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Anthranilamide–Pyrazolo[1,5-*a*]pyrimidine Conjugates as p53 Activators in Cervical Cancer Cells

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A library of new anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates were designed, synthesized, and evaluated for their anticancer activity in cervical cancer cells such as HeLa and SiHa that possess low levels of p53. All 24 conjugates showed antiproliferative activity, while some of them exhibit significant cytotoxicity. In assays related to cell-cycle distribution, these conjugates induced G_2/M arrest in HeLa cells and G_1 cell-cycle arrest in SiHa cells. Immunocytochemistry assays revealed that these compounds cause nuclear translocation of p53, thereby indicating the activation of p53. In cervical cancer cells, the p53 protein is degraded by E6 oncoprotein. Immunoblot and RT-PCR analyses proved the presence of mitochondria-mediated apoptosis with involvement p53 target genes such as BAX, Bcl2, and p21 (CDKI). Moreover, these compounds increased the phosphorylated forms of p53 and provide signals for apoptosis induction. Interestingly, one of the conjugates, (2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone, is the most promising candidate in this series and has the potential to be taken up for further detailed studies.

Introduction

Cervical cancer is the second most prevalent cancer in women worldwide, both in terms of incidence and mortality rates.[1] The occurrence of cervical cancer is higher than that of breast cancer, especially in India.[2] High-risk human papillomavirus (HPV) types 16 and 18 are responsible for more than 70% of cancer cases. Progression of cells from one phase to another is regulated by cyclin-dependent kinases (CDKs) and cyclins.[3] Therefore, various therapeutic strategies that target the major players in cell-cycle regulation have been developed; these include compounds that cause reactivation or overexpression of cellular CDK inhibitors such as p21. Increased expression of CDK inhibitors causes cytostasis and senescence.[4] Although there is no mutation in the p53 gene in human HeLa S3 cervical cancer cells, p53 function and its production are impaired due to the expression of the E6 oncoprotein of HPV, which promotes the degradation of p53.[5,6] Abrogation of functional p53 is responsible for malignant transformation.[7] Therefore, any drug that enhances p53 production apart from cell-cycle control is likely to be of great value in the control and eradication of cervical cancer.[8]

Pyrazolo[1,5-*a*]pyrimidine derivatives have been reported as inhibitors of CDKs that are involved in mediating the transmission of mitogenic signals and numerous other cellular events[9–15] including cell proliferation, migration, differentiation, metabolism, and immune responses. It has also been observed that many of these derivatives may block the proliferation of various cancer cell lines.[16] A number of selective CDK inhibitors have been described, and some of them currently undergoing clinical trials are flavopiridol (**1**),[17] roscovitine (**2**),[18] and BMS 387032 (**3**),[19] as shown in Figure 1. However, there are opportunities to identify and develop additional

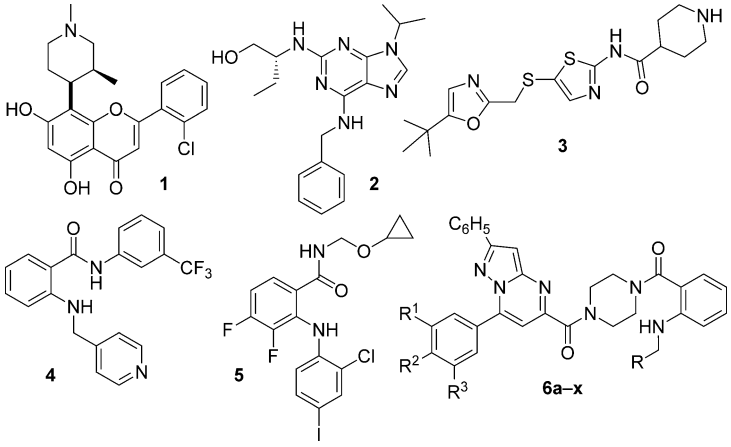

Figure 1. Structures of flavopiridol (**1**), roscovitine (**2**), BMS 387032 (**3**), anthranilamide AAL993 (**4**), Cl-1040 (**5**), and anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates **6a**–**x**.

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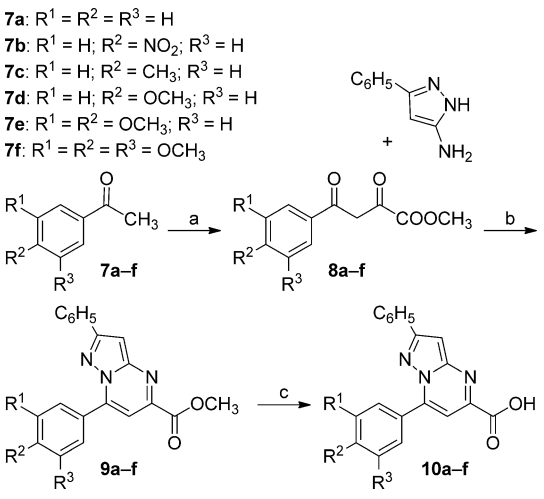
novel CDK inhibitors that possess superior biological profiles than those of the presently known candidates. Hence, a considerable challenge in this area is the identification of new conjugates composed of pharmacophores of known antitumor agents that enhance selectivity and antitumor activity. An anthranilamide moiety is present in agents such as AAL993 (**4**) and PD 184352 (CI-1040; **5**),[20,21] which is considered responsible for their potent antitumor properties[22,23] (Figure 1). Similarly, over the last decade, a number of piperazine derivatives have been synthesized to exploit their chemotherapeutic potential.[24–26] Symmetrical bifunctional agents equipped with a piperazine moiety in the linker have been reported as a promising class of antitumor compounds with remarkable selectivity against colon cancers.[27] Recently, *trans*-diamine dichloroplatinum(II) complexes with piperazine ligands were reported that exhibit significant cytotoxicity.[28] In continuation of these efforts, the present work describes the design and synthesis of a series of anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates (**6a**–**x**) to explore their potential as a new class of anticancer agents. All these compounds were evaluated for their anticancer activity against a panel of human cancer cell lines, and the most promising compounds among them (**6f**, **6l** and **6r**) were evaluated for cytotoxicity, cell-cycle effects, induction of apoptosis, and effects on tumor suppressor genes (p53 and p21). Interestingly, this new class of conjugates has a unique mode of action that differs from that of other known antitumor compounds.

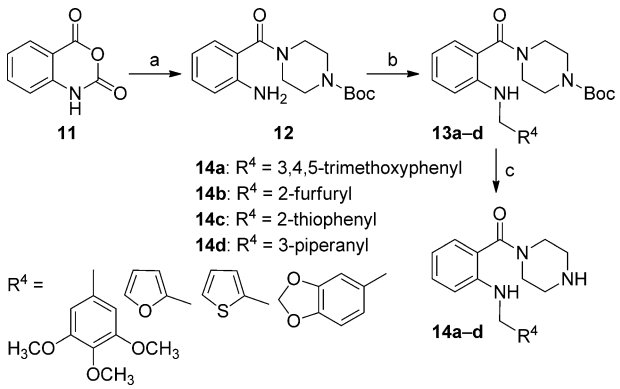
Results and Discussion

Chemistry

Synthesis of the target conjugates was performed in a convenient manner as outlined in Schemes 1, 2 and 3. As shown in Scheme 1, the β-diketo esters **8a**, **8b**, **8d**, and **8e** were synthesized as reported earlier.[29] The various substituted acetophenones were oxylated by dimethyl oxalate in the presence of sodium methoxide to provide the β-diketo esters **8a**–**f**. Heterocycle formation via dehydration coupling with an aza-heterocyclic amine, i.e., 3-amino-5-phenylpyrazole, afforded various substituted 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic esters[30] (**9a**–**f**), which, upon base hydrolysis with sodium hydroxide, provided the corresponding carboxylic acids **10a**–**f**. One of the precursors **14c** was synthesized as reported[31] by us previously; however, the other precursors **14a**, **14b**, and **14d** were prepared by treatment of isatoic anhydride (**11**) with *N*-Boc piperazine at a molar ratio of 1:1.2, and then held at reflux in 1,4-dioxane to give the 4-(2-aminobenzoyl)-*N*-Boc piperazine **12** in nearly quantitative yield. Upon reductive amination of this intermediate with various heteroaryl and aromatic aldehydes using sodium cyanoborohydride in methanol with catalytic amounts of acetic acid afforded compounds **13a**–**d**. These, upon deprotection with triflouroacetic acid, gave the required precursors **14a**–**d** as shown in Scheme 2. Synthesis of anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates **6a**–**x** was carried out by using these precursors (**14a**–**f**). The target conjugates were synthesized by coupling **14a**–**d** to different substituted 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acids (**10a**–**f**) in presence of EDCI and HOBt to afford the corresponding anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates **6a**–**x** (Scheme 3). All the synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, as well as mass spectrometry. Conjugates **6a**–**x** were evaluated for their anticancer activity in selected human cancer cell lines.



Scheme 1. *Reagents and conditions:* a) dimethyl oxalate, NaOMe, THF, RT, 8 h; b) HCl (cat.), EtOH, reflux, 2 h; c) 2 N NaOH, MeOH, reflux, 2 h.



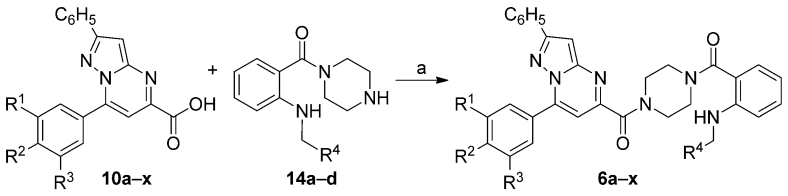
Scheme 2. *Reagents and conditions:* a) *N*-Boc piperazine, 1,4-dioxane, reflux, 4 h; b) R^4-CHO, $NaCNBH_3$, MeOH, CH_3COOH, RT, 22 h; c) TFA, CH_2Cl_2, RT, 5 h.

Biology

Anticancer activity

Some representative anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates were tested against 60 human cancer cell lines derived from nine types of cancer (lung, leukemia, colon, melanoma, ovarian, renal, prostate, breast, and CNS) at a single dose at the National Cancer Institute (NCI; Bethesda, MD, USA). Three anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates—**6f**, **6l**, and **6r**—that exhibited considerable activity in the pri-

mary screen were further evaluated against the panel of 60 human cancer cell lines at five concentrations, and the results are listed in Table 1. These three compounds exhibit a broad spectrum of activity against various cancer cell lines with GI_{50} values in the range of 0.58–53 μM. Specifically, conjugate **6r** exhibits significant activity against most of the cancer cell lines, with GI_{50} values ranging from 0.58–6.8 μM.



