Full Paper

Cell-Based Biological Evaluation of a New Bisamide FMS Kinase Inhibitor Possessing Pyrrolo[3,2-*c*]pyridine Scaffold

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A bisamide compound **1** possessing the pyrrolo[3,2-*c*]pyridine nucleus was synthesized and biologically evaluated. It was tested for kinase inhibitory activity over a panel of 47 kinases, and its selectivity toward the FMS kinase was accidentally discovered. Compound **1** was tested over a panel of seven ovarian, two prostate, and six breast cancer cell lines at a single dose concentration of 10 μM and showed high activity. It was further tested in a 5-dose mode to determine its IC_{50} and total growth inhibition (TGI) values over the 15 cell lines. Compound **1** showed high potency on the submicromolar scale and good efficacy. The cytotoxic effect of compound **1** over peritoneal macrophages was also investigated. Compound **1** demonstrated higher selectivity against different cancer cell lines compared with HS-27 fibroblasts.

Keywords: Anticancer / Bisamide / Diarylamide / FMS kinase / Pyrrolo[3,2-*c*]pyridine

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Introduction

FMS, first discovered as the oncogene responsible for feline McDonough sarcoma, is a type III receptor tyrosine kinase that acts as exclusive receptor for the macrophage or monocyte colony-stimulating factor (M-CSF or CSF-1). Signal transduction through CSF-1/FMS or M-CSF/FMS binding results in survival, proliferation, and differentiation of monocyte/macrophage lineage [1, 2]. Over-expression of CSF-1 and/or FMS has been implicated in a number of disease states such as the growth of metastasis of certain types of cancer, in promoting osteoclast proliferation in bone osteolysis, and in many inflammatory disorders. Inhibition of CSF-1 and/or FMS may help treat these pathological conditions.

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There are direct relationships between macrophage numbers, angiogenesis, and malignancy progression [3]. The growth of several tumor types is associated with over-expression of CSF-1 and FMS receptor in cancer cells and/or tumor stroma [4–7]. CSF-1 and FMS were found to be important in the physiology of the breast and female reproductive tract neoplasms. The expression of FMS in breast cancer has been linked to poor survivability and increased tumor size, where presumably the receptor is involved in local invasion and metastasis [8–10]. It was also reported that macrophages mediate hormone resistance in prostate cancers by a nuclear receptor derepression pathway [11]. High serum levels of CSF-1 in bone metastatic prostate cancer have also been detected. This plasma CSF-1 may play a pivotal role in prostate cancer progression as a clinically useful tumor marker [12].

There are many reported FMS kinase inhibitors possessing a diversity of chemical scaffolds. We have recently reviewed and classified them according to their chemotypes [13]. And we have reported a selective and potent bisamide FMS kinase inhibitor (compound **1**, Fig. 1) [14]. Due to the relationship

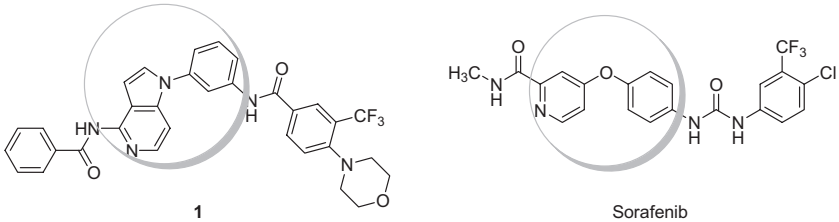


Figure 1. Structures of the target compound **1** and sorafenib.

between FMS kinase and macrophage, and female reproductive system, prostate, and breast cancers, we studied the antiproliferative activities of compound **1** over 15 cancer cell lines of those three cancer types and HS-27 fibroblasts, and its cytotoxicity against macrophages in the present investigation. The results and experimental procedures are reported in detail.

