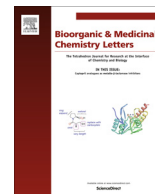




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Synthesis and mechanistic aspects of 2-anilonicotinyl-pyrazolo [1,5-*a*]pyrimidine conjugates that regulate cell proliferation in MCF-7 cells via estrogen signaling

Ahmed Kamal^{a,*}, Shaikh Faazil^a, S. M. Ali Hussaini^{a,†}, M. Janaki Ramaiah^b, M. Balakrishna^a, Nibedita Patel^b, S. N. C. V. L. Pushpavalli^b, Manika Pal-Bhadra^{b,*}

^a Medicinal Chemistry and Pharmacology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India

^b Chemical Biology Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India

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ABSTRACT

A series of anilonicotinyl linked pyrazolo[1,5-*a*]pyrimidine conjugates (**6a–x**) were synthesized and evaluated for their antiproliferative activity. Some of these conjugates exhibited promising cytotoxic effects in the MCF-7 cell line and among these **6a** and **6c** exhibited significant effects, apart from G2/M cell cycle arrest. Interestingly they showed profound effects on cyclin D1, Bcl-2 and survivin proteins that regulate breast cancer cell proliferation. Moreover, ER alpha protein expression was studied to understand regulatory role of these conjugates on estrogen activity in estrogen positive breast cancer cells like MCF-7 and compounds **6a** and **6c** reduced their activity.

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Breast carcinoma is the most common malignancy in women, estrogen receptor α is an important prognosis marker and is considered as predictive of response to endocrine therapy in patients with breast cancer.¹ Estrogen receptors ER α and ER β are the members of nuclear hormone receptor family that acts as transcription factors when bound and activated by their ligand.² In breast cells ER α and ER β are expressed and share 97% identity in their DNA-binding domains and 55% identity in their ligand binding domains. Both these receptors function in antagonistic manner wherein ER α increases tumor growth and ER β inhibits tumor growth.³ Estrogen receptors once activated by estrogen form dimers (both homo as well as hetero) and regulate the genes.⁴ It is interesting to note that more than 70% of breast cancers are estrogen positive.⁵ Thus there is an urgent need to discover the new drugs that effectively controls the ER α -positive cell growth.⁶

Fused heterocycles, pyrazolopyrimidines have been found to possess significant pharmacological activities⁷ such as antibacterial,⁸ antifungal,⁹ antitumor,¹⁰ and antiinflammatory.¹¹ Owing to the structural similarity with purines,¹² and hence exhibits promising antitumor activity by acting as ATP competitive

inhibitor for many kinase enzymes, they are also known to function as CNS depressants,¹³ neuroleptic¹⁴ and tuberculostatic agents.¹⁵ Their cytotoxic activities might be attributed to inhibition of several enzymes such as glutathione peroxidase,¹⁶ tyrosine kinase,¹⁷ mammalian target of rapamycin (mTOR),¹⁸ cyclin dependent kinase (CDK)¹⁹. Various pyrazolo[1,5-*a*]pyrimidine scaffolds and its derivatives have been reported as potential anticancer agents that include inhibition of cyclin-dependent kinases (**1**, **2**),²⁰ checkpoint kinase 1 (CHK1) inhibitors (**3**),²¹ estrogen (ER) selective ligand (**4**)²² (Fig. 1). However, opportunities exist to identify and develop new anticancer agents that may possess superior biological profile compared to the presently identified molecules. Hence, there is a considerable challenge that exists in this area towards the identification of new conjugates comprising of pharmacophores of known antitumor agents for enhancing the selectivity as well as anticancer activity. 2-Anilonicotinyl moiety is present in agents like E7010 (**5**) which inhibit tubulin polymerization and exhibits good in vivo antitumor activity against several rodent as well as human tumours and is presently in phase II clinical trials.²³

Similarly, it is fascinating to observe that in the last 10 years, numerous piperazine linked derivatives have been design and synthesized to exploit their chemotherapeutic potential.²⁴ It is previously reported that piperazine linked bifunctional agents proved to be promising anticancer class of compounds with

* Corresponding authors. Tel.: +91 40 27193157; fax: +91 40 27193189.

E-mail address: ahmedkamal@iict.res.in (A. Kamal).

[†] Presently working at: Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK S7N 5C9, Canada.