6a: $R^1 = R^2 = R^3 = H$; R^4 = 3,4,5-trimethoxyphenyl
6b: $R^1 = H$; $R^2 = NO_2$; $R^3 = H$; R^4 = 3,4,5-trimethoxyphenyl
6c: $R^1 = H$; $R^2 = CH_3$; $R^3 = H$; R^4 = 3,4,5-trimethoxyphenyl
6d: $R^1 = H$; $R^2 = OCH_3$; $R^3 = H$; R^4 = 3,4,5-trimethoxyphenyl
6e: $R^1 = R^2 = OCH_3$; $R^3 = H$; R^4 = 3,4,5-trimethoxyphenyl
6f: $R^1 = R^2 = R^3 = OCH_3$; R^4 = 3,4,5-trimethoxyphenyl
6g: $R^1 = R^2 = R^3 = H$; R^4 = 2-furfuryl
6h: $R^1 = H$; $R^2 = NO_2$; $R^3 = H$; R^4 = 2-furfuryl
6i: $R^1 = H$; $R^2 = CH_3$; $R^3 = H$; R^4 = 2-furfuryl
6j: $R^1 = H$; $R^2 = OCH_3$; $R^3 = H$; R^4 = 2-furfuryl
6k: $R^1 = R^2 = OCH_3$; $R^3 = H$; R^4 = 2-furfuryl
6l: $R^1 = R^2 = R^3 = OCH_3$; R^4 = 2-furfuryl
6m: $R^1 = R^2 = R^3 = H$; R^4 = 2-thiophenyl
6n: $R^1 = H$; $R^2 = NO_2$; $R^3 = H$; R^4 = 2-thiophenyl
6o: $R^1 = H$; $R^2 = CH_3$; $R^3 = H$; R^4 = 2-thiophenyl
6p: $R^1 = H$; $R^2 = OCH_3$; $R^3 = H$; R^4 = 2-thiophenyl
6q: $R^1 = R^2 = OCH_3$; $R^3 = H$; R^4 = 2-thiophenyl
6r: $R^1 = R^2 = R^3 = OCH_3$; R^4 = 2-thiophenyl
6s: $R^1 = R^2 = R^3 = H$; R^4 = 3-piperanyl
6t: $R^1 = H$; $R^2 = NO_2$; $R^3 = H$; R^4 = 3-piperanyl
6u: $R^1 = H$; $R^2 = CH_3$; $R^3 = H$; R^4 = 3-piperanyl
6v: $R^1 = H$; $R^2 = OCH_3$; $R^3 = H$; R^4 = 3-piperanyl
6w: $R^1 = R^2 = OCH_3$; $R^3 = H$; R^4 = 3-piperanyl
6x: $R^1 = R^2 = R^3 = OCH_3$; R^4 = 3-piperanyl

Scheme 3. *Reagents and conditions:* a) EDCI/HOBt, CH_2Cl_2, 0 °C→RT, 8 h.

Cell viability (MTT) assay

In view of the encouraging GI_{50} values, we next evaluated the cytotoxicity of these compounds by MTT assay. Compounds such as **6f**, **6l**, and **6r**, which exhibited efficient antiproliferative activity in the preliminary screen, were evaluated for their cytotoxicity against two representative human cervical cancer cell lines: HeLa and SiHa; MTT assays were performed by treating cells for this compound series.[32] Determination of proliferation by MTT assay clearly showed that all three compounds are highly cytotoxic, more so than roscovitine, a positive control used in the study. Concentrations <1 μM were found to have negligible effects on cytotoxicity. Dose–response analyses were carried out at conjugate concentrations of 1, 2, and 4 μM, and from these data, IC_{50} values were deduced. IC_{50} values for these conjugates were in the ranges of 1.46–2.72 and 1.56–2.70 μM for the HeLa and SiHa cell lines, respectively. From the biological data (inhibition of proliferation) for all the conjugates, it is apparent that compounds **6f**, **6l**, and **6r** are the most effective in both cervical cancer cell lines (Table 2). Interestingly, the substitution pattern (R^1, R^2, and R^3) on the 7-phenyl ring of the pyrazolo[1,5-*a*]pyrimidine subunit considerably affects the anticancer activity of these conjugates. Trisubstituted compounds such as 3,4,5-trimethoxy analogues display higher potency than those of other substitution patterns on the pyrazolo[1,5-*a*]pyrimidine moiety phenyl ring. In contrast, heteroaryl and aromatic aldehyde substitutions on the phenyl ring of the anthranilamide subunit do not exhibit a significant effect on the potency of such analogues. These aspects reveal that 3,4,5-trimethoxy substitution on the 7-phenyl ring of the pyrazolo[1,5-*a*]pyrimidine subunit is essential for maintaining antitumor activity.

Table 1. Anticancer activity of anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates in human cancer cell lines.

Cancer panel Cell lines	GI_{50} [μM] **6f**[b]	**6l**[c]	**6r**[d]	*Cancer panel* Cell lines	GI_{50} [μM] **6f**[b]	**6l**[c]	**6r**[d]
Leukemia				*Ovarian*			
HL-60(TB)	3.7	6.1	1.98	IGROV1	5.58	13.9	7.7
K-562	1.9	1.38	1.5	OVCAR-3	2.5	3.0	2.69
MOLT-4	4.9	43	3.3	OVCAR-4	6.83	5.5	4.37
SR	NT[e]	3.3	0.58	OVCAR-5	12	15	10
CCRF-CEM	3.5	NT[e]	NT[e]	OVCAR-8	4.47	30	4.3
RPMI-8226	4.0	NT[e]	NT[e]	NCI/ADR-RES	1.2	2.6	1.9
				SK-OV-3	2.67	5.5	4.4
Non-small-cell lung				*Renal*			
A549/ATCC	2.8	5.6	3.5	786-0	4.6	11.4	4.49
EKVX	6.0	53	4.9	A498	2.99	8.4	2.1
HOP-62	3.8	11	6.0	ACHN	7.3	4.9	4.2
HOP-92	NT[e]	2.41	NT[e]	CAKI-1	1.9	2.78	1.39
NCI-H226	8.4	6.39	4.44	RXF 393	2.0	2.45	2.3
NCI-H23	4.3	15.7	4.96	SN12C	5.0	29	17
NCI-H322M	5.3	NT[e]	5.6	TK-10	6.0	20	6.8
NCI-H460	2.8	4.26	3.3	UO-31	4.87	6.99	1.5
NCI-H522	1.0	1.67	2.2				
Colon				*Prostate*			
COLO 205	1.3	4.0	4.1	PC-3	2.2	4.18	2.3
HCC-2998	7.3	14.3	5.8	DU-145	3.27	5.0	3.96
HCT-116	3.6	4.49	NT[e]				
HCT-15	3.6	4.43	4.2				
HT29	1.1	3.78	3.5				
KM12	1.2	3.16	2.78				
SW-620	2.98	4.7	3.67				
CNS				*Breast*			
SF-268	5.2	5.9	4.89	MCF7	1.5	2.5	3.0
SF-295	2.3	3.78	2.18	MDA-MB-231/ATCC	3.8	8.67	7.0
SF-539	2.7	9.85	2.5	HS 578T	2.66	36	3.8
SNB-19	5.0	8.9	6.7	BT-549	5.3	6.26	3.27
SNB-75	1.56	3.3	1.98	T-47D	3.2	8.38	NT[e]
U251	3.6	5.76	3.37	MDA-MB-468	2.46	2.71	2.1
Melanoma				*Melanoma*			
LOX IMVI	3.79	6.0	4.8	SK-MEL-28	12.9	9.6	5.2
MALME-3M	7.3	NT[e]	NT[e]	SK-MEL-5	1.46	2.85	1.58
M14	3.8	5.9	4.0	UACC-257	3.9	NT[e]	4.97
MDA-MB-435	0.3	1.16	0.66	UACC-62	2.3	2.8	2.37
SK-MEL-2	3.2	3.7	4.37				

[a] Compound concentration required to decrease cell growth to half that of untreated cells. [b] **6f** (NSC755290). [c] **6l** (NSC755289). [d] **6r** (NSC755288). [e] Not tested.

Effect on cell cycle

Previous studies have demonstrated that roscovitine, a CDK inhibitor, controls cell growth by cell-cycle arrest.[33] Therefore, we considered it important to understand the role of these conjugates (**6a**–**x**) on cell-cycle progression in cells where p53, the main cell-cycle checkpoint, is expressed at negligible levels.[34,35] Treatment of HeLa cells with these compounds at

2 μM (the IC_{50} concentration) caused an increase in the percentage of cells in the G_2/M phase and a decrease of those in the G_1 phase (i.e., G_2/M arrest). However, treatment of SiHa cells with the same compounds caused G_1 arrest (Figure 2; see also Supporting Information tables 3 and 4).

Table 2. In vitro cytotoxicity of compounds.

Compound	IC_{50} [μM][a] SiHa	HeLa
6a	2.08±0.09	2.05±0.05
6b	2.71±0.12	2.42±0.44
6c	2.24±0.22	1.81±2.24
6d	2.23±0.18	1.89±0.13
6e	2.07±0.44	2.46±0.24
6f	1.91±0.17	1.51±0.20
6g	1.93±0.32	2.72±0.02
6h	2.33±0.39	2.02±0.38
6i	2.16±0.11	2.02±0.16
6j	2.19±0.50	1.73±0.18
6k	1.83±0.49	1.56±0.12
6l	1.61±0.30	1.54±0.18
6m	2.06±0.23	2.07±0.36
6n	2.04±0.48	2.38±0.30
6o	2.35±0.05	2.35±0.05
6p	2.34±0.38	2.12±0.15
6q	1.81±0.22	1.99±0.17
6r	1.56±0.30	1.46±0.15
6s	2.14±0.42	1.95±0.19
6t	1.99±0.17	1.99±0.64
6u	2.71±0.39	2.13±0.05
6v	2.40±0.16	1.95±0.42
6w	2.40±0.58	2.35±0.27
6x	2.31±0.54	1.76±0.24
Roscovitine[b]	2.69±0.35	2.20±0.12

[a] Compound concentration required to inhibit cancer cell proliferation by 50%; data are expressed as the mean ±SD from the dose–response curves of at least three independent experiments. [b] Positive control reference compound.

BrdU incorporation assay

Interestingly, compounds **6f**, **6l**, and **6r** showed enhanced cytotoxicity apart from this significant effect on cell cycle, thereby prompting us to take up a detailed investigation on these conjugates. The cell-cycle arrest caused by **6f**, **6l**, **6r**, and roscovitine (ros) was confirmed by a 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay (Figure 3). HeLa cells treated with conjugates showed no change in BrdU incorporation relative to control. This confirms G_2/M arrest for HeLa, whereas SiHa cells treated with conjugates showed a decrease in BrdU incorporation; these data strongly confirm G_1 phase cell-cycle arrest. It is plausible that different cells originated from the same cancer phenotype respond differently to the same conjugates.

