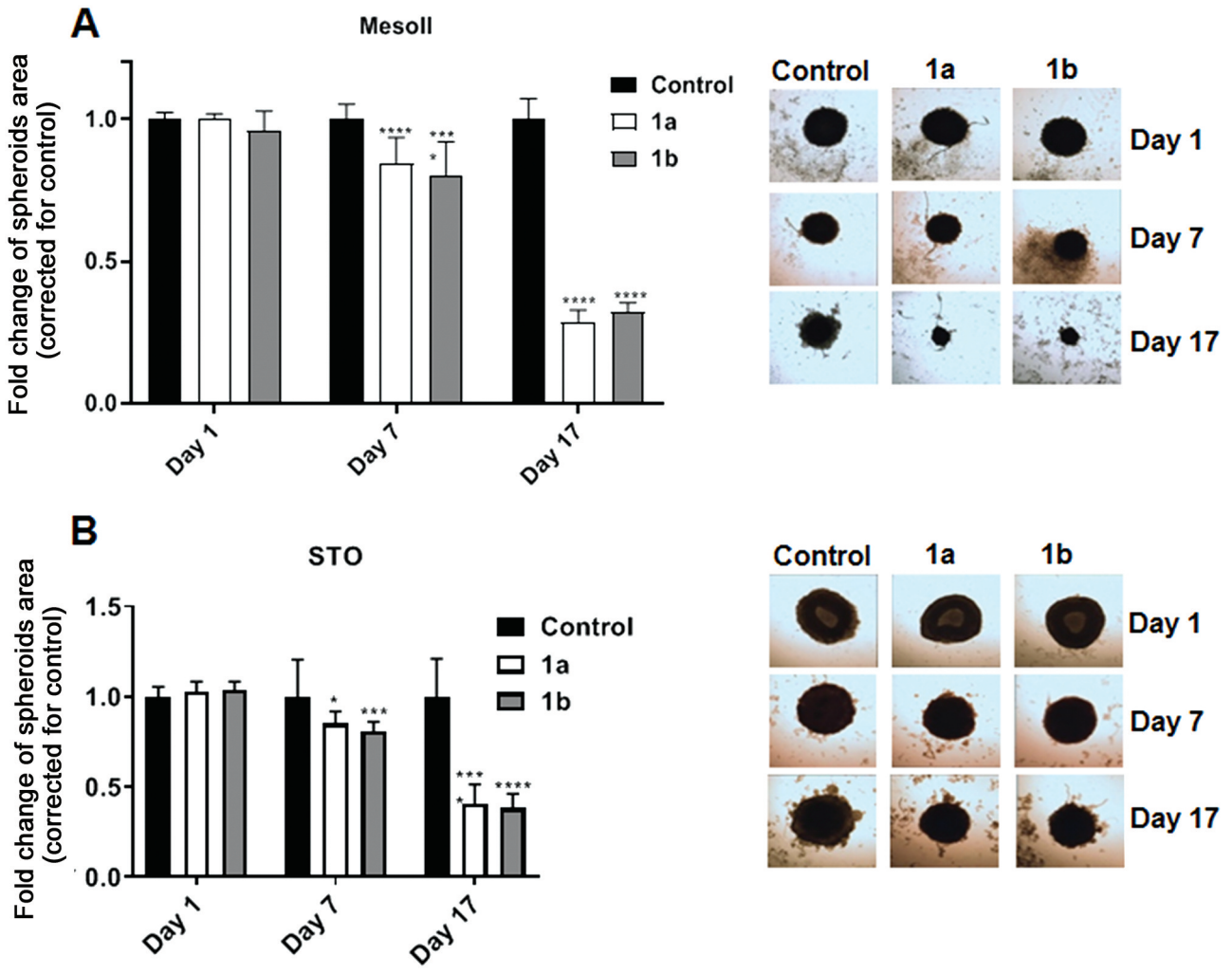

Figure 2. *Size reduction of (A) MesoII and (B) STO spheroids treated with compounds 1a or 1b at 5× IC_{50} concentrations. (Left plot) Fold-change compared to control, on day 1, 7 and 17. (Right pictures) representative images of spheroids, taken with an automated phase-contrast microscope on day 1 of treatment (Original magnification 5×), and after 7 and 17 days. All p-values were determined by Two-way ANOVA followed by Tukey's multiple comparisons test, *p<0.05, ***p<0.001, ****p<0.0001. These values were obtained by taking the mean value of at least ten different spheroids into account.*

pH and nutrients gradients, as well as drug transport (15). Therefore, we evaluated the ability of compounds **1a** and **1b** to affect the size of spheroids of MesoII and STO cells. As shown in Figure 2, the spheroids decreased significantly in size over time in both cell lines. Notably, after 17 days of treatment, we found about 2-fold reduction, compared to the untreated spheroids.