Results and discussion

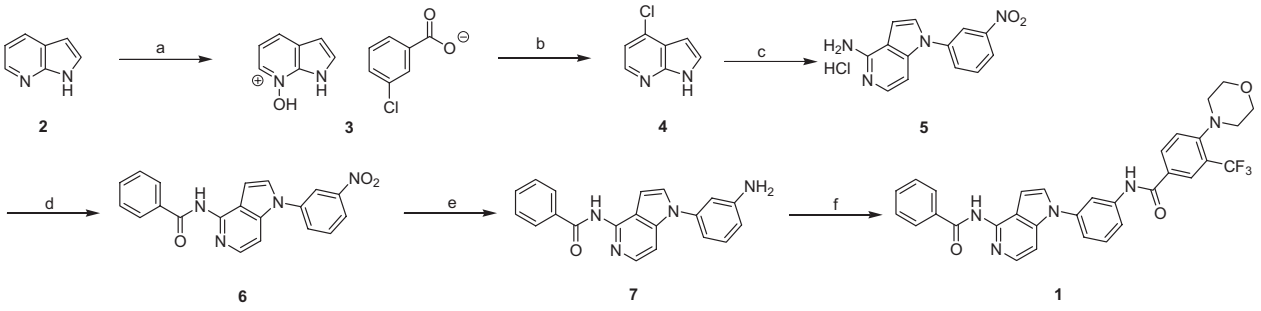
Chemistry

Synthesis of the target compound **1** was successfully achieved as illustrated in Scheme 1. 7-Hydroxy-1*H*-pyrrolo[2,3-*b*]pyridinium 3-chlorobenzoate (**3**) was prepared by reacting 7-azaindole (**2**) with 3-chloroperbenzoic acid [15, 16]. Compound **3** was then heated with phosphorus oxychloride to produce 4-chloro-7-azaindole (**4**) [16]. Compound **4** was prepared according to the literature procedure [14, 17, 18]. Fusion of **4** with *m*-nitroaniline neat led to nucleophilic displacement of the 4-chloro group by the aromatic amino group, followed by rearrangement of the resulting secondary amine to give the amine hydrochloride salt **5**. Reaction of the amino group of 1-(3-nitrophenyl)-1*H*-pyrrolo[3,2-*c*]pyridin-4-amine hydrochloride (**5**) with benzoyl chloride in the presence of diisopropylamine as a base produced the benzamido derivative, *N*-(1-(3-nitrophenyl)-1*H*-pyrrolo[3,2-*c*]-pyridin-4-yl)benzamide (**6**). Reduction of the nitro group of **6** using Pd–C in hydrogen atmosphere gave the corresponding amino compound, *N*-(1-(3-aminophenyl)-1*H*-pyrrolo[3,2-*c*]-pyridin-4-yl)benzamide (**7**). Condensation of compound **7** with 4-morpholino-3-(trifluoromethyl)benzoic acid in the presence of HOBt/EDCI/triethylamine furnished the target compound **1** [14].

Biology

Compound **1** was tested at a single-dose concentration of 1 μM over a panel of 47 kinases. It inhibited the activities of FMS and KDR kinases by 90 and 71%, respectively (Fig. 2). Compound **1** was further tested in a 10-dose testing mode in order to determine its IC_{50} values over FMS and KDR kinases (Table 1). The results showed that compound **1** is 11 times more selective toward FMS kinase than KDR kinase [14].

The colony stimulating factor-1 (CSF-1) and its receptor FMS (encoded by the c-fms proto-oncogene) constitute a reciprocal system that has been linked to several human epithelial cancers including ovarian, breast, and prostate cancers [7, 19–21]. Compound **1** was tested for antiproliferative activities over a panel of seven ovarian, two prostate, and six breast cancer cell lines at the National Cancer Institute (NCI, Bethesda, Maryland, USA) [22]. The results are shown in Table 2 and Fig. 3. At a single-dose concentration of 10 μM, compound **1** exerted more than 100% growth inhibition over SK-OV-3 ovarian cancer cell line and MDA-MB-468 breast cancer cell line. The %inhibition was >90% in cases of IGROV1



Scheme 1. Reagents and conditions: (a) 3-chloroperoxybenzoic acid, DME/heptane (1:2), rt, 2.5 h, 89.2%; (b) $POCl_3$, 55°C, then rt, then 85–90°C, 18 h, 80%; (c) *m*-nitroaniline, 180°C, 2–5 h, 9.5%; (d) benzoyl chloride, diisopropylamine, CH_3CN, rt, 8 h, 15%; (e) Pd/C, H_2, THF, rt, 2 h, 32%; (f) 4-morpholino-3-(trifluoromethyl)benzoic acid, HOBt, EDCI, TEA, DMF, 80°C, 12 h, 16%.