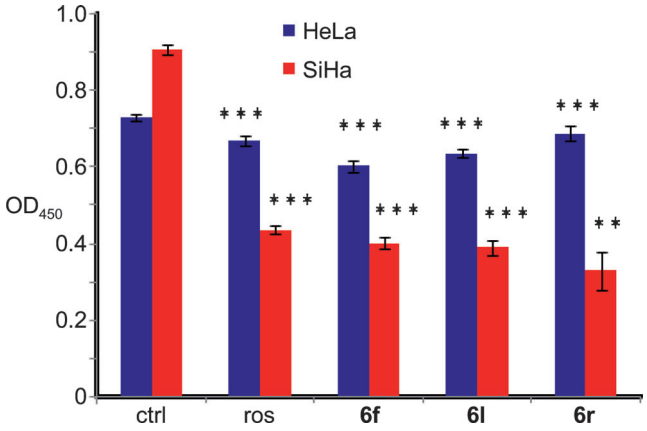


Figure 3. BrdU assay: HeLa and SiHa cells were treated with compounds (ros, **6f**, **6l**, and **6r**) at a final concentration of 2 μM and incubated for 24 h. BrdU incorporation was monitored to confirm G_1 cell-cycle arrest by test compounds in SiHa cells. Statistical significance of effects observed in compound-treated cells relative to control (ctrl): $^{***}p<0.001$, $^{**}p<0.01$.

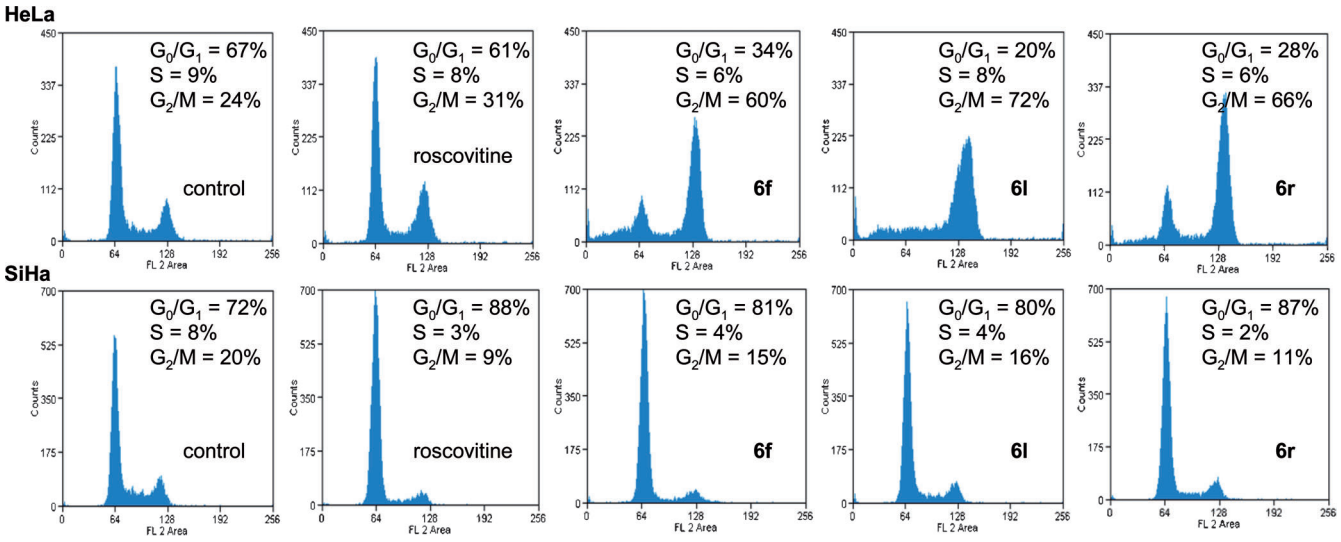


Figure 2. Cell-cycle distribution: HeLa (top row) and SiHa (bottom row) cells treated with roscovitine, **6f**, **6l**, and **6r** were studied immediately following an incubation period of 24 h. HeLa cells show an increased percentage of cells arrested in the G_2/M phase relative to control cells, whereas SiHa cells exhibit G_1 cell-cycle arrest.

Effect on p53 and dependent genes

p53 is a tumor suppressor that plays a crucial role in cell-cycle arrest at G_1 and G_2/M and in apoptotic processes.[36,37] In general, the activation of p53 occurs by phosphorylation at serine residues 46 and 15; it then translocates to the nucleus upon receipt of stress signals induced by anticancer agents.[38] Immediately after activation, p53 further controls genes such as p21, Bcl2, and BAX,[38] each of which contain p53 binding sites in their promoters. Thus HeLa cells were treated with compounds **6f**, **6l**, and **6r** at 2 μM (Figure 4A), and after incubation for 24 h, gene expression patterns were studied by RT-PCR analysis. Interestingly, p53 and p21 transcript levels increased in the compound-treated cells, wherein GAPDH serves as an internal control. To further confirm p53 activation, immunofluorescence experiments were carried out with **6r**-treated HeLa cells, with roscovitine as control. We observed complete nuclear translocation in compound-treated cells, whereas control-untreated cells showed p53 protein localization in both the nucleus and cytoplasm. This strongly suggests the p53-activating nature of these compounds[38] (Figure 4B). We also observed a decrease in the levels of Bcl2 proteins and increased expression of phosphorylated forms of p53 as well as BAX protein levels in compound-treated cells (Figure 4C). These results support the activation of p53 by these conjugates.

Effect on caspase-3 activation

Caspases are cysteine proteases that are known to be the main executors in the process of apoptosis.[39] Among all caspases, caspase-3 is a frequently activated death protease and is indispensible for DNA fragmentation, chromatin condensation, and apoptotic body formation.[40] Because an increase in the levels of p53 protein cause its nuclear translocation, we were interested in investigating downstream signaling events such as induction of apoptosis, which is caused by caspases. Therefore, caspase-3 activity was assayed to understand the induction of apoptosis by these compounds. Interestingly, compound-treated cells showed a near twofold increase in caspase-3 activation relative to control (untreated) cells; the known CDK inhibitor roscovitine was used as a positive control (Figure 5). Thus, this study revealed that the caspase-mediated apoptotic pathway is responsible for the antiproliferative activity of these compounds (Figure 6).

Conclusions

In the present study, a series of anthranilamide–pyrazolo[1,5-*a*]pyrimidine conjugates were synthesized and evaluated for their anticancer activity. Cervical cancer cell lines that express negligible p53 protein levels were selected in this study. These compounds exhibited significant anticancer activity, with IC_{50} values in the ranges of 1.46–2.72 and 1.56–2.7 μM in HeLa and SiHa cells, respectively. Compounds **6f**, **6l**, and **6r** were found to be much more cytotoxic than the other compounds in this series. Studies also showed that **6f**, **6l**, and **6r** caused the highest G_2/M cell-cycle arrest in HeLa cells and G_1 phase arrest in SiHa cells. They not only regulate cell cycle, but also induce expression of p53 as well as p21 transcript levels. These compounds also induce the BAX protein and decrease the Bcl2 protein levels, which are dependent on p53 and cause apoptosis in a caspase-3 dependent manner. Interestingly, molecules

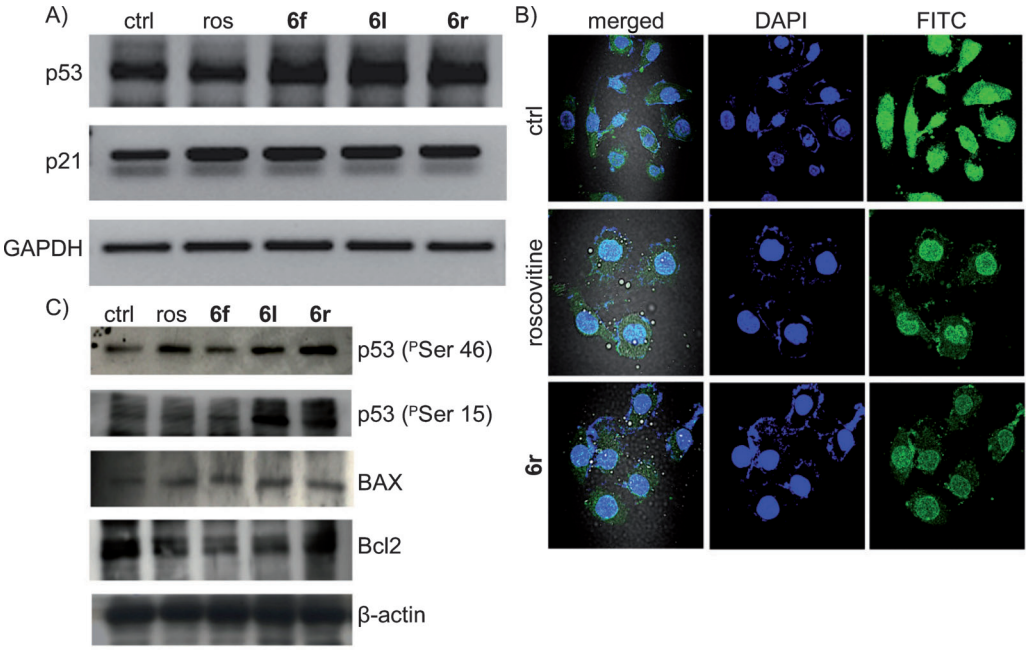


Figure 4. Purine conjugates activate p53 and induce mitochondria-mediated apoptosis: A) RT-PCR analysis was conducted to examine the change in gene expression levels of p53 and its target gene p21 in HeLa cells treated with roscovitine, **6f**, **6l**, and **6r** for 24 h. B) p53 localization was examined in cells treated with conjugate **6r**; roscovitine was used as positive control. C) The activated (i.e., phosphorylated) forms of p53 as well as genes that are tightly associated with apoptosis, such as BAX and Bcl2, were examined by Western blot analysis; β-actin was used as loading control.

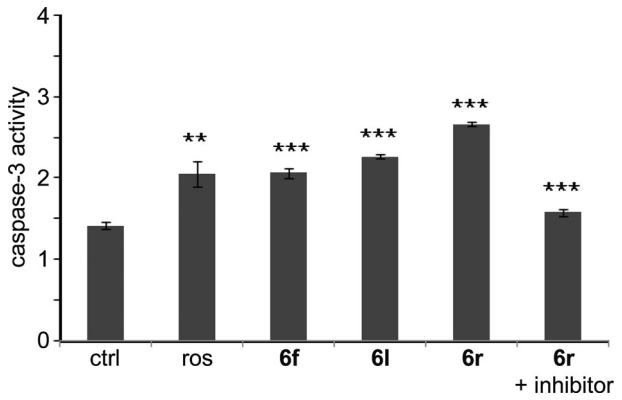


Figure 5. Effect of purine conjugates on caspase-3 activity in HeLa cells: The increased activity of caspase-3 during apoptosis after treatment with conjugates **6f**, **6l** and **6r** at 2 μM was measured by fluorimetry. The cleavage of the peptide substrate by active caspase-3 releases the fluorophore AFC, which was quantified at an excitation wavelength of 400 nm and an emission wavelength of 505 nm (DEVD-CHO is the indicated inhibitor of caspase-3 in the rightmost column). Roscovitine (ros) was used as positive control. Statistical significance of effects observed in compound-treated cells relative to control (ctrl): $^{***}p < 0.001$, $^{**}p < 0.01$.