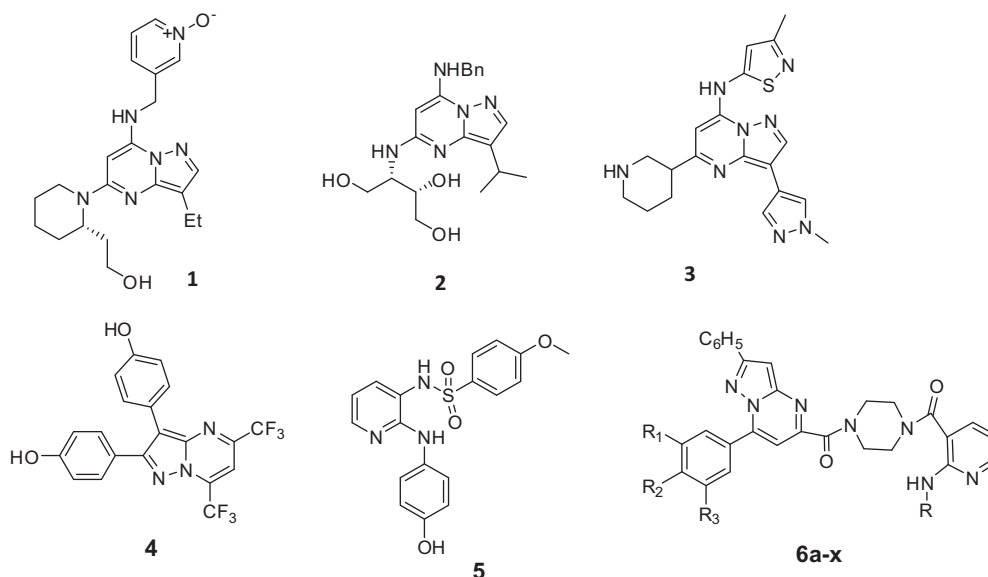


Figure 1. Chemical structures of pyrazolopyrimidines (**1–4**), E7010 (**5**), 2-anilonicotinyl-pyrazolo[1,5-a]pyrimidine conjugates (**6a–x**).

remarkable selectivity against different types of cancers.²⁵ As described here, pyrazolopyrimidines and 2-anilonicotinyl scaffolds are of considerable interest in the development of anticancer compounds. Modifications on the pyrazolo[1,5-a]pyrimidine nucleus and 2-anilonicotinic acids have resulted in a large number of compounds that exhibit significant anticancer activities.

In continuation to our efforts on the development of useful synthetic protocols suitable for applications in the field of combinatorial chemistry and chemistry-driven drug discovery,^{26–28} Additionally, the medicinal importance of these moieties with impressive anticancer profile and their effect on various molecular targets as potential cancer chemotherapeutic agents an attempt has been made in the present study to link the pyrazolo[1,5-a]pyrimidine moiety to the 2-anilonicotinyl structural component of E7010. The conformationally restricted cyclic piperazine amide has been employed as a linker for this purpose resulting in a new class of compounds. These newly synthesized chemical entities have been evaluated for their antiproliferative potential and cell cycle perturbations. Moreover detailed studies have been carried out to understand their mechanistic aspects of regulating the cell proliferation in MCF7 cell line via estrogen signaling.

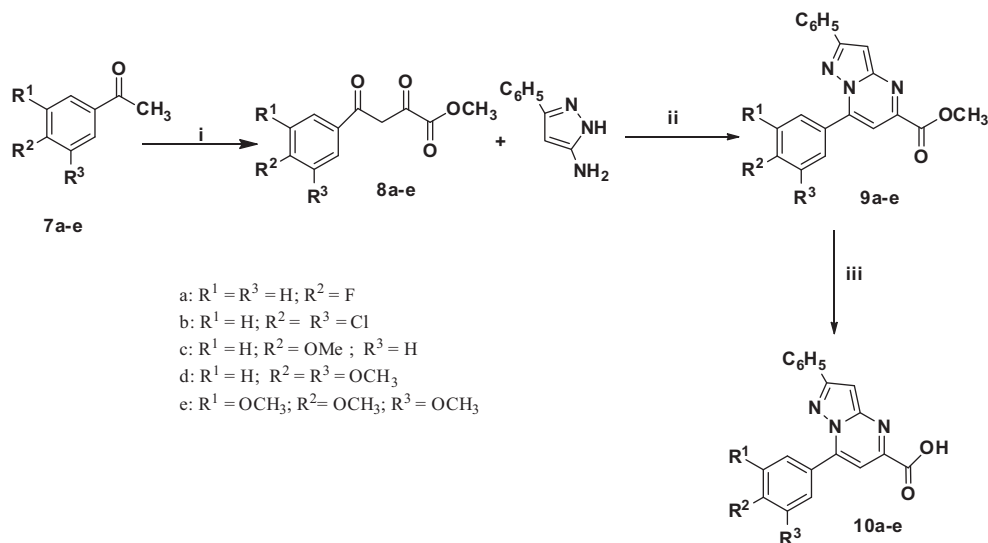
The synthesis of the target conjugates have been performed in a convenient manner as outlined in Schemes 1 and 2. The synthetic route employed starts with the synthesis of β -diketoesters (**8a**, **8b**, **8c**, **8d** and **8e**) with some modification to the one reported in the literature.²⁹ For this purpose, different substituted acetophenones (**7a–e**) on oxylation by dimethyl oxalate in the presence of sodium methoxide provides the β -diketoesters (**8a–e**). Different acetophenones with 4-chloro, 4-fluoro, 3-methoxy, 4-methoxy and 3,4,5-trimethoxy substitutions have been employed. A convenient protocol was employed for the synthesis of fused pyrazolo[1,5-a]pyrimidines compounds. The β -diketoester on cyclization with an aza-heterocyclic amine, i.e., 3-amino-5-phenyl pyrazolo in the presence of catalytic amount of ceric ammonium nitrate (CAN, 5 mol %) in ethanol at room temperature affords various substituted 2,7-diphenylpyrazolo[1,5-a]pyrimidine-5-carboxylic esters (**9a–e**), which upon base hydrolysis with sodium hydroxide provides the corresponding carboxylic acids (**10a–e**). Then with pyrazolo[1,5-a]pyrimidine acid intermediates (**10a–e**) in hand, we proceeded to synthesize the target conjugates.