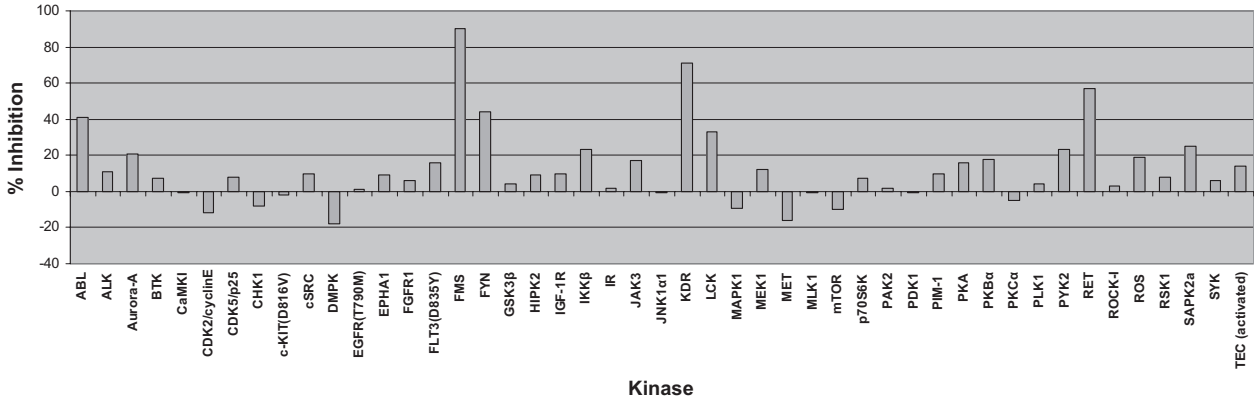


Figure 2. Percentages of enzymatic inhibitions exerted by compound **1** over 47 kinases.

Table 1. IC_{50} of compound **1** over FMS and KDR kinases.

Kinase enzyme	IC_{50} (nM)
FMS	96
KDR	1058

ovarian cancer cell line and T-47D breast cancer cell line, and more than 73% over another eight cell lines of the three tested cancer types.

Compound **1** was further tested in a 5-dose testing mode in order to determine its IC_{50} and total growth inhibition (TGI) concentration values. It showed high potency with IC_{50} values in submicromolar scale over all the tested cell lines. Of special interest, it exhibited 2-digit nanomolar IC_{50} values over five ovarian, two prostate, and three breast cancer cell lines. In addition to its strong potency, compound **1** showed high efficacy also. Its TGI values were less than 100 μM against 12 cancer cell lines. The highest efficacy was expressed against OVCAR-3 ovarian cancer cell line and DU-145 prostate cancer cell line with 100% growth inhibition at a concentration as small as 45 and 98 nM, respectively.

The highest percent inhibition was reported against SK-OV-3 ovarian cancer cell line (106.21%). The IC_{50} and TGI values of compound **1** over this cell line were 33 nM and 3.60 μM, respectively. Woo et al. reported high expression of CSF-1 mRNA level and high level of secreted CSF-1 protein level

Table 2. Inhibition percentages at a single-dose of 10 μM, and IC_{50} and TGI values in μM of compound **1** and sorafenib over ovarian, prostate, and breast cancer cell lines.

