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Design and Synthesis of an Anticancer Diarylurea Derivative with Multiple-Kinase Inhibitory Effect

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A diarylurea compound **1** possessing pyrrolo[3,2-*c*]pyridine nucleus was designed and synthesized with structure similarity to Sorafenib. Compound **1** was tested over 60-cancer cell line panel at a single dose concentration of 10 μM and showed high activity. It was further tested in a five-dose mode to determine its IC_{50}, TGI, and LC_{50} values over the 60 cell lines. Compound **1** showed high potency and good efficacy, and was accordingly tested at a single dose concentration of 10 μM over a panel of 40 kinases. At this concentration, it completely inhibited the enzymatic activities of a number of oncogenic kinases, including ABL, ALK, c-RAF, FLT3, KDR, and TrkB. The target compound was subsequently tested over these 6 kinases in 10-dose testing mode in order to determine its IC_{50} values.

Key Words : Anticancer, Diarylurea, Multiple-kinase, Pyrrolo[3,2-*c*]pyridine

Introduction

Cancer is a leading cause of death worldwide, and it accounted for 7.6 million deaths (around 13% of all deaths) in 2008 according to WHO reports. More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030.[1] Cancer is thought to reflect a multi-step process, resulting from an accumulation of inherited and/or acquired defects in genes involved in the positive or negative regulation of cell proliferation and survival. The classical cancer treatments such as surgery, radiation and cytotoxic chemotherapy are seemed to be no longer effective neither for the complete eradication of disease nor for the improvement of cancer patients' life. For the development of a clinically recognizable human cancer, the activation or inactivation of as many as four or five different genes may be required.[2]

The human genome encodes approximately 500 predicted protein kinases, and many of them are participating in signal transduction pathways that regulate cell growth and survival.[3] Over-expression of many kinases has been implicated in cancer initiation and progression. Much attention has been paid to the development of kinase inhibitors as anticancer agents.

Despite extensive efforts within the oncology field to develop kinase inhibitors, uncertainty remains over the relative merits of selective compounds versus less selective or "multi-targeted" inhibitors.[4] Targeted molecules offer the clearest indication that *in vivo* effects result from the intended *in vitro* activity. Moreover, toxicity derived from additional activity against other kinases is likely to be reduced.[5] However, inhibition of a single kinase may not be sufficient to achieve a clinical benefit, either through the built-in redundancy of signaling pathways, or the ability of tumors to acquire resistance.[6] Inhibitors with activity against multiple kinases may in fact be more effective, and several multi-targeted kinase inhibitors are now commercially available.

Sorafenib is an anticancer diarylurea derivative with multiple-kinase inhibitory effect. It has been approved by the U. S. food and drug administration (FDA) for treatment of advanced renal cancer.[7] It has also been approved in Europe for treatment of hepatocellular carcinoma.[8] Sorafenib is currently subjected to clinical trials for other types of cancer.

In the present study, the diarylurea target compound **1** was designed with structure similarity to Sorafenib (Fig. 1). We recently reported its synthesis and antiproliferative potency against melanoma cell lines.[9] Herein, we report its cytotoxicity profile against a panel of another 60 cell lines of 9 different cancer types. Moreover, in an attempt to examine its mechanism of action at molecular level, compound **1** was tested at a single dose concentration of 10 μM over a panel