*Compounds **1a** and **1b** inhibited cell migration and phospho-FAK in STO cells*. Secondary lesions that originate from DMPM primary site are very uncommon. However, localized and/or regional metastasis with the involvement of lymph nodes have been observed (16-18). Furthermore, the spread of tumor cells to form new metastatic *loci* on distant organs has been reported; particularly, the pancreas and the kidneys are the main organs involved, whereas the lung, the heart and the brain are less commonly affected (19, 20). The interesting antiproliferative activity of compounds **1a** and **1b**, prompted us to investigate their anti-migratory activity by wound-healing assay in the STO cells, which were selected because of their higher sensitivity and a replication time well-above 24 h. In these cells, we observed a reduction of migration rates by 25.8% and 20%, after 20 h from the treatment, compared to control (set at 100%) (Figure 3A). Statistical analyses revealed that the reduction of migration in STO cells

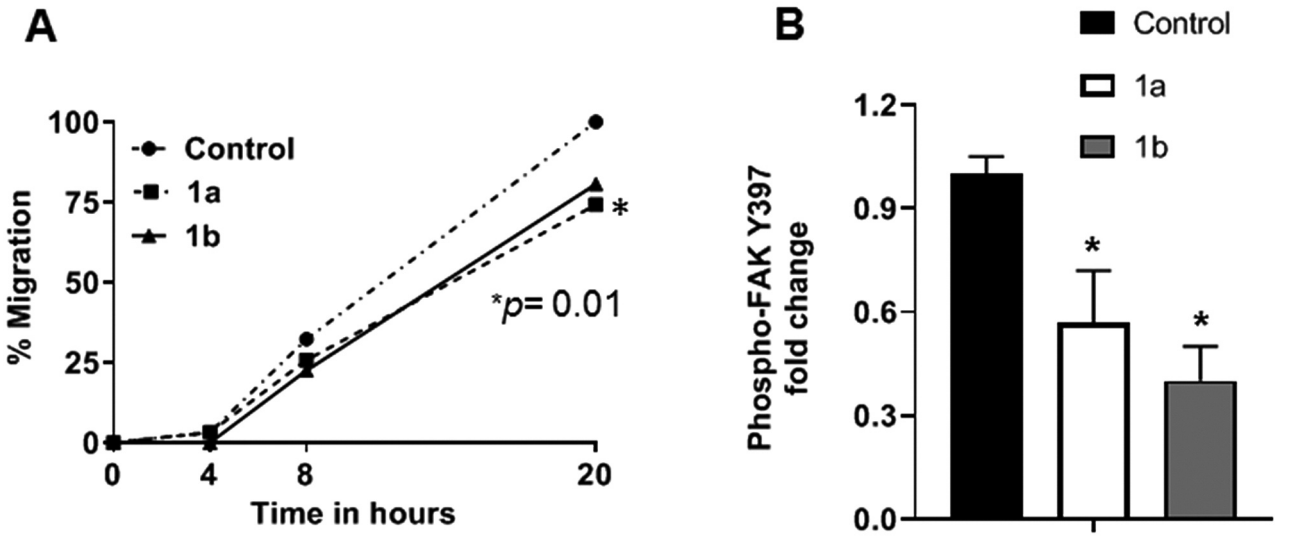

Figure 3. *(A) Modulation of the migration rate of STO cells treated for the indicated times with the compounds 1a and 1b at concentrations of 5× IC_{50}. Mean values were obtained from the means of at least six different scratch areas. SEM were always below 10%. (B) Inhibition of FAK phosphorylation at tyrosine residue 397 by compounds 1a and 1b. *p<0.05.*