			5-Dose results of compound 1		5-Dose results of sorafenib	
	Cell line	%Inhibition (1-dose)	IC_{50}[a)]	TGI[b)]	IC_{50}[a)]	TGI[b)]
Ovarian cancer	IGROV1	91.01	0.057	56.9	2.51	7.94
	OVCAR-3	73.13	0.022	0.045	3.16	10.00
	OVCAR-4	48.34	0.239	50.0	3.16	25.10
	OVCAR-5	58.93	0.200	>100	3.16	15.85
	OVCAR-8	83.66	0.056	>100	3.16	19.95
	NCI/ADR-RES	85.72	0.036	26.4	2.51	15.85
	SK-OV-3	106.21	0.033	3.60	2.51	6.31
Prostate cancer	PC-3	76.33	0.055	>100	2.00	7.94
	DU-145	81.33	0.036	0.098	3.16	12.59
Breast cancer	MCF7	86.03	0.040	54.0	2.51	10.00
	MDA-MB-231/ATCC	79.25	0.158	3.42	1.26	3.98
	HS 578T	44.47	0.990	97.2	2.51	19.95
	BT-549	74.84	0.203	45.8	3.16	7.94
	T-47D	92.43	0.084	7.46	1.58	6.31
	MDA-MB-468	102.66	0.029	0.10	2.00	7.94
HS-27 fibroblasts	–	0.30	–	7.80	–	

[a)] IC_{50} is the concentration producing 50% inhibition. The results are expressed as means of duplicate experiments.
[b)] TGI is the concentration producing 100% inhibition.

