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Biological Evaluation of the Antiproliferative and Anti-migratory Activity of a Series of 3-(6-Phenylimidazo[2,1-*b*][1,3,4]thiadiazol-2-yl)-1*H*-indole Derivatives Against Pancreatic Cancer Cells*

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Abstract. *Heterocyclic rings are recognized as key components of many natural, semi-synthetic and synthetic molecules with a broad spectrum of biological activities. Among these molecules, the indole and imidazo[2,1-b][1,3,4]thiadiazole systems have recently been described as useful scaffolds for the design of anticancer agents. Herein the antitumor activity of a series of 3-(6-phenylimidazo[2,1-b][1,3,4]thiadiazol-2-yl)-1H-indoles, designed as hybrid structures, was assessed. Seven out of 10 compounds (**1a-g**) were submitted to National Cancer Institute (NCI). Remarkably, compound **1g** showed antiproliferative activity against the full panel of sixty human cancer lines, with half-maximal inhibitory concentration of between 1.67 and 10.3 μM. Further studies showed antiproliferative activity of **1a-g** and of three additional compounds **1h**, **1i** and **1l**, with different substituents on the indole nucleus and phenyl ring, against three pancreatic cancer cell lines. In particular, derivatives **1g** and **1h** inhibited both proliferation and migration of SUIT-2 cells at concentrations lower than 10 μM. In conclusion, new indole derivatives are characterized by in vitro antitumor activity, supporting future mechanistic studies.*

The use of heterocyclic ring systems containing oxygen, nitrogen or sulphur heteroatom(s) is attracting much attention in the field of medicinal chemistry for the design and development of new therapeutic agents (1). The incorporation of heterocyclic rings makes it possible to modify some important pharmaceutical parameters, such as lipophilicity, polarity and aqueous solubility, in order to obtain lead compounds with ideal biological and physicochemical features. These characteristics are essential in predicting the selectivity and potency of a candidate drug (2). The indole nucleus has recently emerged as one of the most relevant heterocyclic rings for such an aim, endorsed by the unique ability to mimic peptide derivatives and reversibly bind proteins (3). Many indole derivatives endowed with significant biological activities including anti-inflammatory, analgesic (4), antiviral (5), anticancer (6-10) and antibacterial agents (11) were reported in the past decade. Additionally, the imidazo[2,1-*b*][1,3,4]thiadiazole ring system has been described as an important scaffold for the design and synthesis of compounds with different therapeutic properties, such as anti-tubercular (12), antibacterial (13), anticonvulsant and analgesic (14), antifungal (15) and anticancer (16, 17). On the basis of these findings, as well as on the concept of the "One-Compound-Multi-Target" (18, 19), we synthesized a series of hybrid structures, 3-(6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-2-yl)-1*H*-indoles, in order to obtain active analogs with different biological activities. Interestingly, these compounds were able to inhibit biofilm formation of the Gram-positive bacterial reference strains *Staphylococcus aureus* ATCC 25923,

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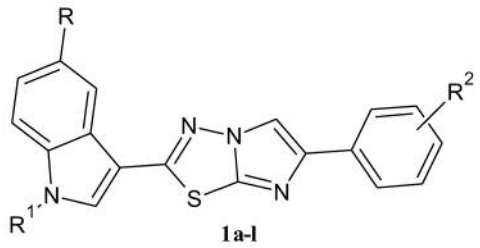
S. aureus ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228 at low micromolar concentration (20). Since previous studies reported that several compounds bearing the imidazo[2,1-*b*][1,3,4]thiadiazole ring system showed potent anticancer activity (17) we decided to assay ten imidazo[2,1-*b*][1,3,4]thiadiazole compounds (Figure 1) for their antiproliferative activity. For this purpose, seven out of these compounds, **1a-g**, (Figure 1) were submitted to National Cancer Institute (NCI; Bethesda, MD, USA) screening (21). This screening is performed for the evaluation of their antitumor activity on a panel of 60 human cancer cell lines derived from nine cancer types grouped into disease subpanels including leukemia, non-small cell lung, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast tumors. Furthermore, we investigated the antiproliferative activity of the imidazo[2,1-*b*][1,3,4]thiadiazoles **1a-l** on three pre-clinical models of pancreatic ductal adenocarcinoma (PDAC), namely SUIT-2, Capan-1 and Panc-1 cells. PDAC is a deadly disease with poor prognosis and high mortality rate. According to Rahib and collaborators, PDAC will become the second-leading cause of cancer death in the United States in the next 10 years (22). Currently, there are no effective treatments for patients with advanced PDAC. Therefore, new drugs to treat this aggressive tumor are urgently needed.