The synthesis of these new conjugates (**6a–x**) has been accomplished by the synthetic route depicted in Scheme 2. The ethyl ester of 2-chloronicotinic acid **11** and different substituted anilines **12a–e** are refluxed in ethylene glycol to give the coupled product of 2-anilonicotinic acid esters **13a–e**. The anilines used are with different substitutions such as 4-fluoro, 4-methoxy, 3,4-dimethoxy and 3,4,5-trimethoxy substitutions with a view to bring diversity in the conjugates for a better understanding of their SAR. The esters **13a–e** on base hydrolysis on treating with 2 N sodium hydroxide solution in ethanol affords 2-anilonicotinic acids (**14a–e**) in quantitative yields. These acids are converted to amides by coupling them with commercially available Boc-protected piperazine in presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and hydroxyl benzotriazole (EDCI/HOBt) in dry dimethylformamide at 0 °C. To effect the coupling of 2-anilonicotinyl piperazineamide (**15a–e**) and pyrazolo[1,5-a]pyrimidine acid intermediates (**10a–e**), the Boc-protection on the piperazine moiety of **15a–e** is removed by treatment with trifluoroacetic acid in dichloromethane. This was followed by the crucial coupling assisted by EDCI/HOBt methodology in dry DMF to yield the desired 2-anilonicotinyl linked pyrazolo[1,5-a]pyrimidine conjugates (**6a–x**) as shown in Scheme 2 (Supplementary information).

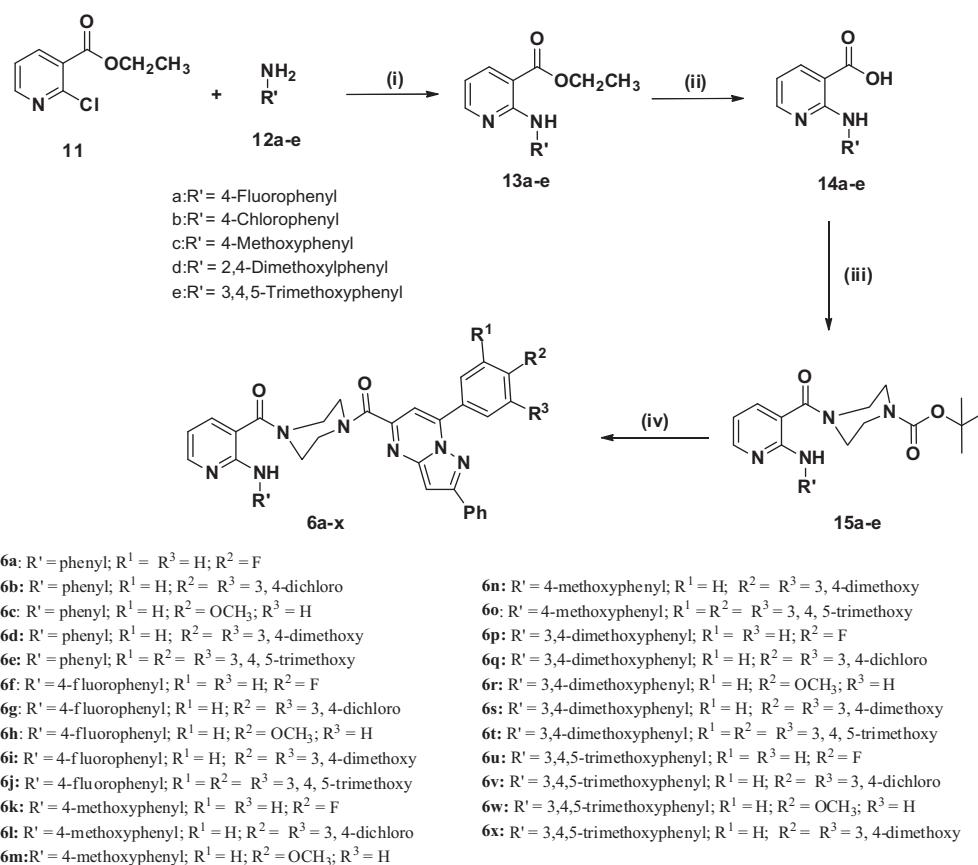
By employing this efficient and convenient synthetic route, we have synthesized twenty four new 2-anilonicotinyl linked pyrazolo[1,5-a]pyrimidine conjugates that are joined through conformationally restricted piperazineamide spacers.