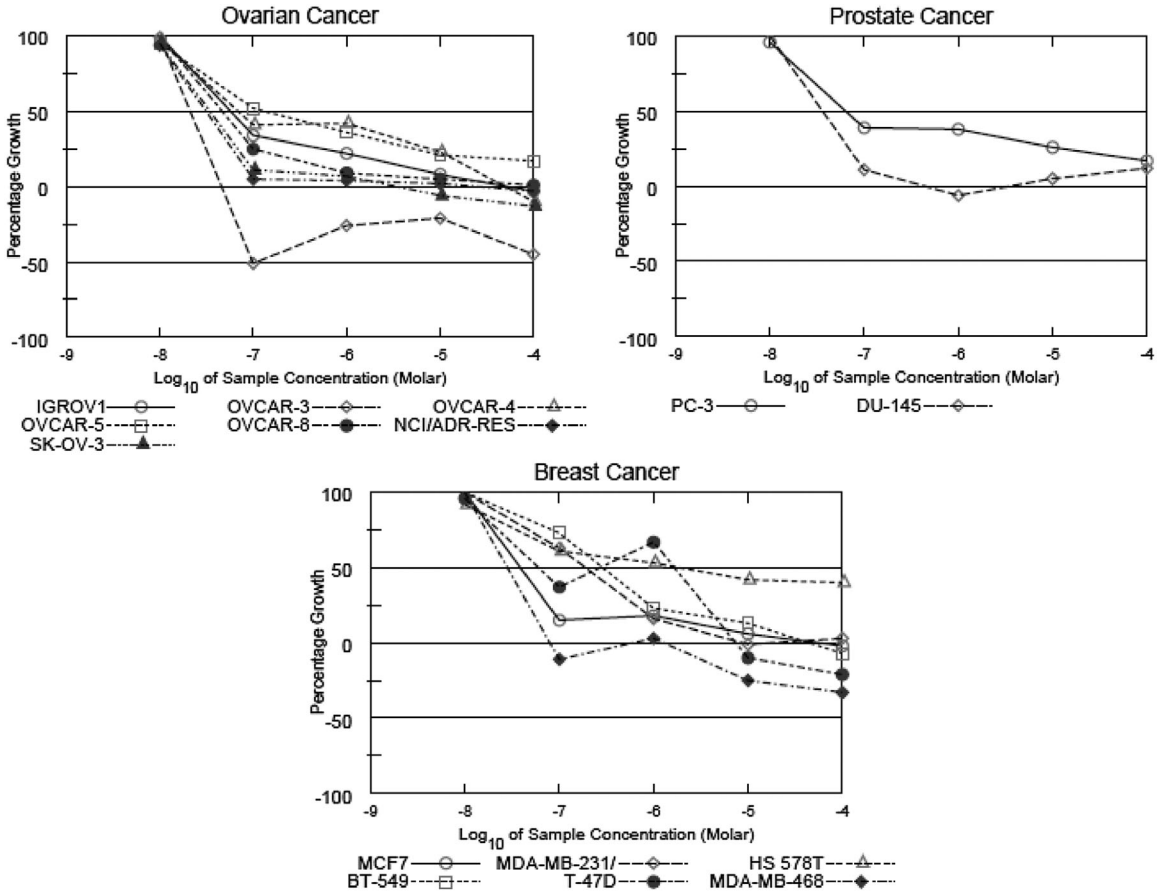


Figure 3. Dose–response curves of compound **1** over the ovarian, prostate, and breast cancer subpanels.

in SK-OV-3 cell line [23]. So it can be assumed that the anticancer activity of compound **1** against this cell line is related to CSF-1 and FMS inhibition.

Sorafenib is a standard anticancer agent. The *N*-phenylpyrrolo[3,2-*c*]pyridine nucleus of compound **1** is considered as a conformationally restricted analog of the 4-phenoxypyridine moiety of sorafenib (Fig. 1). Due to the structural similarity between compound **1** and sorafenib, and the potential anticancer activity of sorafenib, it was utilized as a reference compound in this work to compare the activity of compound **1** with its activity. Upon comparison, it was found that compound **1** showed superior potencies over 14 tested cell lines (in submicromolar range) to those of sorafenib (micromolar scale). The efficacy of compound **1** was higher than that of sorafenib against OVCAR-3 and SK-OV-3 ovarian cancer cell lines and MDA-MB-231/ATCC breast cancer cell line. Sorafenib is a well-known B-RAF kinase inhibitor. But the target compound **1** did not show significant B-RAF kinase inhibitory effect ($IC_{50} > 2\,\mu M$). The restricted conformation of compound **1** compared with sorafenib may be responsible for its enhanced selectivity toward FMS kinase. On the other hand, sorafenib is a multiple-kinase inhibitor.

In order to compare the selectivity of compound **1** and sorafenib toward cancer cells compared with normal cells, they were tested against HS-27 fibroblasts. Their IC_{50} values against HS-27 were 0.30 and 7.8 μM, respectively (Table 2). It was found that compound **1** exerted superior selectivity indices against nine cell lines to sorafenib (Table 3).

FMS kinase binds to macrophage or monocyte colony-stimulating factor (M-CSF or CSF-1). Signal transduction through that binding results in survival, proliferation, and differentiation of monocyte/macrophage lineage [1, 2]. So compound **1** was also tested for inhibitory effect over peritoneal macrophage cells in a 5-dose testing mode (Fig. 4). At 1 μM concentration, it inhibited 52% of macrophage cell viability. Bingle et al. [3] reported a direct relationship between macrophage numbers, angiogenesis and malignancy progression. So we can conclude that the inhibitory effect of compound **1** against macrophage cells

Table 3. Selectivity indices of compound **1** toward cancer cell lines over HS-27 fibroblasts.[a)]

Cell line	Compound 1	Sorafenib
IGROV1	5.26	3.11
OVCAR-3	13.64	2.47
OVCAR-4	1.26	2.47
OVCAR-5	1.50	2.47
OVCAR-8	5.36	2.47
NCI/ADR-RES	8.33	3.11
SK-OV-3	9.09	3.11
PC-3	5.45	3.90
DU-145	8.33	2.47
MCF7	7.50	3.11
MDA-MB-231/ATCC	1.90	6.19
HS 578T	0.30	3.11
BT-549	1.48	2.47
T-47D	3.57	4.94
MDA-MB-468	10.34	3.90

[a)] Selectivity index was calculated by dividing the IC_{50} value against HS-27 by the IC_{50} value against the cell line (Table 2).