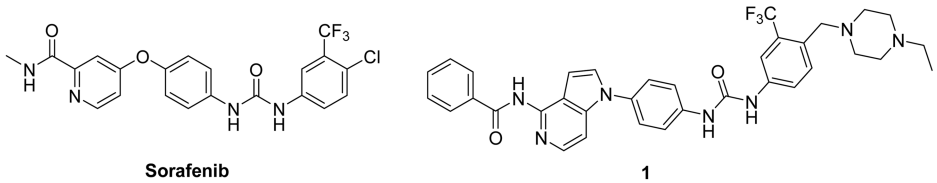
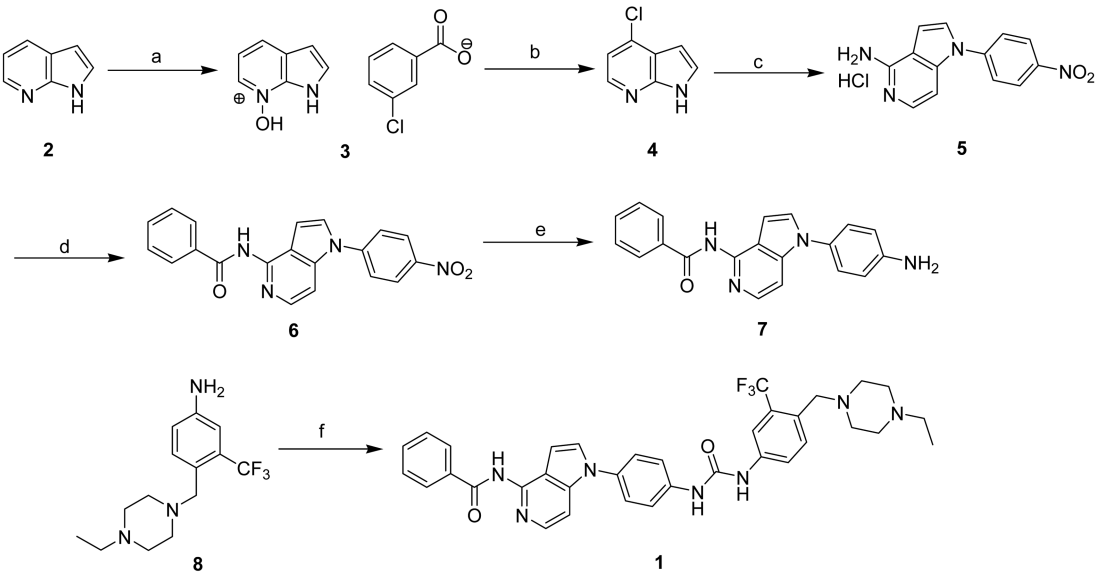


Figure 1. Structures of Sorafenib and the target compound **1**.



Scheme 1. Reagents and conditions: (a) 3-chloroperoxybenzoic acid, DME:heptanes (1:2), rt, 2.5 h, 90%; (b) $POCl_3$, 55 °C then rt then 85-90 °C, 18 h, 80%; (c) 4-nitroaniline, 180 °C, 2-5 h, 18%; (d) benzoyl chloride, diisopropylamine, CH_3CN, rt, 8 h, 85%; (e) Pd/C, H_2, THF, rt, 2 h, 48%; (f) (i) 4-nitrophenyl chloroformate, TEA, 1,4-dioxane, 60 °C, 2 h, (ii) **7** in 1,4-dioxane, 90 °C, overnight, 15%.

of 40 kinases for determination of its kinase inhibition profile. At this concentration, compound **1** showed multiple inhibition over a number of oncogenic kinases. It was further tested in a ten-dose mode over 6 kinases in order to determine its IC_{50} values.

Results and Discussion

Synthesis of the Target Compound 1 (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.[10,11] Compound **3** was heated with phosphorus oxychloride to produce 4-chloro-7-azaindole (**4**).[11] Compound **5** was prepared according to the literature procedure.[12,13] Fusion of **4** with 4-nitroanline 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**, albeit in low yield. The benzamido derivatives **6** was obtained by reaction of the amino group of **5** with benzoyl chloride in the presence of diisopropylamine as a base. Reduction of the nitro group of **6** using Pd-C/H_2 gave the corresponding amino compound **7**. The target compound **1** was prepared through heating 4-((4-ethylpiperazin-1-yl)-methyl)-3-(trifluoromethyl)aniline (**8**) with *p*-nitrophenyl chloroformate in the presence of triethylamine as a base to form the corresponding carbamate intermediate, and subsequent heating with compound **7**.[9]

Biological Screening

***In vitro* Anticancer Screening.** Our target compound was selected by the National Cancer Institute (NCI),[14] Bethesda, Maryland, USA, for *in vitro* anticancer assay against tumor cells in a panel of 60 cell lines taken from nine different tissues (blood, lung, colon, CNS, skin, ovary, kidney, prostate, and breast). The compound was tested at a single dose concentration of 10 μM, and the percentages of growth inhibition over the sixty tested cell lines were determined. The inhibition percentages expressed at this concentration over the full panel of cell lines are illustrated in Table 1. Compound **1** demonstrated a remarkable mean inhibition of 112.86% over the 60 cell lines. As shown in Table 1, compound **1** showed moderate to strong inhibitions over most of the tested cell lines. The inhibitions exceeded 100% to exert a lethal, rather than inhibitory, effect in 35 cell lines.