These conjugates have been evaluated for their biological properties such as cytotoxic activity, cell viability and effects on proteins such as estrogen receptor, Bcl-2, survivin and cyclin D1 in MCF breast cancer cell lines in order to understand the mechanism involved during cell division arrest of cancer cells.

In order to study the possible cytotoxic effect by these series of conjugates (**6a–x**), MTT assay was conducted. Here SiHa, HeLa (cervical cancer cell line), MCF-7 (breast cancer), IMR-32 (neuroblastoma) were treated at concentration ranging from 0.25 to 8 μ M of conjugates for 48 h. It was observed that conjugates **6a**, **6c**, **6j**, **6m**, **6o**, **6q**, **6r**, **6s**, **6t** and **6w** were effective against all the cells. Conjugates **6a**, **6c** were found to be most effective among the series against the cell lines tested (Fig. 2 and Table 1). From cytotoxic data it is also evident that these molecules are specific to MCF-7, HeLa



Scheme 1. Reagents and conditions: (i) dimethyl oxalate, NaOMe, THF, rt, 8 h; (ii) cat. CAN, ethanol, 0.5 h, rt; (iii) 2 N NaOH, MeOH, reflux, 2 h.



Scheme 2. Reagents and conditions: (i) ethylene glycol, 160 °C, 6 h; (ii) 2 N NaOH, ethanol, reflux 2 h; (iii) Boc-piperazine, EDCI/HOBt, dry DMF 0 °C–rt, 8 h; (iv) (a) TFA, DCM, rt, 2 h; (b) substituted 2,7-diphenylpyrazolo[1,5-a]pyrimidine-5-carboxylic acid, EDCI/HOBt, dry DMF 0 °C–rt, 8 h.

and SiHa cells. Conjugates **6r**, **6s** and **6v** have shown specific effects in SiHa; conjugates **6q**, **6r** and **6s** have shown specific effects in MCF-7; conjugates **6a**, **6c**, **6l** and **6m** have shown specific effects in HeLa cells and are found to cause strong cytotoxic effects. However, IMR-32 cells did not respond significantly to these conjugates.

With the help of cell viability studies discussed above, structure activity relationships have been determined. Electron donating substituents on the phenyl ring of pyrazolopyrimidine scaffold as in **6c**, **6j**, **6m**, **6o**, **6r**, **6t**, **6s** and **6w** were found to be fruitful for activity. These compounds exhibited good activity against one or more of the tested cell lines irrespective of the substituents present