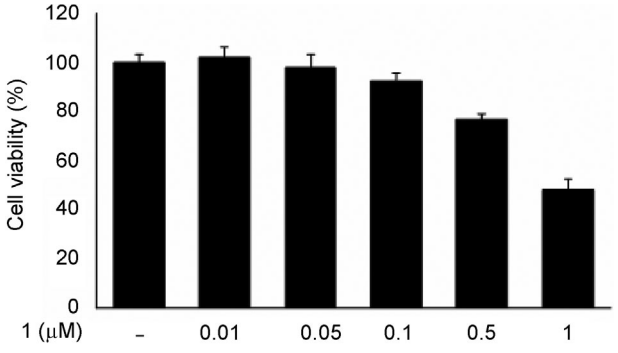


Figure 4. Effect of compound **1** on peritoneal macrophage cell viability.

may contribute, at least in part, to its anticancer activities *in vivo*.

Conclusion

A bisamide compound **1** containing pyrrolo[3,2-*c*]pyridine scaffold was synthesized and reported to possess selective FMS kinase inhibitory activity. It was tested over a panel of seven ovarian, two prostate, and six breast cancer cell lines at the NCI, USA. It exerted strong potency with submicromolar IC_{50} values against all the tested cell lines, and high efficacy over most of them. Its IC_{50} and TGI values were in 2-digit nanomolar scale (22 and 45 nM, respectively) on OVCAR-3 ovarian cancer cell line. Compound **1** was more potent than sorafenib against 14 cell lines, and more efficacious over three cell lines. Compound **1** demonstrated higher selectivity indices than sorafenib against nine cancer cell lines. It also showed inhibitory effect against peritoneal macrophage cellular viability. So it can be concluded that the strong antiproliferative activity of compound **1** may be mainly due to FMS kinase inhibition.

Experimental

General

Melting points were obtained on a Walden Precision Apparatus Electrothermal 9300 apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectral analyses were performed using a Bruker ARX-300, 300 MHz spectrometer and a Bruker ARX-400, 400 MHz spectrometer (Bruker Bioscience, Billerica, MA, USA) with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) and purity of the target compound (>96%) were determined by LC-MS analysis using the following system: Waters 2998 photodiode array detector, Waters 3100 mass detector, Waters SFO system fluidics organizer, Waters 2545 binary gradient module, Waters reagent manager, Waters 2767 sample manager, Sunfire™ C18 column (4.6 mm × 50 mm, 5 μm particle size); solvent gradient = 95% A at 0 min, 1% A at 5 min; solvent A: 0.035% trifluoroacetic acid (TFA) in water; solvent B: 0.035% TFA in CH_3OH; flow rate = 3.0 mL/min; the AUC was calculated using Waters MassLynx 4.1 software. All reagents and solvents were purchased from Aldrich Chemical Co. and Tokyo Chemical Industry (TCI) Co., and used without further purification.

Synthesis

*7-Hydroxy-1H-pyrrolo[2,3-b]pyridinium 3-chlorobenzoate (**3**) [15, 16]*

To a solution of 7-azaindole (**2**, 19.83 g, 167.9 mmol) in DME/heptane (1:2, 294 mL) was added 3-chloroperbenzoic acid (85 wt%, 46.2 g, 194.9 mmol) portionwise at 8–26°C. Precipitation occurred after half of the 3-chloroperbenzoic acid was added. The slurry was stirred at room temperature for 2.5 h. The precipitate was filtered and washed with DME/heptane (1:2, 100 mL). The product was dried to yield an off-white solid (43.58 g, 89.2%). m.p.: 141–143°C (Lit. m.p.: 144.1–146°C) [16].