After the initial single dose screening, compound **1** was further tested in five-dose mode in order to determine its potencies and efficacies over the 60 cancer cell lines. IC_{50} (the concentration producing 50% inhibition), TGI (the concentration producing 100% inhibition) and LC_{50} (the concentration causing 50% lethality or 50% tumor regression) were recorded. The five-dose testing results of compound **1** are shown in Table 1. The compound showed high potency against almost all the cancer types. Its IC_{50} values were in sub-micromolar range over 22 cell lines. Of special interest, the IC_{50} values over KM12 colon cancer cell line and NCI-H460 non-small cell lung cancer were < 10 nM and 95 nM, respectively. In addition, the compound showed high efficacies, being able to induce total growth inhibition (TGI) at 87% of the tested cell lines, and 50% lethality (LC_{50}) in 32% of the tested cell lines at concentrations below 100 μM.

***In vitro* Kinase Screening.** In order to investigate the mechanism of action and the kinase inhibitory profile of the target compound **1**, it was tested at a single dose concentration of 10 μM over a panel of 40 kinases. As illustrated in Figure 2, compound **1** exerted multiple inhibitions over a

Table 1. Inhibition percentages at a single dose of 10 μM, and IC_{50}, TGI, and LC_{50} values in μM of compound **1** over 60 cancer cell lines

	Cell line	%inhibition (one-dose)	5-dose results		
			IC_{50}[a]	TGI[b]	LC_{50}[c]
Leukemia	CCRF-CEM	NA	0.659	>100	>100
	HL-60 (TB)	64.24	2.77	16.60	>100
	K-562	139.18	0.129	NA	>100
	MOLT-4	132.80	0.313	NA	>100
	RPMI-8226	NA	0.942	>100	>100
	SR	130.89	0.159	>100	>100
Non-small cell lung cancer	A549/ATCC	162.84	0.873	NA	NA
	EKVX	12.64	1.34	3.08	NA
	HOP-62	178.39	1.43	3.00	NA
	HOP-92	NA	13.3	59.6	>100
	NCI-H226	28.95	1.48	5.79	28.8
	NCI-H23	151.50	1.35	3.28	NA
	NCI-H322M	19.22	1.53	3.44	NA
	NCI-H460	144.27	0.095	NA	NA
	NCI-H522	187.19	1.89	NA	>100
Colon cancer	COLO 205	187.55	1.25	2.85	6.46
	HCC-2998	31.68	1.02	2.27	5.06
	HCT-116	183.63	0.384	NA	NA
	HCT-15	106.13	0.14	2.22	NA
	HT29	135.26	0.54	NA	NA
	KM12	85.68	<0.01	1.58	NA
	SW-620	111.89	0.495	1.76	NA
CNS cancer	SF-268	58.27	1.09	2.78	NA
	SF-295	118.90	0.503	2.03	NA
	SF-539	148.36	0.629	2.08	NA
	SNB-19	113.96	1.27	NA	NA
	SNB-75	188.19	1.06	2.28	NA
	U251	175.80	4.30	NA	NA
Melanoma	LOX IMVI	184.53	0.781	1.89	NA
	MALME-3M	148.01	1.54	NA	NA
	M14	165.05	1.22	2.65	NA
Melanoma	MDA-MB-435	161.46	0.894	2.54	NA
	SK-MEL-2	131.47	3.62	>100	>100
	SK-MEL-28	196.67	1.29	2.68	NA
	SK-MEL-5	197.65	0.764	2.02	4.53
	UACC-257	169.58	1.59	2.99	NA
	UACC-62	58.65	1.27	NA	NA
Ovarian cancer	IGROV1	56.14	0.866	NA	>100
	OVCAR-3	151.94	1.38	2.71	5.32
	OVCAR-4	21.17	1.17	2.83	NA
	OVCAR-5	69.28	1.67	3.28	NA
	OVCAR-8	128.46	0.968	4.92	>100
	NCI/ADR-RES	72.21	2.18	NA	>100
	SK-OV-3	37.28	1.80	10.5	>100
Renal cancer	786-0	184.62	1.58	3.51	NA
	A498	-4.52	3.81	17.5	52.2
	ACHN	146.31	1.05	2.31	NA
	CAKI-1	52.74	0.663	2.66	NA
	RXF 393	186.80	0.589	2.05	NA
	SN12C	41.74	7.03	>100	>100
	TK-10	74.53	1.50	>100	>100
	UO-31	148.63	1.60	3.42	NA
Prostate cancer	PC-3	45.44	1.16	4.68	>100
	DU-145	158.58	1.11	2.33	4.88
Breast cancer	MCF7	189.98	0.854	2.36	NA
	MDA-MB-231/ ATCC	139.26	1.71	NA	NA
	HS 578T	25.93	1.80	NA	>100
	BT-549	31.49	1.45	3.04	NA
	T-47D	24.39	3.29	12.4	60.4
	MDA-MB-468	39.90	1.45	3.00	NA

[a]IC_{50} is the concentration producing 50% inhibition. [b]TGI is the concentration producing 100% inhibition. [c]LC_{50} is the concentration producing 50% lethality (50% tumor regression), NA means that the datum is not available.