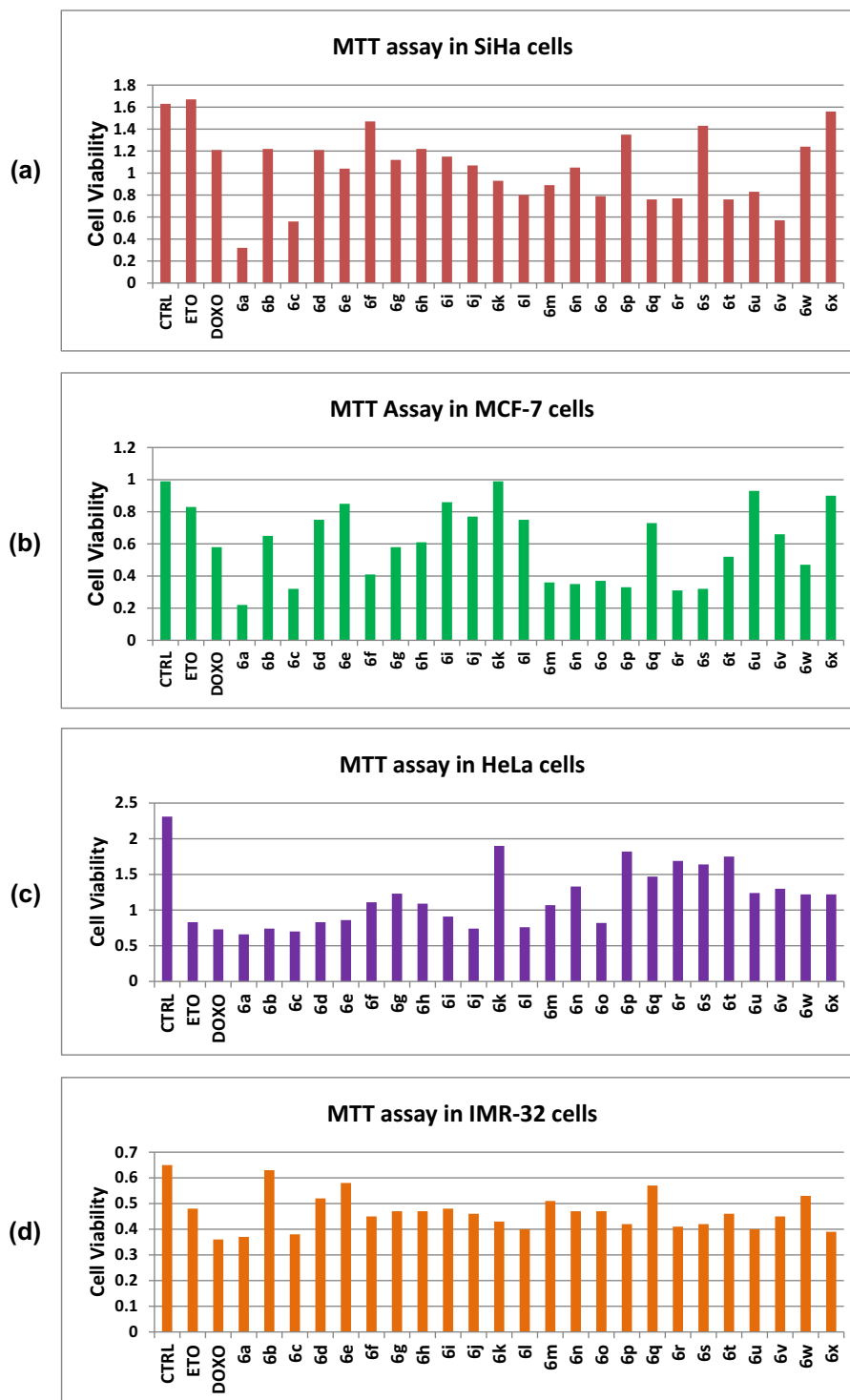


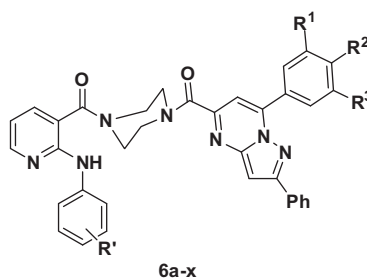
Figure 2. (a–d) SiHa, HeLa (cervical cancer), MCF-7 (breast cancer), IMR-32 (neuroblastoma) were studied at 4 μ M of conjugates for 24 h using MTT assay.

on 2-anilinicotinoyl group. Similarly, **6a** and **6q** containing weak electron withdrawing substituents such as fluoro and chloro on pyrazolopyrimidine scaffold displayed appreciable activity only in presence of certain substituents on 2-anilinicotinoyl group. On the other hand, substituents on 2-anilinicotinoyl moiety displayed random effects on the activity.

Cell cycle analysis has been performed to explore the basis of anti-proliferative properties of these effective conjugates (**6a** and **6c**), wherein doxorubicin was used as the positive control. MCF-7

cells were treated with doxorubicin (Doxo), **6a**, **6c** at 4–32 μ M concentration for 48 h. Control cells have shown only 25% of cells in G2/M phase of cell cycle whereas cells treated by compounds lead to huge accumulation of cells in G2/M phase.

Here doxorubicin caused 48%, 50%, 49%, 46% of G2/M phase cells at 4, 8, 16 and 32 μ M respectively. Whereas compounds **6a** and **6c** treated cells have shown accumulation in the range of 25–39% G2/M phase cells. Surprisingly concentration of 4 μ M and above has given almost the similar percentage of G2/M phase cells as it