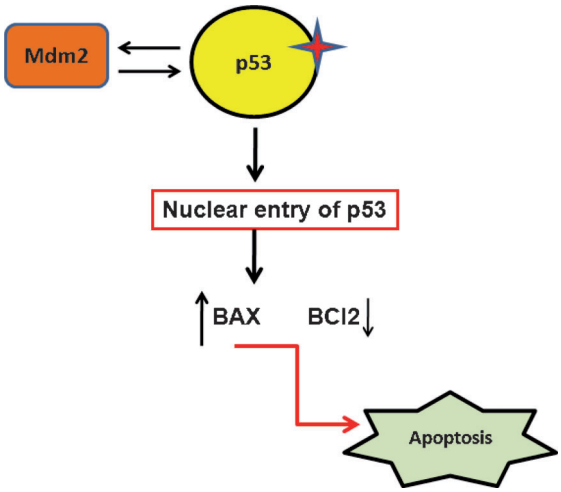


Figure 6. Schematic diagram representing the action of analogues on p53 activation by phosphorylation: Activated p53 translocates to the nucleus and regulates Mdm2 levels and further acts on mitochondria, where a decrease in Bcl2 oncoprotein and an increase BAX protein levels causes caspase-mediated apoptosis.

that can induce p53, particularly in cervical cancer cell lines (HeLa and SiHa), are of great value and are likely to be useful in the treatment of cancer, either alone or in combination with other anticancer agents.

Experimental Section

Chemistry

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA), or Spectrochem Pvt. Ltd. (Mumbai, India), and were used without further purification. Reactions were monitored by TLC performed on glass plates containing silica gel 60 GF_{254}, and visualization was carried out by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H NMR spectra were recorded on Bruker UXNMR/XWIN-NMR (300 MHz) or Inova Varian VXR Unity (400, 500 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from an internal TMS standard. ESIMS data were recorded on a Micromass Quattro LC using ESI+ software at a capillary voltage of 3.98 kV and ESI mode positive ion trap detector. High-resolution MS (HRMS) data were recorded on a QSTAR XL Hybrid MS–MS mass spectrometer. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected. High-performance liquid chromatography (HPLC) analyses for determining the purity of synthesized compounds were performed on a Shimadzu SPD-10A (UV/Vis detector) LC-10AT instrument [column: Luna 5 μm C_{18}(2) 250×4.60 mm Mightysil RP-18 GP 250×4.6 mm (5 μm); mobile phase: 80% A (CH_3CN) and 20% B (H_2O) in 15 min; flow rate: 1 mL min^{-1}; injected sample: 10 μL; column temperature: 27 °C; wavelength: 254 nm]. The purity of all compounds was ≥95% based on analytical HPLC. See the Supporting Information for data for compounds **8c–14d**.

(2,7-Diphenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)methanone (6a): Conjugate **6a** was prepared by amide bond formation between piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7a**, 154 mg, 1.2 mmol) in dry CH_2Cl_2. The coupling reagents EDCI (1.2 mmol) and HOBt (1.2 mmol) were added, and the reaction mixture was stirred at room temperature for 10 h. After completion of reaction as indicated by TLC, the reaction mixture was quenched with $NaHCO_3$ and extracted in EtOAc (4× 25 mL) from the ice-cold aqueous layer and dried over anhydrous Na_2SO_4. The resulting product **6a** was purified by column chromatography to afford a yellow solid: 223 mg, 80% yield. R_f=0.29 (EtOAc/hexane, 7:3); mp: 111–112 °C; IR (KBr) $\tilde{\nu}$=3373, 2932, 1757, 1627, 1550, 1234, 841, 760 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ= 3.78–3.82 (m, 4H), 3.84 (s, 9H), 3.87–3.96 (m, 4H), 4.29 (s, 2H), 5.6 (br s, 1H), 6.58 (d, 2H, J=9 Hz), 6.68–6.74 (m, 2H), 7.07–7.15 (m, 2H), 7.34 (d, 1H, J=5.2 Hz), 7.41–7.53 (m, 5H), 7.61 (t, 2H, J= 2.2 Hz), 8.01 (dd, 2H, J=1.5, 8.3 Hz), 8.20–8.25 ppm (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ=42.9, 46.9, 47.9, 56, 60.7, 94.2, 94.6, 103.8, 107.1, 112.2, 116.1, 116.2, 118.5, 126.4, 126.5, 127.1, 127.8, 128.5, 128.9, 129.2, 129.6, 130.7, 131.5, 132.4, 134.5, 147.2, 149, 151.1, 156.8, 165.8, 170.7 ppm; MS (ESI): 683 $[M+H]^+$; HRMS (ESI) calcd for $C_{40}H_{39}N_6O_5$ $[M+H]^+$ 683.2740, found: 683.2732; anal. calcd for $C_{40}H_{38}N_6O_5$: C 70.36, H 5.61, N 12.31, O 11.72, found: C 70.32, H 5.55, N 12.28, O 11.70; purity 95.10% (HPLC).

(7-(4-Nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)methanone (6b): Compound **6b** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7b**, 176 mg, 0.49 mmol) to afford pure compound **6b** as a yellow solid in 244 mg, 82% yield. R_f= 0.31 (EtOAc/hexane, 7:3); mp: 113–114 °C; IR (KBr) $\tilde{\nu}$=3380, 3065, 2925, 1629, 1604, 1549, 1257, 831, 755 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ=3.69–3.79 (m, 4H), 3.80 (s, 3H), 3.83 (s, 6H), 3.84–3.91 (m, 2H), 3.92–4.0 (m, 2H), 4.27 (s, 2H), 5.69 (br s, 1H), 6.55 (s, 2H), 6.66 (t, 2H, J=8.3 Hz), 7.03 (s, 1H), 7.09 (d, 1H, J=6.0 Hz), 7.17–7.24 (m, 2H), 7.34 (s, 1H), 7.36–7.48 (m, 3H), 7.59 (t, 2H, J=3.7 Hz), 7.97 (d, 2H, J=7.5 Hz), 8.19–8.25 ppm (m, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ=42.8, 47.5, 47.9, 56.4, 60.9, 94.4, 103.1, 106.9, 112, 114,

116.2, 118.2, 126.3, 126.9, 127.4, 128.3, 128.7, 128.9, 129.3, 129.8, 130.5, 131.7, 132.6, 134.7, 148, 149.2, 151.3, 152.4, 156.9, 162.2, 165.5, 170.8 ppm; MS (ESI): 728 $[M+H]^+$; HRMS (ESI) calcd for $C_{40}H_{38}N_7O_7$ $[M+H]^+$ 728.2432, found: 728.2413; anal. calcd for $C_{40}H_{37}N_7O_7$: C 66.01, H 5.12, N 13.47, O 15.39, found: C 65.98, H 5.09, N 13.46, O 15.37; purity 95.30% (HPLC).

(2-Phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)methanone (6c): Compound **6c** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7c**, 161 mg, 0.49 mmol) to afford pure compound **6c** as a yellow solid in 214 mg, 75% yield. $R_f=0.33$ (EtOAc/hexane, 7:3); mp: 116–117 °C; IR (KBr) $\tilde{\nu}=3371$, 2927, 2835, 1631, 1605, 1550, 1239, 838, 755 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=2.49$ (s, 3H), 3.75–3.83 (m, 4H), 3.85–3.91 (m, 4H), 3.92 (s, 3H), 3.93 (s, 6H), 4.36 (s, 2H), 5.47 (br s, 1H), 6.74 (t, 1H, $J=4.4$ Hz), 7.06–7.14 (m, 3H), 7.19 (t, 1H, $J=8.8$ Hz), 7.27–7.29 (m, 2H), 7.31 (s, 1H), 7.38–7.42 (m, 3H), 7.46 (t, 2H, $J=7.7$ Hz), 8.01 (d, 2H, $J=6.6$ Hz), 8.15 ppm (d, 2H, $J=7.7$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=21.6$, 42.9, 43.1, 47.5, 56.1, 56.2, 61, 94.5, 105.9, 106.7, 116.9, 117.6, 118.7, 118.9, 126.6, 127.6, 127.9, 128.7, 128.9, 129.3, 129.4, 130.3, 130.4, 131, 132.6, 142, 145.8, 147.3, 149.2, 151.2, 153.5, 156.8, 161.2, 166.0 ppm; MS (ESI): 697 $[M+H]^+$; HRMS (ESI) calcd for $C_{41}H_{41}N_6O_5$ $[M+H]^+$ 697.3109, found: 697.3118; anal. calcd for $C_{41}H_{40}N_6O_5$: C 70.67, H 5.79, N 12.06, O 11.48, found: C 70.62, H 5.74, N 12.04, O 11.46; purity 95.10% (HPLC).

(7-(4-Methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)methanone (6d): Compound **6d** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 7-(4-methoxyphenyl)-2-phenylpyrazolo-[1,5-*a*]pyrimidine-5-carboxylic acid (**7d**, 169 mg, 0.49 mmol) to afford pure compound **6d** as a yellow solid in 221 mg, 76% yield. $R_f=0.28$ (EtOAc/hexane, 7:3); mp: 104–105 °C; IR (KBr) $\tilde{\nu}=3392$, 2931, 2837, 1630, 1601, 1549, 1256, 831 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=3.64$–3.73 (m, 4H), 3.74 (s, 3H), 3.77 (s, 6H), 3.78–3.84 (m, 2H), 3.86 (s, 3H), 3.87–3.93 (m, 2H), 3.78–3.84 (m, 2H), 4.20 (s, 2H), 5.62 (br s, 1H), 6.49 (s, 2H), 6.55–6.63 (m, 2H), 6.94 (s, 1H), 6.99–7.05 (m, 3H), 7.09–7.21 (m, 2H), 7.28–7.41 (m, 3H), 7.92 (d, 2H, $J=6.9$ Hz), 8.21 ppm (d, 2H, $J=8.8$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=42.9$, 47.5, 48, 55.5, 56.1, 60.8, 94.4, 104.1, 106.2, 112.3, 114, 116.3, 122.7, 126.6, 127.9, 128.8, 129.2, 131.3, 131.4, 131.5, 132.6, 134.6, 146.9, 147.1, 149.3, 151.1, 152, 153.4, 156.8, 162.2, 166.1, 170.8 ppm; MS (ESI): 713 $[M+H]^+$; HRMS (ESI) calcd for $C_{41}H_{41}N_6O_6$ $[M+H]^+$ 713.3087, found: 713.3120; anal. calcd for $C_{41}H_{40}N_6O_6$: C, 69.09; H, 5.66; N, 11.79; O, 13.47, found: C 69.03, H 5.69, N 11.80, O 13.44; purity 99.50% (HPLC).