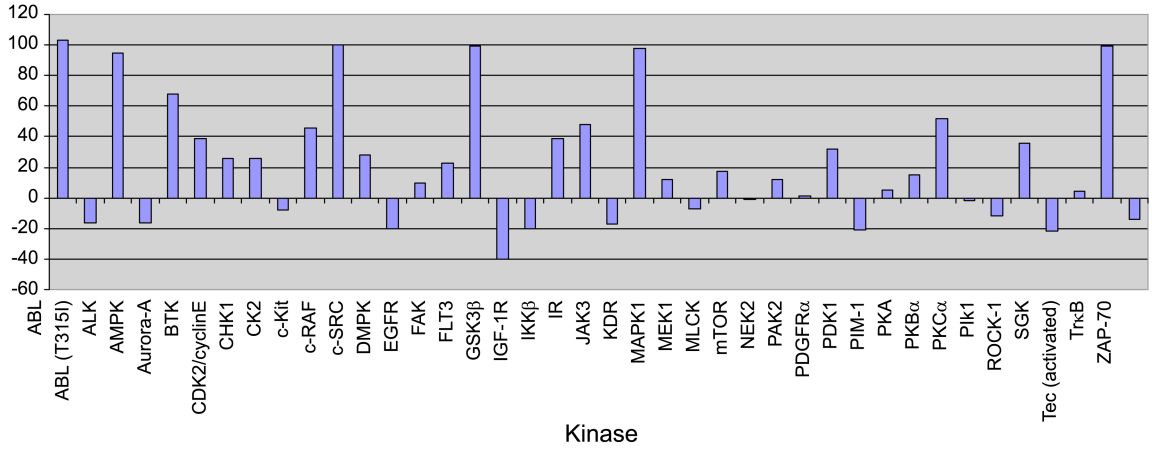


Figure 2. Inhibition percentages of compound **1** at a single dose concentration of 10 μM over 40 kinases.

number of oncogenic serine/threonine and tyrosine kinases at the test concentration. It completely inhibited the enzymatic activities of ABL, ALK, c-RAF, FLT3, KDR, and TrkB kinases. Compound **1** inhibited Aurora-A kinase also

but with a milder degree, 68%, at the same concentration. But the inhibitions were below 53% in the other tested 33 kinases.

ABL is a fusion tyrosine kinase responsible for 90% of chronic myeloid leukemia (CML) cases.[15] Anaplastic Lymphoma Kinase (ALK) is a member of the insulin receptor tyrosine kinase family. Nucleoplasmin-ALK (NPM-ALK) is constitutively active and plays an oncogenic role in 70-80% of all anaplastic large cell lymphomas.[16,17] Two other ALK fusion mutants of relevance in cancer pathogenesis were reported, tropomyosin 3 gene (TPM3-ALK) in inflammatory myofibroblastic tumors[18] and echinoderm microtubule-associated protein-like 4 gene (EML4-ALK)[19,20] in non-small cell lung cancers. Over-expression of ALK was also observed in glioblastoma[21] and neuroblastoma.[22] Dysregulated signaling through RAF kinase isoforms has been detected in ~30% of human cancers.[23] Wild type c-RAF is hyperactivated in a wide range of human solid tumors.[7] It is significantly associated with disease progression and cell proliferation in a subset of melanoma.[24-26] Mutations of the RAF protein were found in approximately 7% of human cancers[27,28] with particularly high frequency in melanoma (50-70%), ovarian (35%), thyroid (30%), and colorectal (10%) cancers. FLT3 kinase is a type III receptor tyrosine kinase whose mutations were reported in about 30% of cases of acute myeloid leukemia (AML).[29] KDR (VEGFR2) kinase plays a critical role in tumor angiogenesis in solid cancers.[30] Trk kinases are important for regulation of development and for the correct functioning of the neural system. Normally, they are expressed at low levels outside the CNS in adults. Pathological over-expression, activation, and amplification of Trk may lead to numerous cancer types including neuroblastoma,[31-34] ovarian,[35,36] prostate,[37] and colorectal cancer.[38] In addition, several studies have reported the implication of TrkB receptor with Alzeheimer's disease.[39,40] Aurora-A is a putative oncogene that is over-expressed in a number of human malignancies including colon, gastric, breast, pancreatic, and ovarian cancers.[41] The multiple inhibitions expressed by compound **1** over this group of oncogenic kinases might synergize together to give the strong and broad-spectrum anticancer activity of the compound.