Table 1Cytotoxicity of the compounds **6a–x** expressed in μM 

Compound	R'	R ¹	R ²	R ³	SiHa	MCF-7	HeLa	IMR-32
6a	H	H	F	H	2.65	1.79	2.29	4.65
6b	H	H	Cl	Cl	5.93	5.30	2.57	7.87
6c	H	H	OCH ₃	H	3.33	2.16	2.43	4.75
6d	H	H	OCH ₃	OCH ₃	5.93	6.12	2.88	6.5
6e	H	OCH ₃	OCH ₃	OCH ₃	5.10	6.93	2.99	7.25
6f	4-F	H	F	H	7.21	3.34	3.86	5.62
6g	4-F	H	Cl	Cl	5.27	4.73	4.27	5.87
6h	4-F	H	OCH ₃	H	5.93	4.97	3.79	5.87
6i	4-F	H	OCH ₃	OCH ₃	5.64	7.02	3.16	5.87
6j	4-F	OCH ₃	OCH ₃	OCH ₃	5.25	6.28	2.57	5.75
6k	4-OCH ₃	H	F	H	4.56	8.08	6.60	5.37
6l	4-OCH ₃	H	Cl	Cl	3.92	6.12	2.64	5
6m	4-OCH ₃	H	OCH ₃	H	4.36	2.93	3.72	6.37
6n	4-OCH ₃	H	OCH ₃	OCH ₃	5.10	2.85	4.62	5.87
6o	4-OCH ₃	OCH ₃	OCH ₃	OCH ₃	3.87	3.02	2.85	5.87
6p	3,4-diOCH ₃	H	F	H	6.62	2.69	6.33	5.25
6q	3,4-diOCH ₃	H	Cl	Cl	3.73	5.95	5.11	7.15
6r	3,4-diOCH ₃	H	OCH ₃	H	3.73	2.53	5.87	5.12
6s	3,4-diOCH ₃	H	OCH ₃	OCH ₃	5.93	2.61	5.70	5.25
6t	3,4-diOCH ₃	OCH ₃	OCH ₃	OCH ₃	3.73	4.24	6.08	5.75
6u	3,4,5-triOCH ₃	H	F	H	4.07	7.59	4.31	5.0
6v	3,4,5-triOCH ₃	H	Cl	Cl	3.65	5.38	4.52	5.62
6w	3,4,5-triOCH ₃	H	OCH ₃	H	6.08	3.83	4.24	6.62
6x	3,4,5-triOCH ₃	H	OCH ₃	OCH ₃	7.65	7.34	4.24	4.88
Doxorubicin (Doxo)	—	—	—	—	3.93	0.473	1.25	4.5

may be the saturation concentration for inducing cell cycle effects. In addition the increased concentration resulted in increased percentage of cells that undergo apoptosis as indicated by cells accumulated in G0 phase (Fig. 3).

Since breast cancer cells are highly resistant to anticancer drugs due to multidrug resistance (MDR), we were interested to examine the molecular mechanism of action of these molecules in ER positive MCF-7 cells. In breast cells estrogen receptors such as ER α , ER β are expressed and are activated by estrogen hormone.³⁰ High ER α expression is associated with tumor growth in breast and high levels ER β inhibit tumor growth.³

It is well established fact that ligands such as 17- β -estradiol and genistein bind to estrogen receptor and cause breast cancer.

The activated ER α regulate gene expression by binding to consensus and non-consensus **ERE** (5'-GGTCAnnnTGACC-3') on target promoters and thus acts as transcription factor that regulate the breast cancer cell proliferation.³¹ Thus we have examined the effect of these conjugates on ERE and ER α protein expression by conducting luciferase based studies by treating MCF-7 cells with the effective compounds (**6a**, **6c**, **6j**, **6m**, **6o**, **6q**, **6r**, **6s**, **6t**, **6w**) obtained from MTT assay at 1 μM concentration for 24 h, wherein etoposide (ETO) and doxorubicin (Doxo) are used as standard drugs as shown in Figures 4 and 5.

Small molecules that target ERE play a crucial role in controlling breast cancer cell proliferation. Bcl-2 is the major anti-apoptotic protein that confers resistance to apoptosis by reducing the effectiveness of chemotherapy and inhibits the mitochondrial apoptosis pathway. Further studies also indicate that over expression of Bcl-2

inhibits apoptosis induced by anticancer drugs, radiation, and other DNA-damaging agents.³² Moreover 17- β -estradiol induces bcl-2 gene transcription in human breast cancer cell line MCF-7 via two estrogen response element (ERE's)³³ and also Bcl-2 and survivin proteins are responsible for rapid cell proliferation by functioning as anti-apoptotic proteins (IAPs). It was considered of interest to examine the protein levels in the compound treatment scenario. To our surprise these molecules inhibits the expression of Bcl-2 and survivin. This can function by inhibiting the interaction between ERE and ER alpha at Bcl-2 and effect the downstream surviving protein. Further we have also examined the possibility of effect of compounds on cyclin D1, the protooncogene that is functions at G1-S phase transition, no significant changes were observed in the level of this protein (Fig. 6).