(7-(3,4-Dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)-methanone (6e): Compound **6e** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 7-(3,4-dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7e**, 183 mg, 0.49 mmol) to afford pure compound **6e** as a yellow solid in 222 mg, 73% yield. $R_f=0.27$ (EtOAc/hexane, 7:3); mp: 115–116 °C; IR (KBr) $\tilde{\nu}=3393$, 2836, 1626, 1597, 1552, 1237, 813, 761 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=3.78$–3.83 (m, 4H), 3.85 (s, 9H), 3.87–3.97 (m, 4H), 4.02 (s, 6H), 4.29 (s, 2H), 5.61 (br s, 1H), 6.60 (s, 2H), 6.71 (t, 2H, $J=7.9$ Hz), 7.05–7.16 (m, 3H), 7.24 (d, 1H, $J=8.3$ Hz), 7.35 (s, 1H), 7.41–7.52 (m, 3H), 7.89 (dd, 1H, $J=2.2$, 8.3 Hz), 7.98–8.06 ppm (m, 3H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=42.9$, 47.4, 48, 56, 60.8, 94.4, 104, 106.2, 110.8, 112.2, 112.3, 116.2, 118.2, 122.7, 123.2, 126.4, 127.9, 128.7, 129.2, 131.4, 132.5, 134.5, 136.9, 146.8, 147, 148.5, 149.2, 151, 151.6, 153.3, 156.6, 166, 170.7 ppm; MS (ESI): 742 $[M^+]$; purity 98.20% (HPLC).

(2-Phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(3,4,5-trimethoxybenzylamino)benzoyl)piperazin-1-yl)-methanone (6f): Compound **6f** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(3,4,5-trimethoxybenzylamino)phenyl)methanone (**14a**, 160 mg, 0.41 mmol) and 2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7f**, 198 mg, 0.49 mmol) to afford pure compound **6f** as a yellow solid in 221 mg, 70% yield. $R_f=0.23$ (EtOAc/hexane, 7:3); mp: 114–115 °C; IR (KBr) $\tilde{\nu}=3384$, 2934, 2834, 1627, 1581, 1551, 1239, 832, 761 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=3.72$–3.82 (m, 4H), 3.84 (s, 9H), 3.86–3.95 (m, 4H), 3.97 (s, 6H), 3.99 (s, 3H), 4.29 (s, 2H), 5.59 (br s, 1H), 6.59 (d, 2H, $J=4.4$ Hz), 6.69–6.74 (m, 2H), 7.09–7.15 (m, 2H), 7.36 (s, 1H), 7.40–7.52 (m, 4H), 7.57 (s, 2H), 8.0 ppm (d, 2H, $J=7.7$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=42.9$, 44.2, 47.9, 56, 56.3, 60.7, 61, 94.6, 104.1, 106.7, 107, 107.3, 112.3, 116.2, 118.2, 125.3, 126.4, 127.8, 127.9, 128.8, 129.2, 131.5, 132.8, 134.5, 137, 140.8, 146.8, 147.4, 149.2, 151, 153.3, 155.8, 165.8, 170.7 ppm; MS (ESI): 773 $[M+H]^+$; HRMS (ESI) calcd for $C_{43}H_{45}N_6O_8$ $[M+H]^+$ 773.3293, found: 773.3289; anal. calcd for $C_{43}H_{44}N_6O_8$: C, 66.83; H, 5.74; N, 10.87; O, 16.56 found: C, 66.85, H 5.70, N 10.84, O 16.54; purity 98.40% (HPLC).

(2,7-Diphenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(furan-2-ylmethylamino)benzoyl)piperazine-1-yl)methanone (6g): Compound **6g** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)-phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7a**, 211 mg, 0.67 mmol) to afford pure compound **6g** as a yellow solid in 231 mg, 71% yield. $R_f=0.56$ (EtOAc/hexane, 7:3); mp: 110–111 °C; IR (KBr) $\tilde{\nu}=3334$, 3062, 2922, 1654, 1611, 1581, 1224, 847, 767 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=3.70$–3.99 (m, 8H), 4.33 (s, 2H), 5.5 (br s, 1H), 6.21 (d, 1H, $J=2.2$ Hz), 6.29 (s, 1H), 6.64–6.77 (m, 2H), 7.01–7.11 (m, 2H), 7.17–7.24 (m, 1H), 7.34 (s, 2H), 7.36–7.48 (m, 3H), 7.59 (d, 3H, $J=2.8$ Hz), 7.97 (d, 2H, $J=7.1$ Hz), 8.22 ppm (d, 2H, $J=3.0$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=40.8$, 42.9, 47.4, 94.6, 107.1, 110.3, 112, 116.5, 118.8, 126.6, 127.1, 127.9, 128.5, 128.7, 129, 129.2, 129.4, 130.5, 131.2, 131.3, 132.4, 141.9, 146.4, 147.2, 149, 151.2, 152.2, 156.8, 165.8, 170.5 ppm, MS (ESI): 583 $[M+H]^+$; HRMS (ESI) calcd for $C_{35}H_{31}N_6O_3$ $[M+H]^+$ 583.2890, found: 583.2884; anal. calcd for $C_{35}H_{30}N_6O_3$: C 72.15, H 5.19, N 14.42, O 8.24, found: C 72.06, H 5.22, N 14.44, O 8.22; purity 98.85% (HPLC).

4-(2-(Furan-2-ylmethylamino)benzoyl)piperazin-1-yl)(7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6h): Compound **6h** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7b**, 241 mg, 0.67 mmol) to afford pure compound **6h** as a yellow solid in 245 mg, 70% yield. $R_f=0.5$ (EtOAc/hexane, 7:3); mp: 107–108 °C; IR (KBr) $\tilde{\nu}=3383$, 3012, 2962, 1625, 1585, 1556, 1238, 841, 760 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta=$ 3.73–3.81 (m, 4H), 3.84–3.88 (m, 2H), 3.89–3.93 (m, 2H), 4.33 (s, 2H), 5.45 (br s, 1H), 6.23 (s, 1H), 6.31 (s, 1H), 6.72 (t, 1H, $J=6.9$ Hz),

6.78 (d, 1H, $J=8.9$ Hz), 7.07–7.15 (m, 2H), 7.27–7.29 (m, 1H), 7.32 (s, 1H), 7.36 (s, 1H), 7.41 (d, 1H, $J=5.9$ Hz), 7.46 (t, 2H, $J=6.9$, 7.9 Hz), 7.6 (t, 2H, $J=2.0$, 2.9 Hz), 8.0 (d, 2H, $J=7.9$ Hz), 8.22 ppm (t, 2H, $J=2.9$, 3.9 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=40.9$, 42.9, 47.4, 94.6, 107.1, 110.3, 112, 116.6, 118.8, 119.1, 126.6, 127.9, 128.5, 128.7, 129.2, 129.4, 130.5, 131.1, 131.3, 132.4, 141.9, 146.4, 147.2, 149.1, 151.2, 152.2, 156.9, 165.8, 170.5 ppm; MS (ESI): 728 $[M+H]^+$; HRMS (ESI) calcd for $C_{35}H_{30}N_7O_5$ $[M+H]^+$ 628.2289, found: 628.2283; anal. calcd for $C_{35}H_{29}N_7O_5$: C 66.98, H 4.66, N 15.62, O 12.75 found: C 66.91, H 4.62, N 15.64, O 12.73; purity 95.13% (HPLC).

(4-(2-(Furan-2-ylmethylamino)benzoyl)piperazin-1-yl)(2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6i): Compound **6i** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7c**, 220 mg, 0.67 mmol) to afford pure compound **6i** as a yellow solid in 226 mg, 68% yield. $R_f=0.57$ (EtOAc/hexane, 7:3); mp: 116–117 °C; IR (KBr) $\tilde{\nu}=3374$, 2927, 1742, 1612, 1577, 1254, 856, 764 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=2.48$ (s, 3H), 3.69–3.96 (m, 8H), 4.36 (s, 2H), 5.66 (br s, 1H), 6.59 (s, 1H), 6.74 (d, 2H, $J=$ 8.1 Hz), 7.05–7.15 (m, 2H), 7.20 (t, 1H, $J=7.5$ Hz), 7.29 (d, 2H, $J=$ 8.5 Hz), 7.35–7.53 (m, 6H), 8.02 (d, 2H, $J=6.9$ Hz), 8.15 ppm (d, 2H, $J=7.9$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=21.5$, 42.8, 42.9, 47.7, 94.4, 106.4, 112, 116.7, 119.1, 124.6, 125, 126.7, 127, 127.6, 127.9, 128.7, 129.4, 129.5, 129.8, 132.4, 140.1, 142.3, 142.7, 146.2, 147.5, 148.9, 151.6, 156.9, 166.4, 170.7 ppm; MS (ESI): 597 $[M+H]^+$; HRMS (ESI) calcd for $C_{36}H_{33}N_6O_3$ $[M+H]^+$ 597.2461, found: 597.2469; anal. calcd for $C_{36}H_{32}N_6O_3$: C 72.47, H 5.41, N 14.08, O 8.04 found: C 72.51, H 5.38, N 14.06, O 8.02; purity 98.63%, (HPLC).

(4-(2-(Furan-2-ylmethylamino)benzoyl)piperazin-1-yl)(7-(4-methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6j): Compound **6j** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 7-(4-methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7d**, 231 mg, 0.67 mmol) to afford pure compound **6j** as a yellow solid in 250 mg, 73% yield. $R_f=0.36$ (EtOAc/hexane, 7:3); mp: 110–111 °C; IR (KBr) $\tilde{\nu}=3377$, 2923, 1624, 1604, 1549, 1257, 832, 760 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=$ 3.73–3.80 (m, 4H), 3.82–3.88 (m, 4H), 3.92 (s, 3H), 4.32 (s, 2H), 5.58 (br s, 1H), 6.20 (d, 1H, $J=2.2$ Hz), 6.27 (d, 1H, $J=3.3$ Hz), 6.67 (t, 1H, $J=7.7$ Hz), 6.74 (d, 1H, $J=8.8$ Hz), 7.0 (s, 1H), 7.05–7.10 (m, 3H), 7.20–7.25 (m, 1H), 7.30 (s, 1H), 7.33–7.39 (m, 2H), 7.43 (t, 2H, $J=7.7$ Hz), 7.97 (d, 2H, $J=7.7$ Hz), 8.27 ppm (d, 2H, $J=8.8$); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=40.8$, 42.8, 47.4, 55.4, 94.4, 106.1, 107.1, 110.3, 112.0, 114.0, 116.6, 118.8, 122.6, 126.6, 127.9, 128.7, 129.1, 131.3, 131.7, 132.6, 141.9, 146.4, 146.9, 148.9, 149.2, 151.1, 155, 156.7, 162, 166, 170.5 ppm; MS (ESI): 613 $[M+H]^+$; HRMS (ESI) calcd for $C_{36}H_{33}N_6O_4$ $[M+H]^+$ 613.2563, found: 613.2580; anal. calcd for $C_{36}H_{32}N_6O_4$: C 70.57, H 5.26, N 13.72, O 10.45, found: C 70.52, H 5.28, N 13.75, O 10.42; purity 95.70% (HPLC).