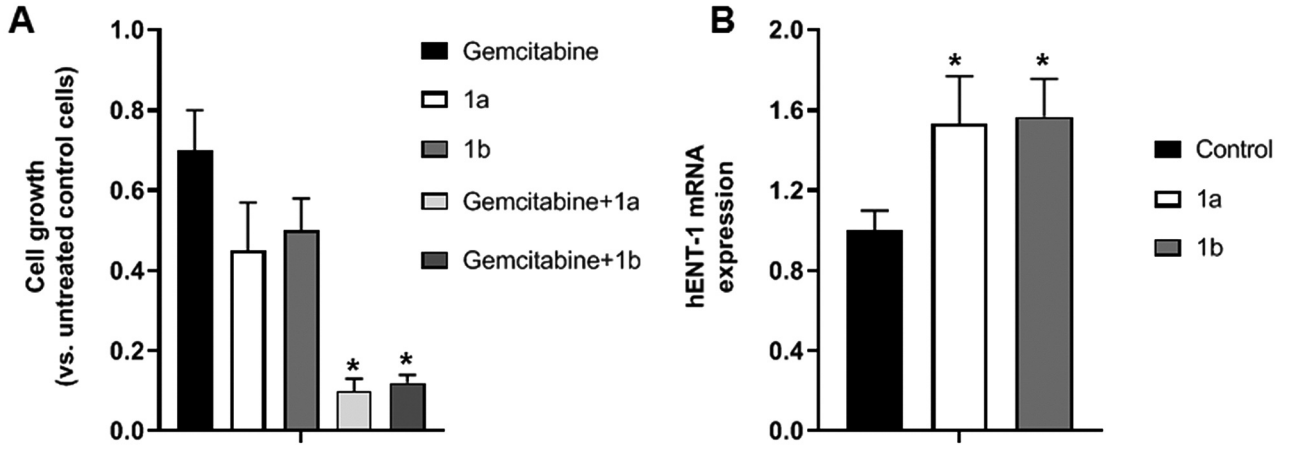

Figure 4. *The combination of compounds 1a and 1b with gemcitabine led to a significant reduction in cell growth and increased hENT-1 expression. (A) Effect of the combination of gemcitabine, at its IC_{25} concentration, with the compounds 1a or 1b, at their IC_{50} concentrations, on the growth of STO cells. (B) Modulation of hENT-1 mRNA levels in STO cells. Expression was determined with quantitative-PCR by normalization with the GAPDH housekeeping gene, and the values are expressed in arbitrary units, as described previously (12); Columns, mean values obtained from triplicate experiments. Bars, SEM; *p<0.05.*

treated with compound **1a** was significant, compared to the untreated control cells. Parallel ELISA studies revealed that both compounds **1a** and **1b** significantly reduced the phosphorylation of FAK (Figure 3B).

*Compounds **1a** and **1b** increased hENT-1 expression and gemcitabine cytotoxicity.* Since previous data showed synergistic effects of gemcitabine with the new anticancer agents in mesothelioma cells (7, 21) we tested whether the addition of compounds **1a** and **1b** at their IC_{50} concentrations would increase the antiproliferative effects of gemcitabine. These experiments were performed in STO cells because of the strongest antiproliferative and antimigratory effects. Interestingly, the combination of both compounds **1a** and **1b** at their IC_{50} concentration with gemcitabine at its IC_{25} concentration led to a significant reduction in cell growth, which reached values around 10% compared to untreated cells (Figure 4A).

Finally, to investigate the molecular mechanisms underlying the interaction of gemcitabine with compounds **1a** and **1b**, we measured the modulation of the gene expression of hENT-1, which is a key determinant of gemcitabine transport. Both compounds increased hENT-1 expression significantly (Figure 4B), suggesting its potential role in the increased activity of gemcitabine in combination with compounds **1a** and **1b**.

Discussion

Multimodal treatment including the combination of surgery and chemotherapy represents the standard of care for the treatment of patients with peritoneal mesothelioma; indeed, unlike radiotherapy, palliative surgery combined with chemotherapy showed a longer survival rate of patients, as demonstrated in a retrospective study conducted on Finnish patients with DMPM between 1 January 2000 and 31 December 2012 (22).

Ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds, which inhibited FAK protein expression in the treatment of pancreatic cancer (6), were tested for their antiproliferative activity on two primary cell cultures of diffuse malignant peritoneal mesothelioma, namely MesoII and STO. Four compounds **1a, b, g** and **h** showed promising antitumor activity with IC_{50}s in the range from 0.59 to 5.9 μM. In particular, the compounds **1a** and **1b** showed the lowest IC_{50} in both cell lines. Similar results were observed in spheroids, inhibiting their area by approximately 2-fold compared to the controls. These are very interesting results since spheroids of mesothelioma cells are resistant to different treatments, including conventional chemotherapeutic drugs.

Moreover, the lowest IC_{50} values were also associated with the ability of compounds **1a** and **1b** to reduce cell migration of STO cells by 25.8% and 20%, respectively. These results gave more insight in the mechanism of action and led us to investigate the ability of these compounds to inhibit FAK phosphorylation, as reported previously (7). Remarkably, both compounds were able to reduce the phosphorylation of FAK, which is a potential target in mesothelioma (23).

Moreover, these compounds potentiated the activity of gemcitabine and we might hypothesize that this effect is due to the increased mRNA expression of hENT-1, which has been associated with gemcitabine activity in different cancer cell types (24). Of note, a previous study showed that inhibition of hENT-1-mediated transport may result from p42/44 MAPK activation in HUVEC cells after short periods of hypoxia (25). Therefore, we hypothesize that the inhibition of FAK might cause an inhibition of its downstream target MAPK and this might in turn lead to an increase in the expression of hENT-1.

In conclusion, our novel findings should prompt further studies on imidazo[2,1-*b*][1,3,4]thiadiazole compounds as well as on the role of the modulation of FAK and hENT-1 for the rational development of new drug combinations in DMPM.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

GLP, OR and CP performed chemical synthesis, experimental work and wrote the manuscript. SZ, SMC, DC, BP, and BEH, assisted with experimental work. AC, NZ, and GC provided essential material and helped to revise the manuscript. PD, GJP and EG were responsible for experimental design and helped to write the manuscript.

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Notes

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