Model proposed: Conjugates inhibited the expression of ER alpha and down regulated the proteins that are vital for breast cancer cell proliferation such as Bcl-2, cyclin D and survivin that finally result in cell cycle perturbations and apoptosis (see Fig. 7)

To summarise, in this work new chemical entities based on the conjugation of potent moieties pyrazolo[1,5-a]pyrimidine and 2-anilinicnicotinic acid linked by conformationally restricted piperazineamide spacers were synthesized by a convenient synthetic route. These chemical entities are tested for their anticancer properties followed by detailed biological studies to understand their mechanism of action. Some of the conjugates like **6a**, **6c**, **6r** and **6s** are found to be promising antiproliferative agents by regulating antiapoptotic proteins and proto-oncogene. Thus it could be concluded that conjugates **6a** and **6c** that effects ERE-ER α interaction

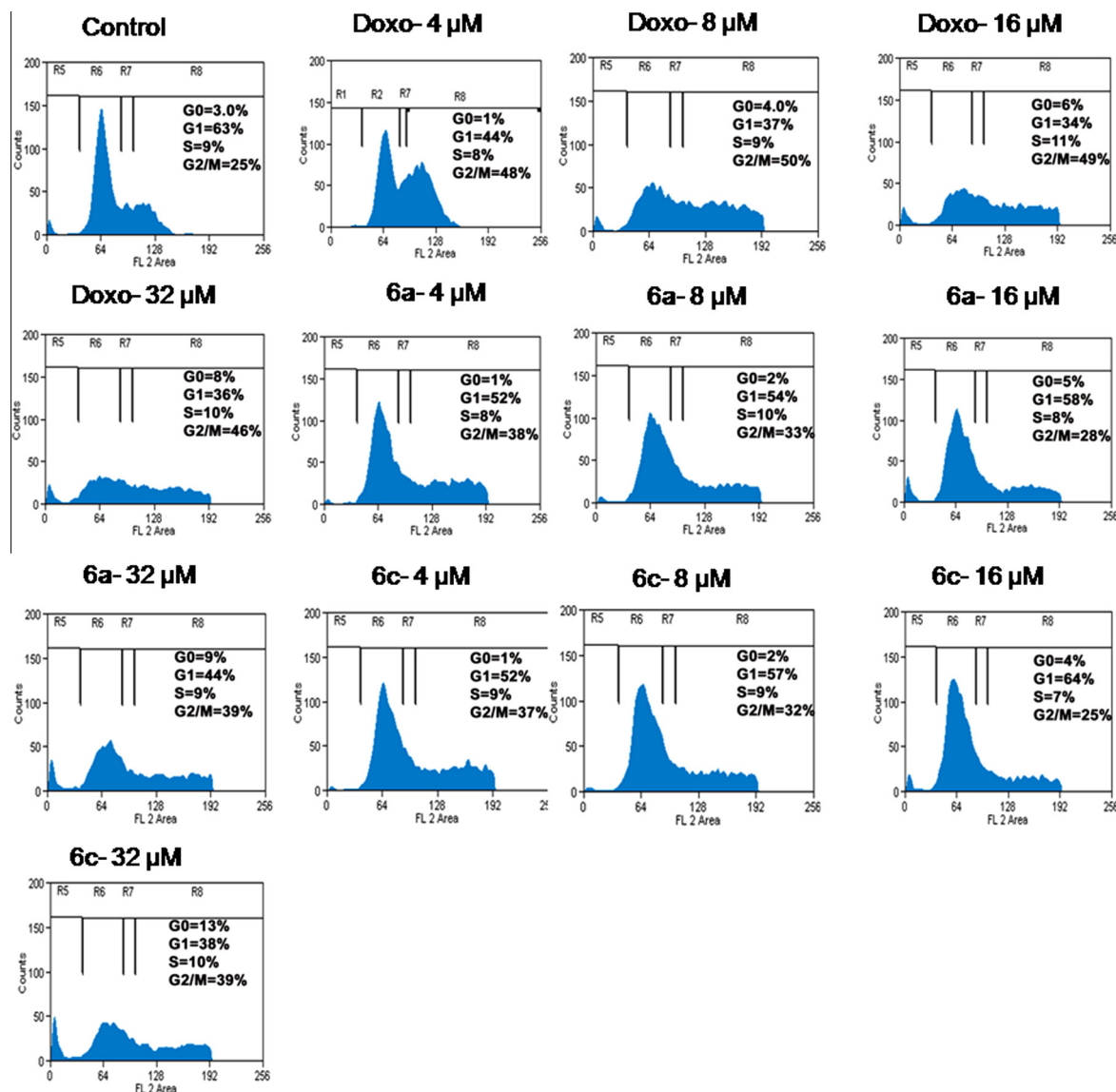


Figure 3. The cell cycle analysis was conducted in MCF-7 cells treated with **6a**, **6c** at concentrations of 4, 8, 16, 32 μM for 48 h. Here doxorubicin (Doxo), the anthracycline antibiotic as well as one of most effective anticancer agent used to treat against breast cancer was used as positive control. Conjugates induced G2/M cell cycle arrest. Increased concentration of drug caused apoptosis as indicated by percentage of cells in G0 phase.