(7-(3,4-Dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(furan-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6k): Compound **6k** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 7-(3,4-dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7e**, 251 mg, 0.67 mmol) to afford pure compound **6k** as a yellow solid in 251 mg, 70% yield. $R_f=0.35$ (EtOAc/hexane, 7:3); mp: 102–103 °C; IR (KBr) $\tilde{\nu}=3373$, 3000, 2927, 1627, 1577, 1550, 1262, 852, 808 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.58$–3.95 (m, 8H), 3.99 (s, 6H), 4.33, (s, 2H), 5.61 (br s, 1H), 6.24 (d, 2H, $J=2.9$ Hz), 6.60–6.78 (m, 2H), 6.99–7.11 (m, 3H), 7.25 (s, 1H), 7.31–7.48 (m, 5H), 7.84 (d, 1H, $J=8.8$ Hz), 7.98 ppm (d, 3H, $J=6.2$ Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=42.7$, 42.9, 47.5, 56, 56.1, 94.4, 106.3, 107.1, 110.7, 110.8, 112.5, 112.8, 116.6, 118.7, 118.9, 122.8, 122.9, 123.2, 126.3, 126.5, 127.9, 128.8, 129.2, 131.4, 132.6, 142, 146.4, 146.8, 148.6, 151.7, 152.2, 156.7, 166.1, 170.6 ppm; MS (ESI): 643 $[M+H]^+$; HRMS (ESI) calcd for $C_{37}H_{35}N_6O_5$ $[M+H]^+$ 643.2668, found: 643.2685; anal. calcd for $C_{37}H_{34}N_6O_5$: C 69.14, H 5.33, N 13.08, O 12.45, found: C 69.17, H 5.30, N 13.06, O 12.42; purity 95.40% (HPLC).

(4-(2-(Furan-2-ylmethylamino)benzoyl)piperazin-1-yl)(2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6l): Compound **6l** was prepared by following the method described for the preparation of compound **6a**, employing (2-(furan-2-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14b**, 160 mg, 0.56 mmol) and 2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7f**, 271 mg, 0.67 mmol) to afford pure compound **6l** as a yellow solid in 278 mg, 74% yield. $R_f=0.3$ (EtOAc/hexane, 7:3); mp: 107–108 °C; IR (KBr) $\tilde{\nu}=3375$, 3054, 2932, 1627, 1578, 1550, 1245, 837, 762 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.73$–3.83 (m, 4H), 3.85–3.95 (m, 4H), 3.97 (s, 6H), 3.98 (s, 3H), 4.34 (s, 2H), 5.51 (br s, 1H), 6.24 (d, 1H, $J=3.0$ Hz), 6.33 (dd, 1H, $J=2.2$, 3.7 Hz), 6.7–6.82 (m, 2H), 7.11 (s, 1H), 7.25–7.32 (m, 2H), 7.37 (d, 2H, $J=6.0$ Hz), 7.41–7.51 (m, 3H), 7.56 (s, 2H), 8.02 ppm (dd, 2H, $J=1.5$, 8.3 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=40.9$, 42.9, 47.5, 56.4, 61, 94.6, 106.7, 107, 107.1, 110.3, 112, 116.6, 118.8, 125.4, 126.4, 127.9, 128.8, 129.3, 130.5, 131.3, 132.5, 140.8, 141.9, 146.4, 146.8, 149.3, 151.2, 152.2, 153.1, 156.8, 165.9, 170.5 ppm; MS (ESI): 673 $[M+H]^+$; HRMS (ESI) calcd for $C_{38}H_{37}N_6O_6$ $[M+H]^+$ 673.2774, found: 673.2777; anal. calcd for $C_{38}H_{36}N_6O_6$: C 67.84, H 5.39, N 12.49, O 14.27, found: C 67.86,H 5.33, N 12.50, O 14.25; purity 98.26% (HPLC).

(2,7-Diphenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6m): Compound **6m** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7a**, 198 mg, 0.63 mmol) to afford pure compound **6m** as a yellow solid in 250 mg, 79% yield. $R_f=0.50$ (EtOAc/hexane, 7:3); mp: 117–118 °C; IR (KBr) $\tilde{\nu}=3012$, 2931, 1757, 1684, 1631, 1588, 1234, 831, 767 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.75$–3.84 (m, 4H), 3.85–3.96 (m, 4H), 4.54 (s, 2H), 5.49 (br s, 1H), 6.71–6.81 (m, 2H), 6.95–6.99 (m, 1H), 7.0–7.03 (m, 1H), 7.19 (d, 1H, $J=2.4$ Hz), 7.13 (dd, 1H, $J=7.5$, 7.7 Hz), 7.21 (dd, 1H, $J=5.0$, 4.9) Hz, 7.24–7.28 (m, 1H), 7.33 (d, 1H, $J=2.2$ Hz), 7.40–7.51 (m, 3H), 7.58–7.63 (m, 3H), 7.99–8.04 (m, 2H), 8.20–8.25 ppm (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta=42.8$, 42.9, 47.5, 94.6, 107.1, 112.1, 116.8, 119.2, 124.4, 124.8, 126.6, 126.8, 127.1, 127.7, 128.5, 128.7, 128.9, 129.2, 129.4, 130.5, 131.2, 131.3, 132.4, 142.6, 146.1, 147.2, 149, 151.2, 156.8, 165.8, 170.5 ppm; MS (ESI): 599 $[M+H]^+$; HRMS (ESI) calcd for $C_{35}H_{31}N_6O_2S$ $[M+H]^+$ 599.2228, found: 599.2223; anal. calcd for $C_{35}H_{30}N_6O_2S$: C 70.21, H 5.05, N 14.04, O 5.34, S 5.36, found: C 70.18, H5.08, N 14.01, O 5.32, S 5.38; purity 95.80% (HPLC).

(7-(4-Nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethyl)benzoyl)piperazin-1-yl)methanone (6n): Compound **6n** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimi-

dine-5-carboxylic acid (**7b**, 226 mg, 0.63 mmol) to afford pure compound **6n** as a yellow solid in 272 mg, 80% yield. R_f = 0.48 (EtOAc/hexane, 7:3); mp: 109–110 °C; IR (KBr) $\tilde{\nu}$ = 3378, 2937, 2887, 1627, 1607, 1584, 841, 752 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.74–4.03 (m, 8H), 4.53 (s, 2H), 5.64 (br s, 1H), 6.65–6.75 (m, 2H), 6.91–6.95 (m, 1H), 6.98 (d, 1H, J = 3.0 Hz), 7.04 (s, 1H), 7.09 (d, 1H, J = 7.5 Hz), 7.14–7.27 (m, 2H), 7.34 (s, 1H), 7.36–7.48 (m, 3H), 7.58 (t, 2H, J = 3.0 Hz), 7.97 (d, 2H, J = 8.3 Hz), 8.22 (dd, 1H, J = 3.0, 6.7 Hz), 8.3 ppm (d, 1H, J = 8.3 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 42.8, 47.4, 94.6, 106.7, 107.1, 112.1, 116.7, 119.1, 124.4, 124.8, 126.5, 126.8, 127.3, 127.7, 128.5, 128.7, 129.1, 129.4, 130.5, 131.2, 131.3, 132.3, 142.6, 146, 147.2, 149, 156.8, 165.8, 170.4 ppm; MS (ESI): 644 $[M+H]^+$; HRMS (ESI) calcd for $C_{35}H_{30}N_7O_4S$ $[M+H]^+$ 644.2049, found: 644.2068; anal. calcd for $C_{35}H_{29}N_7O_4S$: C, 65.30; H, 4.54; N, 15.23; O, 9.94; S, 4.98, found: C 65.25, H 4.57, N 15.25, O 9.91, S 4.96; purity 95.50%, (HPLC).

(2-Phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6o): Compound **6o** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7c**, 207 mg, 0.63 mmol) to afford pure compound **6o** as a yellow solid in 253 mg, 78% yield. R_f = 0.52 (EtOAc/hexane, 7:3); mp: 112–113 °C; IR (KBr) $\tilde{\nu}$ = 3375, 2941, 1684, 1628, 1580, 883, 803 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 2.51 (s, 3H), 3.75–3.82 (m, 4H), 3.84–3.90 (m, 2H), 3.92–3.99 (m, 2H), 4.52 (s, 2H), 5.63 (br s, 1H), 6.65–6.75 (m, 2H), 6.91–6.95 (m, 1H), 6.97–7.00 (m, 1H), 7.03 (s, 1H), 7.09 (d, 1H, J = 7.5 Hz), 7.15–7.23 (m, 2H), 7.32 (s, 1H), 7.36–7.44 (m, 5H), 7.95–8.0 (m, 2H), 8.14 ppm (d, 2H, J = 8.3 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 21.6, 42.9, 43, 47.5, 94.5, 106.7, 112.3, 116.8, 119.2, 124.5, 124.9, 126.6 126.9, 127.7, 127.8, 128.7, 129.2, 129.3, 129.5, 131.3, 132.6, 140.7, 142, 142.6, 146.1, 147.3, 149.2, 151.2, 156.8, 166, 170.5 ppm; MS (ESI): 613 $[M+H]^+$; HRMS (ESI) calcd for $C_{36}H_{33}N_6O_2S$ $[M+H]^+$ 613.2382, found: 613.2374; anal. calcd for $C_{36}H_{32}N_6O_2S$: C 70.57, H 5.26, N 13.72, O 5.22, S 5.23, found: C 70.61, H 5.23, N 13.69, O 5.24, S 5.21; purity 98.80% (HPLC).

(7-(4-Methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6p): Compound **6p** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 7-(4-methoxyphenyl)-2-phenylpyrazolo-[1,5-*a*]pyrimidine-5-carboxylic acid (**7d**, 217 mg, 0.63 mmol) to afford pure compound **6p** as a yellow solid in 269 mg, 81% yield. R_f = 0.40 (EtOAc/hexane, 7:3); mp: 98–99 °C; IR (KBr) $\tilde{\nu}$ = 3376, 3065, 2997, 2933, 1757, 1684, 1625, 1577, 1245, 893, 840 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.72–3.83 (m, 4H), 3.84–3.91 (m, 4H), 3.92 (s, 3H), 4.53 (s, 2H), 5.48 (br s, 1H), 6.72 (t, 1H, J = 7.4 Hz), 6.77 (d, 1H, J = 7.4 Hz), 6.94–7.02 (m, 2H), 7.05–7.13 (m, 4H), 7.19 (d, 1H, J = 5.3 Hz), 7.24 (d, 1H, J = 9.5 Hz), 7.30 (s, 1H), 7.38–7.43 (m, 1H), 7.47 (t, 2H, J = 7.4 Hz), 8.02 (d, 2H, J = 7.4 Hz), 8.27 ppm (d, 2H, J = 8.4 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 42.8, 42.9, 47.5, 55.5, 94.4, 106.2, 112.2, 114, 116.8, 119.2, 122.7, 123.5, 124.5, 124.9, 126.6, 126.9, 127.8, 128.8, 129.2, 131.2, 131.3, 132.6, 146.1, 146.9, 149.3, 151.2, 156.7, 162.1, 166.1, 170.5 ppm; MS (ESI): 629 $[M+H]^+$; HRMS (ESI) calcd for $C_{36}H_{33}N_6O_3S$ $[M+H]^+$ 629.2381, found: 629.2362; anal. calcd for $C_{36}H_{32}N_6O_3S$: C 68.77, H 5.13, N 13.37, O 7.63, S 5.10, found: C 68.74, H 5.15, N 13.39, O 7.61, S 5.11; purity 97.40% (HPLC).