1a-l

				IC_{50} (μM)		
Compound	R	R^1	R^2	SUIT-2	Capan-1	Panc-1
1a	H	H	H	10.7 ± 0.23	>20	>20
1b	H	H	3-OCH_3	9.57 ± 0.54	>20	>20
1c	H	H	4-CF_3	5.90 ± 0.46	>20	>20
1d	H	CH_3	H	5.16 ± 0.10	9.73 ± 0.82	10.56 ± 0.11
1e	H	CH_3	3-OCH_3	4.30 ± 0.29	>20	>20
1f	H	CH_3	2,5-OCH_3	5.00 ± 0.38	>20	>20
1g	Br	H	2,5-OCH_3	8.40 ± 0.16	>20	9.84 ± 0.24
1h	Br	CH_3	2,5-OCH_3	5.96 ± 0.28	>20	>20
1i	Br	CH_3	4-CF_3	>20	>20	>20
1l	Cl	H	4-F	>20	13.75 ± 0.84	>20

Figure 1. *Antiproliferative activity of compounds **1a-l** against pancreatic cancer cell lines SUIT-2, Capan-1 and Panc-1. Upper panel: Chemical backbone structure of compounds **1a-l**. Lower panel: Table listing the chemical structure of the R, R1 and R2 components and the half-maximal inhibitory concentrations (IC_{50}) for each compound against the PDAC cell lines. Values are reported as the means±standard error of the mean of three separate experiments.*

Materials and Methods

Drugs and chemical. The imidazo[2,1-b][1,3,4]thiadiazole compounds were synthesized as previously described (20). The drugs were dissolved in dimethyl sulfoxide (DMSO). The medium, fetal bovine serum (FBS), penicillin (50 IU ml^{-1}) and streptomycin (50 μg ml^{-1}) were from Gibco (Gaithersburg, MD, USA). All other chemicals were from Sigma (Zwijndrecht, the Netherlands).

Cell culture. Capan-1 and Panc-1 cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), while SUIT-2 cells were a generous gift from Dr. Adam Frampton (Imperial College, London, UK), The cell lines were tested for their authentication by short tandem repeat-polymerase chain reaction, performed by BaseClear (Leiden, the Netherlands). The cells were cultured in RPMI-1640 (Roswell Park Memorial Institute 1640) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, or in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated FBS, and 1% HEPES. The cells were kept in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C and harvested with trypsin-EDTA.