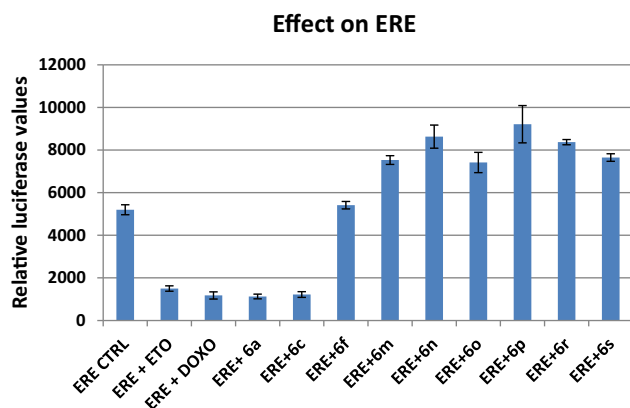


Figure 4. Effect on ERE: the effect compound on ERE was studied in MCF-7 cells at 1 μM conc of **6a**, **6c**, **6j**, **6m**, **6o**, **6q**, **6r**, **6s**, **6t** and **6w** for 24 h. Here ETO: etoposide. Doxo: doxorubicin. Initially the transfection with ERE-Luc is carried out for 48 h followed by compound treatment for 24 h.

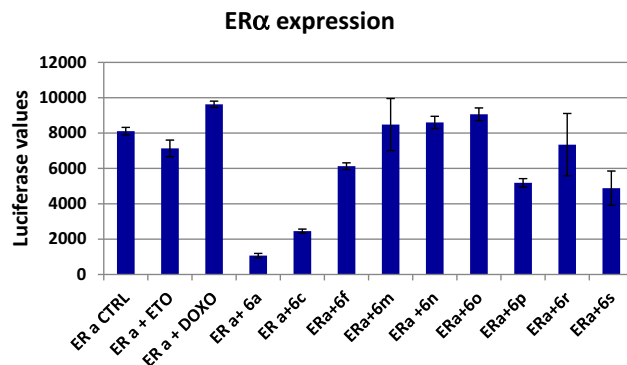


Figure 5. Effect on ER alpha: the effect of compounds on ER alpha was studied in MCF-7 cells at 4 μM conc of **6a**, **6c**, **6f**, **6m**, **6n**, **6o**, **6q**, **6r**, **6s** for 24 h. Here ETO: etoposide. Doxo: doxorubicin. Initially the transfection with ERα is carried out for 48 h followed by compound treatment for 24 h.

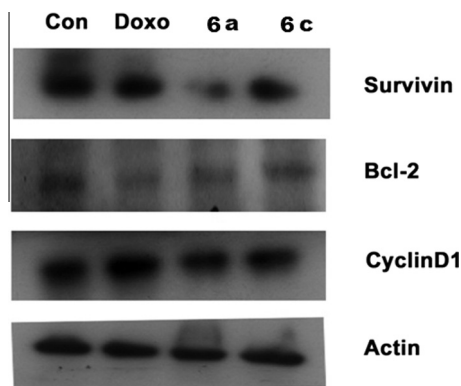


Figure 6. Effect on Bcl-2, survivin and cyclin D1: the effect compound on oncogenic (Bcl-2 and cyclin D1) as well as survivin proteins was studied in MCF-7 cells by treatment at 1 μ M conc of **6a** and **6c** for 24 h. Doxo: doxorubicin was used as standard.

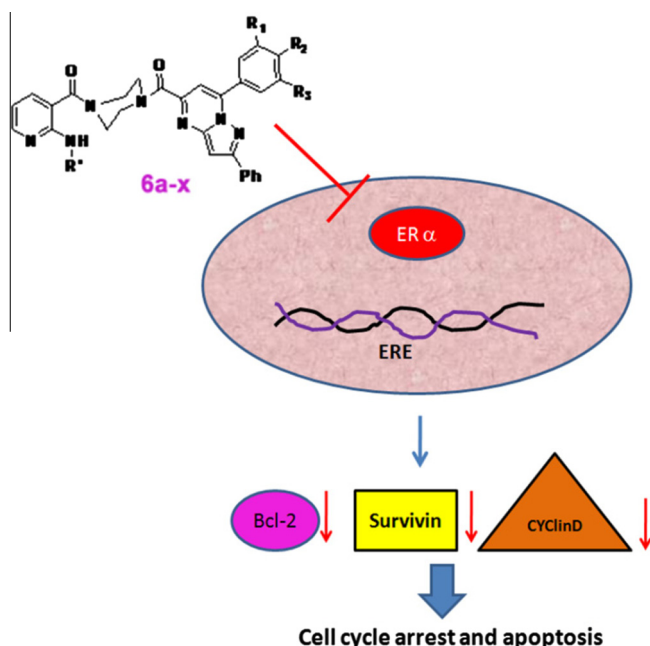


Figure 7. Model proposed displaying the effect of compounds **6a–x** on ER α .

as well as **6r** and **6s** that affects ER α could be considered as effective molecules and have the potential to be taken up for preclinical studies as promising therapeutics for the treatment of breast cancer.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2016.02.072>.

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