*4-Chloro-7-azaindole (**4**) [16]*

To compound **3** (43.4 g, 149.3 mmol) was added $POCl_3$ (170.4 g) at room temperature. The solution was heated to 55°C, and then heating was removed. The temperature slowly went up to 74°C in about 1 h without external heating. The mixture was further heated to 85–90°C for 18 h. *Caution: Two exothermic events were observed, one at 50–60°C and the other at 105–110°C. To prevent a runaway reaction on scale, the solution was first heated to 55°C and then heated slowly to 85–90°C.* The solution was cooled to 50°C, and $POCl_3$ was distilled off *in vacuo*. The residue was dissolved in acetonitrile (100 mL) and quenched by slow addition of water (100 mL) while keeping the temperature under 50°C. The mixture was basified to pH 9 with 50% NaOH solution. The slurry was allowed to cool to room temperature, and the precipitates were filtered. The wet cake was reslurried with H_2O (200 mL), filtered, and dried to afford the product (18.15 g, 80%). m.p.: 176–177°C (Lit. m.p.: 175.3–177°C) [16].

*1-(3-Nitrophenyl)-1H-pyrrolo[3,2-c]pyridin-4-amine hydrochloride (**5**)*

A mixture of compound **4** (230 mg, 2.0 mmol) and 3-nitroaniline (1.38 g, 10.0 mmol) was fused at 180°C for 2–5 h with stirring. The reaction mixture was cooled to room temperature and dissolved in ethanol (150 mL). The resulting suspension was filtered to remove the insoluble material, and the filtrate was evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, ethyl acetate/methanol 10:1 v/v then switching to ethyl acetate/methanol 5:1 v/v) to obtain the desired purified product. Yield 9.5%; ^{1}H NMR (300 MHz, CD_3OD) δ 8.33 (t, 1H, $J = 2.0$ Hz), 8.25 (dd, 1H, $J = 1.1$, 6.1 Hz), 7.94–7.91 (m, 1H), 7.81–7.76 (m, 1H), 7.63 (d, 1H, $J = 2.2$ Hz), 7.45 (d, 1H, $J = 3.4$ Hz), 6.90 (d, 1H, $J = 3.4$ Hz), 6.85 (d, 1H, $J = 6.3$ Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 155.1, 150.4, 144.5, 140.3, 132.2, 131.0, 127.2, 122.6, 119.7, 115.0, 113.3, 104.5, 98.6; MS *m/z*: 255.95 $(M+2)^+$, 254.65 $(M)^+$.

*N-(1-(3-Nitrophenyl)-1H-pyrrolo[3,2-c]pyridin-4-yl)-benzamide (**6**)*

To a stirred solution of compound **5** (0.465 g, 1.6 mmol) in acetonitrile (25 mL) at room temperature, diisopropylamine (0.7 mL, 4.0 mmol) was slowly added under nitrogen atmosphere. Benzoyl chloride (0.2 mL, 1.9 mmol) was slowly added and the reaction mixture was stirred at room temperature for 8 h. The reaction mixture was concentrated under reduced pressure, and then water (20 mL) and CH_2Cl_2 (20 mL) were added to the residue. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2×20 mL). The combined organic layer extracts were washed with brine, 1 N HCl, and then aqueous $NaHCO_3$, dried over anhydrous $MgSO_4$, and filtered. The organic solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (silica gel, hexane/ethyl acetate 3:1 v/v) to give the target product. Yield 15%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.90 (brs, 1H), 8.41 (t, 1H, $J = 2.1$ Hz), 8.30 (d, 1H, $J = 8.0$ Hz), 8.16–8.09 (m, 4H), 7.93–7.82 (m, 2H), 7.62–7.52 (m, 4H), 6.74 (brs, 1H); MS *m/z*: 359.11 $(M+1)^+$, 358.24 $(M)^+$.

*N-(1-(3-Aminophenyl)-1H-pyrrolo[3,2-c]pyridin-4-yl)-benzamide (**7**)*

A mixture of compound **6** (0.466 g, 1.3 mmol) and Pd/C (10%) in anhydrous THF (20 mL) was stirred in hydrogen atmosphere at room temperature for 2 h. The reaction mixture was filtered through celite, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate 1:2 v/v) to give the purified desired product. Yield 32%; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.84 (brs, 1H), 8.11–8.09 (m, 3H), 7.62 (d, 2H, $J = 3.2$ Hz), 7.54 (d, 2H, $J = 7.5$ Hz), 7.42 (brs, 1H), 7.22 (t, 1H, $J = 7.9$ Hz), 6.77 (brs, 1H), 6.69–6.62 (m, 3H), 5.50 (brs, 2H); MS *m/z*: 330.24 $(M+2)^+$, 329.21 $(M+1)^+$.