(7-(3,4-Dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6q): Compound **6q** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 7-(3,4-dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7e**, 236 mg, 0.63 mmol) to afford pure compound **6q** as a yellow solid in 265 mg, 76% yield. R_f = 0.32 (EtOAc/hexane, 7:3); mp: 96–97 °C; IR (KBr) $\tilde{\nu}$ = 3372, 2925, 1626, 1576, 1551, 1261, 805 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.60–3.77 (m, 4H), 3.78–3.88 (m, 4H), 3.94 (s, 6H), 4.46, (s, 2H), 5.44 (br s, 1H), 6.59–6.77 (m, 2H), 6.86–7.10 (m, 5H), 7.11–7.24 (m, 2H), 7.28 (s, 1H), 7.32–7.46 (m, 3H), 7.81 (d, 1H, J = 6.7 Hz), 7.95 ppm (t, 3H, J = 6.7 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 42.8, 42.9, 47.4, 56, 56.1, 94.4, 106.2, 110.8, 112.1, 112.5, 116.7, 119.2, 122.8, 123.2, 124.4, 124.8, 126.4, 126.8, 127.7, 128.7, 129.1, 131.1, 132.5, 142.6, 146.1, 146.7, 148.6, 149.3, 151.1, 151.7, 156.6, 165.9, 170.4 ppm; MS (ESI): 659 $[M+H]^+$; HRMS (ESI) calcd for $C_{37}H_{35}N_6O_4S$ $[M+H]^+$ 659.2440, found: 659.2432; anal. calcd for $C_{37}H_{34}N_6O_4S$: C, 67.46; H, 5.20; N, 12.76; O, 9.71; S, 4.87, found: C 67.49, H 5.18, N 12.73, O 9.73, S 4.85. purity 96.80% (HPLC).

(2-Phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)(4-(2-(thiophen-2-ylmethylamino)benzoyl)piperazin-1-yl)methanone (6r): Compound **6r** was prepared by following the method described for the preparation of compound **6a**, employing piperazin-1-yl-(2-(thiophen-2-ylmethylamino)phenyl)methanone (**14c**, 160 mg, 0.53 mmol) and 2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7f**, 255 mg, 0.63 mmol) to afford pure compound **6r** as a yellow solid in 270 mg, 74% yield. R_f = 0.3 (EtOAc/hexane, 7:3); mp: 120–121 °C; IR (KBr) $\tilde{\nu}$ = 3378, 3056, 2935, 1632, 1587, 1565, 1263, 843, 761 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 3.75–3.85 (m, 4H), 3.86–3.95 (m, 4H), 3.97 (s, 6H), 3.98 (s, 3H), 4.54 (d, 2H, J = 3.0 Hz), 5.50 (br s, 1H), 6.70–6.81 (m, 2H), 6.96 (dd, 1H, J = 3.7, 5.2 Hz), 7.0–7.03 (m, 1H), 7.10–7.15 (m, 2H), 7.19–7.24 (m, 1H), 7.28 (s, 1H), 7.36 (s, 1H), 7.39–7.52 (m, 3H), 7.56 (s, 2H), 8.02 ppm (dd, 2H, J = 1.5, 8.3 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 42.8, 42.9, 47.5, 56.3, 60.9, 68.2, 94.6, 106.7, 107, 107.2, 112.1, 116.8, 119.1, 120.2, 124.4, 124.8, 125.3, 126.2, 126.4, 126.8, 127.7, 128.6, 128.8, 129.2, 131.2, 132.4, 152.8, 153, 156.7, 165.8, 170.4 ppm; MS (ESI): 689 $[M+H]^+$; HRMS (ESI) calcd for $C_{38}H_{37}N_6O_5S$ $[M+H]^+$ 689.2542, found: 689.2547; anal. calcd for $C_{38}H_{36}N_6O_5S$: C 66.26, H, 5.27, N 12.20, O 11.61, S 4.66, found: C 66.28, H 5.29, N 12.17, O 11.59, S 4.64; purity 97.90% (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-4-ylmethylamino)benzoyl)piperazin-1-yl)(2,7-diphenylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6s): Compound **6s** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*]-[1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 2,7-diphenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7a**, 176 mg, 0.56 mmol) to afford pure compound **6s** as a yellow solid in 233 mg, 78% yield. R_f = 0.56 (EtOAc/hexane, 7:3); mp: 118–119 °C; IR (KBr) $\tilde{\nu}$ = 3376, 2930, 2834, 1667, 1607, 1560, 1244, 841, 760 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 3.76–3.84 (m, 4H), 3.85–3.96 (m, 4H), 4.26 (s, 2H), 5.56 (br s, 1H), 5.94 (s, 2H), 6.65–6.86 (m, 5H), 7.12 (d, 2H, J = 9.63), 7.19–7.25 (m, 1H), 7.33 (s, 1H), 7.40–7.51 (m, 3H), 7.57–7.65 (m, 3H), 8.02 (d, 2H, J = 6.7 Hz), 8.23 ppm (dd, 2H, J = 2.0, 6.9 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 42.5, 42.9, 47.5, 94.7, 107.7, 108.3, 112.3, 116.1, 117.9, 120.3, 124.2, 126.51, 126.6, 127.9, 128.4, 128.6, 128.9, 129, 129.3, 129.5, 130.7, 131.5, 132.6, 132.7, 146.7, 146.9, 147.2, 147.9, 149.8, 151.2, 156.95, 165.9, 170.8 ppm; MS (ESI): 637 $[M+H]^+$ HRMS (ESI)

calcd for $C_{38}H_{33}N_6O_4$ $[M+H]^+$ 637.2556, found: 637.2547; anal. calcd for $C_{38}H_{32}N_6O_4$: C, 71.68, H 5.07, N 13.20, O 10.05, found: C 71.71, H 5.05, N 13.17, O 10.02; purity 95.30%, (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-5-ylmethylamino)benzoyl)piperazin-1-yl)(7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6t): Compound **6t** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*][1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 7-(4-nitrophenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7b**, 201 mg, 0.56 mmol) to afford pure compound **6t** as a yellow solid in 256 mg, 80% yield. R_f=0.54 (EtOAc/hexane, 7:3); mp: 108–109 °C; IR (KBr) $\tilde{\nu}$=3380, 2927, 1634, 1587, 1556, 1243, 884, 803 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ=3.74–3.98 (m, 8H), 4.26 (s, 2H), 5.57 (br s, 1H), 5.94 (s, 2H), 6.64–6.88 (m, 5H), 7.08–7.18 (m, 1H), 7.33 (s, 2H), 7.4–7.51 (m, 4H), 7.59–7.65 (m, 2H), 8.02 (dd, 2H, J=1.7, 6.9 Hz), 8.23 ppm (dd, 2H, J=2.2, 6.0 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ=42.7, 42.9, 47.5, 94.7, 101, 106.7, 107.2, 107.8, 108.3, 112.2, 116.2, 118.3, 119.1, 120.4, 123.6, 126.6, 128, 128.6, 128.8, 129.2, 129.3, 129.5, 130.6, 131.2, 131.4, 132.5, 132.7, 146.8, 147.1, 151.1, 156.9, 165.9, 170.7 ppm; MS (ESI): 682 $[M+H]^+$; anal. calcd for $C_{38}H_{31}N_7O_6$: C 66.95, H 4.58, N 14.38, O 14.08, found: C 66.91, H 4.62, N 14.36, O 14.11; purity 95.50% (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-5-ylmethylamino)benzoyl)piperazin-1-yl)(2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6u): Compound **6u** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*][1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 2-phenyl-7-*p*-tolylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7c**, 184 mg, 0.56 mmol) to afford pure compound **6u** as a yellow solid in 214 mg, 70% yield. R_f= 0.57 (EtOAc/hexane, 7:3); mp: 109–110 °C; IR (KBr) $\tilde{\nu}$=3379, 2937, 1628, 1603, 1565, 1234, 851, 760 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ=2.49 (s, 3H), 3.75–3.84 (m, 4H), 3.86–3.94 (m, 4H), 4.26 (s, 2H), 5.56 (br s, 1H), 5.93 (d, 2H, J=6.0 Hz), 6.69 (t, 1H, J=7.5 Hz), 6.75–6.84 (m, 3H), 7.04–7.14 (m, 2H), 7.19–7.25 (m, 1H), 7.32 (t, 2H, J= 4.5 Hz), 7.38–7.51 (m, 5H), 8.0 (dd, 2H, J=3.7, 12 Hz), 8.16 ppm (d, 2H, J=8.3 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ=21.7, 42.7, 42.9, 47.8, 94.6, 100.7, 106.6, 107.0, 112.1, 116.4, 117.2, 118.0, 119.8, 123.5, 125.8, 126.3, 127.5, 128.2, 128.5, 129.0, 129.4, 129.7, 130.2, 130.7, 131.3, 132.4, 146.2, 147.4, 149.6, 151.1, 156.4, 166.0, 170.6 ppm; MS (ESI): 651 $[M+H]^+$; purity 97.50% (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-5-ylmethylamino)benzoyl)piperazin-1-yl)(7-(4-methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl-methanone (6v): Compound **6v** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*][1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 7-(4-methoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7d**, 193 mg, 0.56 mmol) to afford pure compound **6v** as a yellow solid in 256 mg, 82% yield. R_f=0.36 (EtOAc/hexane, 7:3); mp: 122–123 °C; IR (KBr) $\tilde{\nu}$=3380, 2897, 1628, 1603, 1549, 1253, 929, 832 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ=3.75–3.82 (m, 4H), 3.84–3.89 (m, 2H), 3.93 (s, 3H), 3.94–3.97 (m, 2H), 4.24 (s, 2H), 5.68 (br s, 1H), 5.93 (s, 2H), 6.62–6.67 (m, 2H), 6.79 (d, 2H, J=12.7 Hz), 7.08 (d, 3H, J= 7.2 Hz), 7.18 (t, 1H, J=8.2 Hz), 7.25 (s, 2H), 7.31 (s, 1H), 7.38 (d, 1H, J=7.2 Hz), 7.44 (t, 2H, J=7.2 Hz), 7.98 (d, 2H, J=7.2 Hz), 8.28 ppm (d, 2H, J=8.2 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ=42.9, 47.4, 55.3, 94.6, 100.8, 106.1, 107.6, 108.2, 112.1, 113.9, 116.1, 118.3, 120.2, 122.6, 124.2, 126.5, 127.8, 128.2, 128.6, 129, 129.7, 131.2, 132.5, 132.7, 146.6, 146.8, 147.8, 149.2, 151.1, 156.6, 162, 165.9, 170.6 ppm; MS (ESI): 667 $[M+H]^+$; HRMS (ESI) calcd for $C_{39}H_{35}N_6O_5$ $[M+H]^+$ 667.2663, found: 667.2657; anal. calcd for $C_{39}H_{34}N_6O_5$: C 70.26, H 5.14, N, 12.60, O, 12.00, found: C 70.29, H 5.12, N 12.57, O 11.98; purity 95% (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-5-ylmethylamino)benzoyl)piperazin-1-yl)(7-(3,4-dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6w): Compound **6w** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*][1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 7-(3,4-dimethoxyphenyl)-2-phenylpyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7e**, 210 mg, 0.56 mmol) to afford pure compound **6w** as a yellow solid in 222 mg, 68% yield. R_f=0.35 (EtOAc/hexane, 7:3); mp: 115–116 °C; IR (KBr) $\tilde{\nu}$=3395, 2927, 1627, 1550, 1505, 1256, 853, 808 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ=3.76–3.84 (m, 4H), 3.84–3.98 (m, 4H), 3.99 (s, 6H), 4.24 (s, 2H), 5.69 (br s, 1H), 5.92 (s, 2H), 6.59–6.82 (m, 4H), 7.0–7.10 (m, 3H), 7.18 (t, 1H, J=7.5 Hz), 7.25 (s, 1H), 7.34 (s, 1H), 7.36–7.48 (m, 3H), 7.85 (d, 1H, J=8.3 Hz), 7.98 ppm (t, 3H, J=2.2 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ=42.8, 47.3, 47.4, 55.9, 56, 94.4, 100.8, 106.2, 107.6, 108.2, 110.7, 112.3, 112.7, 116.1, 118.2, 120.2, 122.7, 123.1, 126.1, 126.4, 127.8, 128.7, 129.1, 131.3, 132.4, 132.6, 146.6, 146.7, 146.8, 146.9, 147.8, 148.5, 149.2, 151, 156.6, 165.9, 170.7 ppm; MS (ESI): 697 $[M+H]^+$; HRMS (ESI) calcd for $C_{40}H_{37}N_6O_6$ $[M+H]^+$ 697.2770, found: 697.2781; anal. calcd for $C_{40}H_{36}N_6O_6$: C 68.95, H, 5.21, N 12.06, O 13.78, found: C 68.93, H 5.23, N 12.09, O 13.80; purity 97.60% (HPLC).