Cell growth inhibition. The *in vitro* antiproliferative activity of imidazothiadiazole compounds **1a-g** was evaluated by the NCI against a panel of human cancer cells, including cells derived from different tumor types, using the NCI-validated protocol: https://dtp.cancer.gov/discovery_development/nci-60/methodology.htm. Furthermore, the *in vitro* antiproliferative activity of imidazothiadiazole compounds **1a-l** was assessed on the PDAC cell lines SUIT-2, Capan-1 and Panc-1, by the sulforhodamine-B (SRB) assay, as previously described (23). In short, cells were seeded into 96-well flat-bottom plates in a volume of 100 μl at a density of 3×10^3 cells/well for SUIT-2 and Panc-1, while 5×10^3 cells/well were seeded for Capan-1. Cells were incubated for 24 hours at 37˚C to create a monolayer and then they were treated with 100 μl of the compounds dissolved in DMSO at different concentrations in the nano- and micro-molar range. After 72 h treatment, the cells were fixed with 25 μl of 50% cold trichloroacetic acid and kept for at least 60 minutes at 4˚C. The plates were then 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 dried tissues and the plates were washed with a 1% acetic acid solution and dried at RT overnight. 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 wavelength of 490 nm and 540 nm. Cell growth inhibition was calculated as the percentage OD of drug-treated cells versus that of vehicle-treated cells (negative control) (corrected for OD before drug addition, day-0). Finally, the half maximal-inhibitory concentration (IC_{50}) was calculated with GraphPad Prism 7 (Intuitive Software for Science, San Diego, CA, USA). The IC_{50} was calculated by non-linear least-squares curve fitting. In the NCI protocol, IC_{50} is denoted as GI_{50} (50% growth-inhibitory concentration).

Wound-healing assay. The *in vitro* scratch wound-healing assay was performed as previously described (24). SUIT-2 cells were seeded in 96-well flat-bottom plates at a density of 5×10^4 cells/well in 100 μl.

Table I. *Concentrations leading to 50% growth inhibition (GI_{50}) and total growth inhibition (TGI) by compound **1g** against cell lines of the National Cancer Institute panel.*

Panel/cell line	GI_{50} (μM)	TGI (μM)	Panel/cell line	GI_{50} (μM)	TGI (μM)
Leukemia			Melanoma		
CCRF-CEM	3.73	>100	MALME-3M	2.25	4.93
HL-60(TB)	9.21	>100	M14	2.10	4.75
K-562	2.51	–	MDA-MB-435	2.42	5.82
RPMI-8226	3.19	20.4	SK-MEL-2	3.33	13.4
NSCLC			SK-MEL-28	3.29	11.7
A549/ATCC	2.31	5.31	SK-MEL-5	3.86	15.2
EKVX	2.83	11.4	UACC-257	7.45	30.7
HOP-62	2.70	13.3	UACC-62	3.15	13.2
HOP-92	2.01	5.68	Ovarian cancer		
NCI-H226	10.3	39.8	IGROV1	2.27	11.5
NCI-H23	1.99	4.82	OVCAR-3	1.96	4.32
NCI-H322M	3.17	20.0	OVCAR-4	1.80	3.80
NCI-H460	2.04	4.17	OVCAR-5	3.52	15.6
NCI-H522	1.98	4.81	OVCAR-8	3.01	9.30
Colon cancer			NCI/ADR-RES	3.38	10.9
HCC-2998	2.67	54.9	SK-OV-3	9.01	22.8
HCT-116	1.67	64.3	Renal cancer		
HCT-15	2.11	>100	786-0	2.16	4.57
HT29	2.28	9.90	A498	10.2	24.6
KM12	2.19	>100	ACHN	2.02	6.49
SW-620	2.26	>100	CAKI-1	2.04	6.22
CNS cancer			RXF 393	3.19	8.82
SF-268	2.70	7.60	SN12C	2.74	10
SF-295	4.70	1.63	TK-10	3.18	6.55
SF-539	1.78	3.41	UO-31	1.67	11.2
SNB-19	4.51	18.6	Breast cancer		
SNB-75	3.72	24.3	MCF7	1.77	5.13
U251	2.02	4.09	MDA-MB-231/ATCC	1.73	4.05
Prostate cancer			HS 578T	2.87	14.5
PC-3	1.87	4.66	BT-549	2.90	11.2
DU-145	2.80	7.71	MDA-MB-468	2.94	8.41

NSCLC: Non-small cell lung cancer; CSN: central nervous system.