*N-(3-(4-Benzamido-1H-pyrrolo[3,2-c]pyridin-1-yl)phenyl)-4-morpholino-3-(trifluoromethyl)benzamide (**1**)*

A mixture of compound **7** (17.0 mg, 0.05 mmol), 3-(trifluoromethyl)-4-morpholinobenzoic acid (28.5 mg, 0.1 mmol), HOBt (15.4 mg, 0.11 mmol), and EDCI (24.83 mg, 0.13 mmol) in dry DMF (1.0 mL) was cooled to 0°C under nitrogen atmosphere. To the reaction mixture, TEA (0.02 mL, 0.01 mmol) was added at 0°C. The mixture was then stirred at 80°C for 12 h. The reaction mixture was cooled and then partitioned between water and ethyl acetate and the organic layer was separated. The aqueous layer was then extracted with ethyl acetate and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the residue was purified by column chromatography (silica gel, hexane/ethyl acetate 1:1 v/v) to yield compound **1** as a yellow crystalline solid (5.0 mg, 16%); m.p. 130–132°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.85 (brs, 1H), 10.65 (brs, 1H), 8.28–8.27 (m, 1H), 8.13–8.09 (m, 4H), 7.88–7.83 (m, 1H), 7.75 (d, $J = 3.3$ Hz, 2H), 7.67–7.54 (m, 6H), 7.41–7.37 (m, 1H), 6.69–6.67 (m, 1H), 3.75–3.72 (m, 4H), 2.97–2.89 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.3, 158.2, 141.0, 139.5, 133.3, 133.1, 132.1, 129.3, 128.5, 128.1, 127.8, 127.3, 126.9, 126.1, 125.3, 124.5, 124.0, 123.5, 123.3, 123.0,121.4, 120.8, 119.6, 116.9, 114.4, 104.5, 67.4, 53.6; IR (KBr) 3418, 2919, 1607, 1496, 1323, 1121, 850, 709 cm^{-1}; MS *m/z* 586.2 $(M+1)^+$.

Biological screening

Cancer cell line screening at the NCI

Screening against the 15 cancer cell lines was carried out at the National Cancer Institute (NCI), Bethesda, Maryland, USA [22] applying the standard protocol of the NCI [24, 25].

MTT assay for peritoneal macrophage cell viability

Peritoneal macrophages were plated at a density of 10^5 cells/well in 96-well plates. Cytotoxicity studies were performed 24 h after treating cells with various concentrations of the test compound **1**. Cell viabilities were determined using colorimetric MTT assays, as described previously [26].

Evaluation of the antiproliferative activity against HS-27 fibroblasts

HS-27 fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Republic of Korea) supplemented with bovine calf serum (BCS; Welgene) and 1% penicillin/streptomycin (Welgene) in a humidified atmosphere with 5% CO_2 at 37°C. HS-27 fibroblasts were taken from culture substrate with 0.05% trypsin–0.02% EDTA and plated at a density of 5×10^3 cells/well in 96-well plates and then incubated at 37°C for 24 h in a humidified atmosphere with 5% CO_2 prior to treatment with various concentrations (threefold serial dilution, 12 points) of the tested compounds. The cells were incubated for 48 h after treatment with the test compounds. The HS-27 cell viability was assessed by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. MTT assays were carried out with CellTiter 96® (Promega) according to the manufacturer's instructions. The absorbance at 590 nm was recorded using EnVision 2103 (Perkin Elmer, Boston, MA, USA). The IC_{50} values were calculated using GraphPad Prism 4.0 software. Triplicate testing was performed for each test compound.

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