Subsequently, compound **1** was tested in 10-dose testing mode in order to determine its IC_{50} values over six kinases; ABL, ALK, c-RAF, FLT3, KDR, and TrkB. Over those 6 kinases, compound **1** showed the highest inhibitions in single dose testing . The IC_{50} values are presented in Table 2. For instance, the compound demonstrated 2-digit nanomolar IC_{50} values over TrkB, KDR, and FLT3 kinases.

Table 2. IC_{50} values of compound **1** over six kinases

Kinase	IC_{50} (nM) of compound **1**	Reference compound	IC_{50} (nM) of reference compound
ABL	237	Staurosporine	213
ALK	1490	Staurosporine	11
c-RAF	259	SB203580	144
FLT3	68	Staurosporine	1.9
KDR	56	Staurosporine	13
TrkB	35	JNK Inh II	899

Conclusions

The target compound **1** showed good anticancer potencies and efficacies over a wide range of cancer cell lines. At 10 μM concentration, it exerted lethal effect over 35 cell lines of all the nine tested cancer types. In addition, it showed high potency with IC_{50} values in sub-micromolar scale over 22 cell lines of eight different cancer types. Compound **1** showed high efficacies also against a wide range of cancer cell lines. Its TGI and LC_{50} values were less than 100 μM over 39 and 8 cell lines, respectively. Due to extremely high potencies over KM12 colon cancer cell line and NCI-H460 non-small cell lung cancer, along with very high efficacy against KM12, compound **1** may be a promising candidate for treatment of colon and non-small cell lung cancers. In *in vitro* kinase screening, compound **1** demonstrated multiple inhibitions over a number of oncogenic kinases. The kinase inhibitions are anticipated to be responsible for anticancer activity of this compound. And multiple-kinase inhibitory effect of compound **1** may produce synergistic effect, and hence broad-spectrum anticancer activity. Compound **1** can be utilized as a promising lead for development of new anticancer agents with kinase inhibitory effect.

Experimental

Synthetic Experimental. The target compound was synthesized by the previously reported method.[9]

1-(4-(4-Benzamido-1*H*-pyrrolo[3,2-*c*]pyridin-1-yl)phenyl)-3-(4-((4-ethylpiperazin-1-yl)methyl)-3-trifluoromethylphenyl)urea (1). mp 265-267 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 10.85 (brs, 1H), 9.19-9.14 (m, SH), 8.11-8.08 (m, 3H), 8.02-7.97 (m, 2H), 7.72-7.66 (m, 3H), 7.63-7.53 (m, SH), 7.42 (dd, 1H, J = 6.5 Hz, J = 5.6 Hz), 2.73 (s, 4H), 2.38-2.27 (m, 6H), 0.98 (q, 2H, J = 7.2 Hz), 0.83 (t, 3H, J = 7.3 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 165.0, 152.8, 141.0, 139.8, 139.3, 133.6, 133.0, 132.9, 129.9, 129.2, 128.9, 128.8, 127.7, 126.8, 125.5, 125.2, 124.4, 123.6, 122.0, 121.7, 120.2, 119.9, 117.9, 104.8, 54.5, 53.4, 51.7, 45.1, 12.5; MS *m/z*: 642.75 (M^+ + 1, 38%), 641.72 (M^+, 100%).

NCI-60 Cell Line Screening. Cell line screening was applied at the NCI, Bethesda, Maryland, USA,[14] applying the following procedure. The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96-well microtiter plates in 100 μL at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line

are fixed in situ with trichloroacetic acid (TCA), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additional four, 10-fold or 1/2 log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions are added to the appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentrations. Following drug addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are kept for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

- $[(Ti-Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti \geq Tz$
- $[(Ti-Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$

Three dose response parameters are calculated for each experimental agent. Growth inhibition of 50% (IC_{50}) is calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from $Ti = Tz$. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from $[(Ti-Tz)/Tz] \times 100 = -50$. Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

Kinase Profiling. Reaction Biology Corp. Kinase HotSpot[SM] service[42] was used for screening of compound **1**, and IC_{50} Proler Express for IC_{50} measurement. Assay protocol: In a final reaction volume of 25 μL, kinase (5-10 mU) is incubated with 25 mM Tris pH 7.5, 0.02 mM EGTA, 0.66 mg/mL myelin basic protein, 10 mM magnesium acetate and [γ^{33}P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required). The reaction is initiated by the addition of the Mg-ATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 5 μL of a 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 ltermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

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