After 24 h of pre-incubation at 37˚C, 5% CO_2 and 100% humidity, the cell monolayer was scratched with a specific scratch tool to create a scratch of constant width. After removal of detached cells by washing with phosphate-buffered saline, medium containing the compounds of interest was added to the experimental wells. The wound confluence was monitored by phase-contrast microscopy using Universal Grab 6.3 software (Digital Cell Imaging Labs, Keerbergen, Belgium) integrated into a Leica DMI300B migration station (Leica Microsystems, Eindhoven, the Netherlands) and images were captured immediately after scratch and at intervals after treatment (T=0, and 4, 8, 20 and 24 h). The results were analyzed with Scratch Assay 6.2 software (Digital Cell Imaging Labs).

Statistical analysis. All SRB assays were carried out in triplicate and repeated at least three times, whereas the percentages of cell migration were calculated taking into account at least six scratch areas. The data were evaluated using GraphPad Prism 7 (Intuitive Software for Science, San Diego, CA, USA). Data were expressed as mean values±SEM and analyzed by Student's *t*-test.

Results and Discussion

*Antiproliferative activity of imidazo[2,1-b][1,3,4]thiadiazole derivatives **1a-l***. Compounds **1a-g** were submitted to the NCI and pre-screened, according to the NCI protocol, at one concentration (10 μM) in a full panel of 60 human cancer cell lines. The derivative **1g** satisfied the threshold inhibition criteria established by the NCI and was further selected for full evaluation at five concentration levels (10^{-4}-10^{-8} M). Remarkably, its GI_{50}s were in the range from 1.67 to 10.3 μM (Table I). The antiproliferative effect of the compound **1g** was then evaluated on PDAC cells, using eight increasing concentrations (from 0.125 nM to 20 μM). The PDAC cells were also used to explore the activity of compounds **1h-l** in order to investigate the specific role of a different halogen on the indole moiety (compound **1l**), as well as the effects of N-methyl-indole (compound **1h**) or by different