(4-(2-(Benzo[*d*][1,3]dioxol-5-ylmethylamino)benzoyl)piperazin-1-yl)(2-phenyl-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-*a*]pyrimidin-5-yl)methanone (6x): Compound **6x** was prepared by following the method described for the preparation of compound **6a**, employing (2-(benzo[*d*][1,3]dioxol-5-ylmethylamino)phenyl)(piperazin-1-yl)methanone (**14d**, 160 mg, 0.47 mmol) and 2-phenyl-7-(3,4,5-trimethoxyphenyl)-pyrazolo[1,5-*a*]pyrimidine-5-carboxylic acid (**7f**, 226 mg, 0.56 mmol) to afford pure compound **6x** as a yellow solid in 235 mg, 69% yield. R_f=0.29 (EtOAc/hexane, 7:3); mp: 113–114 °C; IR (KBr) $\tilde{\nu}$=3389, 2934, 1627, 1549, 1508, 1242, 831, 803 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ=3.75–3.84 (m, 4H), 3.86–3.94 (m, 4H), 3.97 (s, 6H), 3.98 (s, 3H), 4.26 (s, 2H), 5.55 (br s, 1H), 5.93 (s, 2H), 6.69 (t, 2H, J=6.9 Hz), 6.75–6.85 (m, 2H), 7.09–7.13 (m, 2H), 7.23 (t, 1H, J=6.9 Hz), 7.36 (s, 1H), 7.39–7.50 (m, 4H), 7.57 (s, 2H), 8.02 ppm (d, 2H, J=7.9 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ= 42.9, 47.4, 47.5, 56.3, 61, 94.6, 100.9, 106.7, 107, 107.7, 108.3, 112.2, 116.1, 118.2, 120.3, 125.4, 126.4, 127.9, 128.6, 128.8, 129.3, 130.3, 131.4, 132, 132.4, 132.7, 146.8, 146.9, 149.3, 151.1, 153.1, 156.8, 165.9, 170.7 ppm; MS (ESI): 727 $[M+H]^+$ HRMS (ESI) calcd for $C_{41}H_{39}N_6O_7$ $[M+H]^+$ 727.2864, found: 727.2869; anal. calcd for $C_{41}H_{38}N_6O_7$: C 67.76, H 5.27, N 11.56, O 15.42, found: C 67.78,H 5.24, N 11.53, O 15.40; purity 95.30%, (HPLC).

Biology

Cell culture: Human cervical epithelial cancer cell lines HeLa and SiHa, purchased from the American Type Culture Collection (ATCC), were maintained in RPMI and Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), respectively, and were supplemented with 2 mM Glutamax (Invitrogen), 10% fetal calf serum, and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin sulfate (Sigma). Cell cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

MTT assay: Cell viability was assessed by MTT assay, which assays mitochondrial function based on the ability of viable cells to reduce MTT to insoluble formazan crystals by mitochondrial dehydrogenase activity. HeLa and SiHa cells were seeded in a 96-well

plate at a density of 1×10^4 cells per well. After overnight incubation, cells were treated with compounds roscovitine (ros) and **6a–x** at concentrations of 1, 2, 4, and 8 μM and incubated for 24 h. The cell culture medium was then discarded and replaced with 10 μL MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubilized in 100 μL extraction buffer. The optical density (OD) was read at λ 570 nm with a microplate reader (Multi-mode Varioskan instrument, Themo Scientific).

Cell cycle analysis: HeLa and SiHa cells (5×10^5) were seeded in 60 mm dishes and allowed to grow for 24 h. Compounds (**6a–x**) were added at a final concentration of 2 μM to the culture media, and cells were incubated for an additional 24 h. Cells were harvested with Trypsin–EDTA, fixed with ice-cold 70% EtOH at 4 °C for 30 min, washed with phosphate-buffered saline (PBS) and incubated with 1 mg mL^{-1} RNase A solution (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm (313 *g*, Sorvall swinging-bucket rotor, model #75002000, Heraeus Multifuge 1S-R, Thermo Scientific) for 5 min and further stained with 250 μL DNA staining solution [10 mg propidium iodide (PI), 0.1 mg trisodium citrate, and 0.03 mL Triton X-100 dissolved in 100 mL sterile Milli-Q-purified water at room temperature for 30 min in the dark]. The DNA contents of 20000 events were measured by flow cytometry (Dako Cytomation, Beckman Coulter, Brea, CA, USA). Histograms were analyzed using Summit Software V4.3.02.

BrdU cell proliferation assay: This assay was carried out by using the 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay kit (Millipore) to assess the effect of compounds such as **6f, 6l**, and **6r** on the proliferation of HeLa and SiHa cells. Roscovitine was used as positive control. Cells (1×10^4) were seeded and allowed to grow for 24 h. BrdU was added and allowed to incorporate for 5 h followed by the addition of test compounds (ros, **6f**, **6l**, and **6r**) at a concentration of 2 μM for 24 h. Fixation was done for 30 min at room temperature. The cells were then washed, anti-BrdU antibody was added; this binds BrdU that was incorporated in the cell. After incubation for 1 h, anti-BrdU goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (100 μL, 1:2000) was added and incubated for 30 min. Washing procedures were followed according to the manufacturer's instructions. TMB substrate (100 μL) was added and incubated for another 30 min at room temperature. A decrease in OD_{450} indicates that the cells are arrested in the G_1 phase.

Semi-quantitative reverse transcription PCR (RT-PCR): Total RNA was extracted using an RNeasy mini kit (Qiagen, USA) and reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen Life Technologies). PCR was carried out with specific primers (Table 3) in a PCR thermocycler (Takara Bioscience). The products were separated by agarose gel (1%) electrophoresis followed by staining with ethidium bromide and visualization under UV light. The signal intensity of respective bands was measured by means of Quantity One software (version 4.1.1) using the Bio-Rad image analysis system (CA, USA).

Caspase-3 assay: A caspase-3 fluorescence assay kit (Clonetech, CA, USA) was used to evaluate caspase-3 activity, following procedures provided by the manufacturer. HeLa cells were treated with compounds ros, **6f**, **6l**, and **6r** at 2 μM as obtained from FACS analysis. Cell lysates were added to the 2× reaction buffer containing DTT and caspase substrate. Incubation was carried out at 37 °C for 1 h. Readings were taken at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Immunoflouroscence microscopy to study p53 localization: HeLa cells were seeded on glass cover slips, incubated for 24 h in the presence or absence of test compounds ros and **6l** at 2 mM. At the end of incubation, cells were fixed with 4% paraformaldehyde, 0.02% glutaraldehyde in PBS, and permeabilized by dipping the cells in 100% methanol (−20 °C). Cover slips were then blocked with 1% BSA in PBS for 1 h followed by incubation with a primary antibody against p53 followed by secondary antibody. At the end of experiments, cells were washed and fixed for confocal microscopy (Olympus).

Protein extraction and Western blot analysis: HeLa cells (5×10^5) were seeded in a 60 mm dish and allowed to grow for 24 h. Compounds ros, **6f**, **6l**, and **6r** were added to the culture media at 2 mM, and the cells were incubated for an additional 24 h. Total cell lysates were obtained by lysing the cells in ice-cold RIPA buffer (1×PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing 100 mg mL^{-1} PMSF, 5 mg mL^{-1} aprotinin, 5 mg mL^{-1} leupeptin, 5 mg mL^{-1} pepstatin, and 100 mg mL^{-1} NaF. After centrifugation at 12000 rpm (6349 *g*, fixed-angle rotor, model #75003348, Heraeus Multifuge 1S-R, Thermo Scientific) for 10 min, the protein in supernatant was quantified by the Bradford method (Bio-Rad) using a Multimode Varioskan instrument (Thermo-Fischer Scientific). Protein (50 μg per lane) was applied to a 12% SDS–polyacrylamide gel. After electrophoresis, the protein was transferred to a poly(vinylidine difluoride) (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in 1×TBS + 0.1% Tween 20 (TBST) containing 5% blocking powder (Santa Cruz Biotechnology). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight. p53 Ser46 and p53 Ser15 were purchased from Cell Signaling, and β-actin, Bcl2, and BAX were purchased from Imgenex. Membranes were washed with TBST three times for 15 min, and the blots were visualized with chemiluminescence reagent (Thermo-Fischer Scientific). X-ray films were developed and fixed with reagents purchased from Kodak.

Statistical analysis: Statistical analyses were performed using GraphPad software to evaluate significant differences between control and test samples. All variables were tested in three independent experiments. Results are reported as the mean ± SD; $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table 3. Primers used in RT-PCR.

Primer	Sequence	Product size [bp]
GAPDH forward	5′-GGG AAG GTG AAG GTC GGA GT-3′	110
GAPDH reverse	5′-TTG AGG TCA ATG AAG GGG TCA-3′	
p21 gene forward	5′-CAC CGA GAC ACC ACT GGA GG-3′	260
p21 gene reverse	5′-GAG AAG ATC AGC CGG CGT TT-3′	
p53 gene forward	5′-AGG TTG GCT CTG ACT GT-3′	220
p53 gene reverse	5′-TTG ACG TGG TGA GGC TC-3′	

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