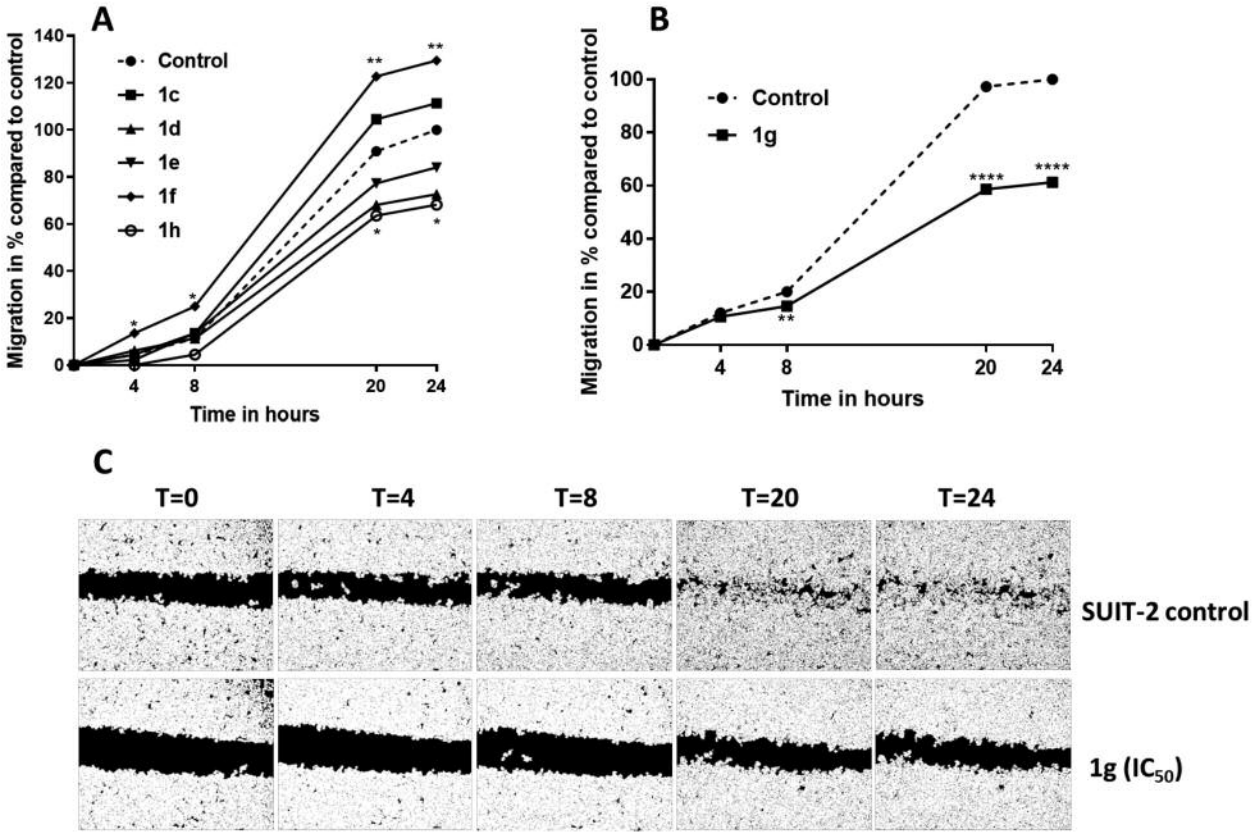


Figure 2. *Modulation of migration rate of SUIT-2 pancreatic cancer cells treated with imidazo[2,1-b][1,3,4]thiadiazole derivatives **1c-f** and **1g** and **1h**. The modulation of migration rate was monitored over time (0, 4, 8, 20 and 24 hours) in SUIT-2 cells treated with compounds **1c-f** and **1h** at concentrations of threefold the half-maximal inhibitory concentration (IC_{50}) value (A), and **1g** (B) at its IC_{50}. Points represent the means of at least six different assessed scratch areas. Standard errors of the mean were always below 10%. p-Values were calculated with the Student's t-test. Significantly different at *p<0.05, **p<0.01 and ****p<0.0001. C: Representative images of the scratch areas of SUIT-2 cell monolayers monitored over time (T=0 and at 4, 8, 20, and 24 hours from the start of the treatment). Upper panel: Untreated cells; lower panel: cells treated with compound **1g**. Original magnification 5×.*

substituents on the phenyl ring (compounds **1i-l**). As shown in Table I and in the lower panel of Figure 1, compounds **1a-h** showed relevant antiproliferative activity, mostly against SUIT-2 cells, with IC_{50} values in the range of 4.3 to 10.7 μM. In particular, the lowest values of IC_{50} were observed for SUIT-2 cells exposed to **1e** and **1f** (4.3-5.0 μM). Conversely, the IC_{50}s of **1i** was above 20 μM in all cell lines. Capan-1 and Panc-1 cells were more resistant to all the compounds, with IC_{50}s>20 μM, except to compound **1l**, which was more active against Capan-1 cells (with IC_{50} of 13.8 μM compared to >20 μM for both SUIT-2 and Panc-1 cells). Compound **1g** was active against both SUIT-2 and Panc-1 cells, with IC_{50}s of 8.4 and 9.8 μM, respectively. Notably, compound **1d** showed a strong antiproliferative activity against SUIT-2 cells with IC_{50} of 5.16 μM. Instead, in Capan-1 and Panc-1 cells, it displayed moderate antiproliferative activity with IC_{50}s of 9.73 and 10.56 μM, respectively.

*Anti-migratory activity of compounds **1c-f**, and **1g** and **1h** against SUIT-2 cells*. Cell migration and invasion are essential for spreading cells from the primary tumor to distant sites and creating metastases. This is a relevant aspect that contributes to the poor prognosis of many cancer types. In particular, PDAC is an aggressive metastatic disease and to date, the molecular mechanisms that drive metastatic events are still unknown. Certainly, a key role is played by the stromal components that maintain cell growth and facilitate the acquisition of aggressive and invasive features (25). The human cell line SUIT-2 was derived from a metastatic lesion in the liver of a patient with PDAC and we selected this cell line as an optimal model for studying the anti-migratory activity of our new compounds (26). Considering the interesting *in vitro* antiproliferative activities of the compounds **1c-f** and **1h**, together with the NCI results for compound **1g**, we selected these compounds for the analysis of the inhibition of migration using a high-throughput screening scratch wound-

healing assay. The SUIT-2 cells were then treated with **1c-f**, and **1h** at threefold the IC_{50}, whereas compound **1g** was used at its IC_{50} value. The percentage of migration was monitored over time through a series of images captured immediately after scratch and treatment (T=0) and after 4, 8, 20 and 24 hours. As shown in Figure 2A, unexpectedly, compounds **1c** and **1f** supported cell migration. Previous studies showed the ability of indoles to reduce migration in cancer cells (27), but a seminal study on the oral oxindole multitargeted kinase inhibitor sunitinib reported metastatic acceleration depending on treatment schedule and tumor model (28).

However, compounds **1d**, **1e**, **1g** and **1h** slowed the migration of cells compared to the controls (Figure 2A and B). In particular, compared to untreated cells (set at 100%), the percentages of migration in cells treated for 24 hours with the compounds **1d**, **1e**, **1g** and **1h** were 72.7%, 84.1%, 61.3% and 68.2%, respectively.

These anti-migratory effects were more evident at 20 and 24 hours from the start of the treatment, and statistical analyses showed that the inhibition of migration compared to that of the control was significant for compounds **1g** and **1h**.

Importantly, in order to optimize the experimental conditions for the wound-healing assay, we determined the doubling time of SUIT-2 cells, which was above 24 hours. Moreover, we measured the area of the wound track (as shown in representative images in Figure 2C), which was approximately 10^6 μm^2. This is too large an area to be covered as a result of cell proliferation alone within 24 h, since the average tumor adherent cell surface is around 100-150 μm^2. Ultimately, we did not observe detached cells after 24-hour treatment, which demonstrates that exposure did not cause cell death at that timepoint.

Finally, these results were comparable to previous data showing that indole-3-carbinol and indole[3,2-*b*]carbazole suppress epithelial–mesenchymal transition and migration of breast cancer cells through the repression of focal adhesion kinase (29), and will prompt future studies on molecular mechanisms underlying the anti-migratory activity of our new compounds.

Conclusion

The imidazo[2,1-*b*][1,3,4]thiadiazole derivatives **1a-l**, endowed with antibiofilm activity, also showed antiproliferative activity. Among the seven 3-(6-phenylimidazo[2,1-*b*][1,3,4]thiadiazol-2-yl)-1*H*-indole derivatives, **1a-g**, screened on a panel of 60 human cancer cells by the NCI at a concentration of 10 μM, compound **1g** displayed relevant antiproliferative activity, with GI_{50} values ranging from 1.67 to 10.3 μM. However, compounds **1a-h** showed interesting *in vitro* antiproliferative activity against SUIT-2 cells, whereas compound **1d** was active against the other two PDAC preclinical models, Capan-1 and Panc-1. Finally, a wound-healing assay demonstrated the anti-migratory ability of compounds **1d**, **1e**, **1g** and **1h**, which reduced cell migration by 20-40%, compared to the control.

Overall, the results of cytotoxicity and cell migration will prompt further studies on imidazo[2,1-*b*][1,3,4]thiadiazole derivatives **1d**, **e**, **g** and **h**, which could be used as interesting lead compounds to facilitate the drug-discovery process using indole derivatives.

Conflicts of Interest

The Authors have declared that no conflict of interest exists in regard to this study.

Authors' Contributions

GLP was the principal investigator and takes primary responsibility for the article; SC, BEH, DC, and BP provided essential material and participated in the research design; GLP, SC, and EG wrote the article; GJP, GC and PD edited the paper. All Authors read and approved the final article.

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