European Journal of Medicinal Chemistry 45 (2010) 3924–3937

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis and biological evaluation of 3,5-diaryl-isoxazoline/isoxazole-pyrrolobenzodiazepine conjugates as potential anticancer agents

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ARTICLE INFO

Article history:
Received 16 April 2010
Received in revised form
19 May 2010
Accepted 21 May 2010
Available online 31 May 2010

Keywords:
Pyrrolobenzodiazepine
3,5-Diaryl-isoxazoline/isoxazole
p53
Cytochrome *c*
Procaspase-3

ABSTRACT

A series of 3,5-diaryl-isoxazoline/isoxazole linked pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) conjugates were prepared. These conjugates showed potent anticancer activity with GI_{50} values in the range of <0.1–3.6 μM. Some of these PBD conjugates (**6a**–**c**) with promising anticancer activity were further investigated on the cell cycle distribution. Moreover, these PBD conjugates exhibited G0/G1 arrest, enhancement in the levels of p53 protein as well as mitochondrial-mediated intrinsic pathway, leading to release of cytochrome *c*, activation of caspase-3, cleavage of PARP and subsequent apoptotic cell death. Hence these PBD conjugates with **6a** being the most potent one could be be taken up for preclinical studies either alone or in combination with existing therapies.

1. Introduction

Chemotherapy is often the treatment of choice for many types of cancer as a result the search for newer chemotherapeutic agents still plays a major role in the fight against cancer. In recent years there has been increasing interest in the design of conjugate molecules that could act in a specific manner on more than one target. The development of such conjugates lowers the risk of drug–drug interaction in comparison to cocktails but could also enhance the efficacy as well as improve the safety aspects in relation to the drugs that interact on a single target, [1–3] and one such recent example is bleomycin [4].

Combretastatins, naturally occurring stilbenes, are isolated from *Combretum caffrum* by Pettit's group [5]. Among them, combretastatin A-4 (CA-4) showed most potent cytotoxicity against a variety of human cancer cell lines including multiple drug-resistant cancer cell lines [6]. From the previous comparative studies of the combretastatins it appears that the *cis* orientation of the two aromatic rings plays an important role in cytotoxicity. Accordingly, a number of *cis*-restricted analogues of CA-4 have been prepared using five-membered heterocycles [7–9] to avoid the stability problem. Many of them showed potent cytotoxicity against various cancer cells compared to CA-4. In a recent study, *cis*-restricted analogues of CA-4 (diaryl substituted isoxazoline/isoxazole derivatives) are reported to possess potent apoptosis-inducing activity [10,11].

Similarly, pyrrolo[2,1-*c*][1,4]benzodiazepines (PBD's) like anthramycin, tomaymycin and DC-81 belong to a family of potent tricyclic anticancer antibiotics, that are isolated from various *Streptomyces* species and they bind in the minor groove of double-stranded DNA forming a covalent bond to the exocyclic amino group of a central guanine within a three base pair recognition site [12–14]. The cytotoxicity and antitumour effects of these compounds are believed to arise from this modification of DNA, which leads to inhibition of nucleic acid synthesis and production of excision dependent single and double strand breaks in cellular DNA (Fig. 1).

In the past few years, several hybrid compounds, in which a known antitumour compound or some simple active moiety

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doi:10.1016/j.ejmech.2010.05.047

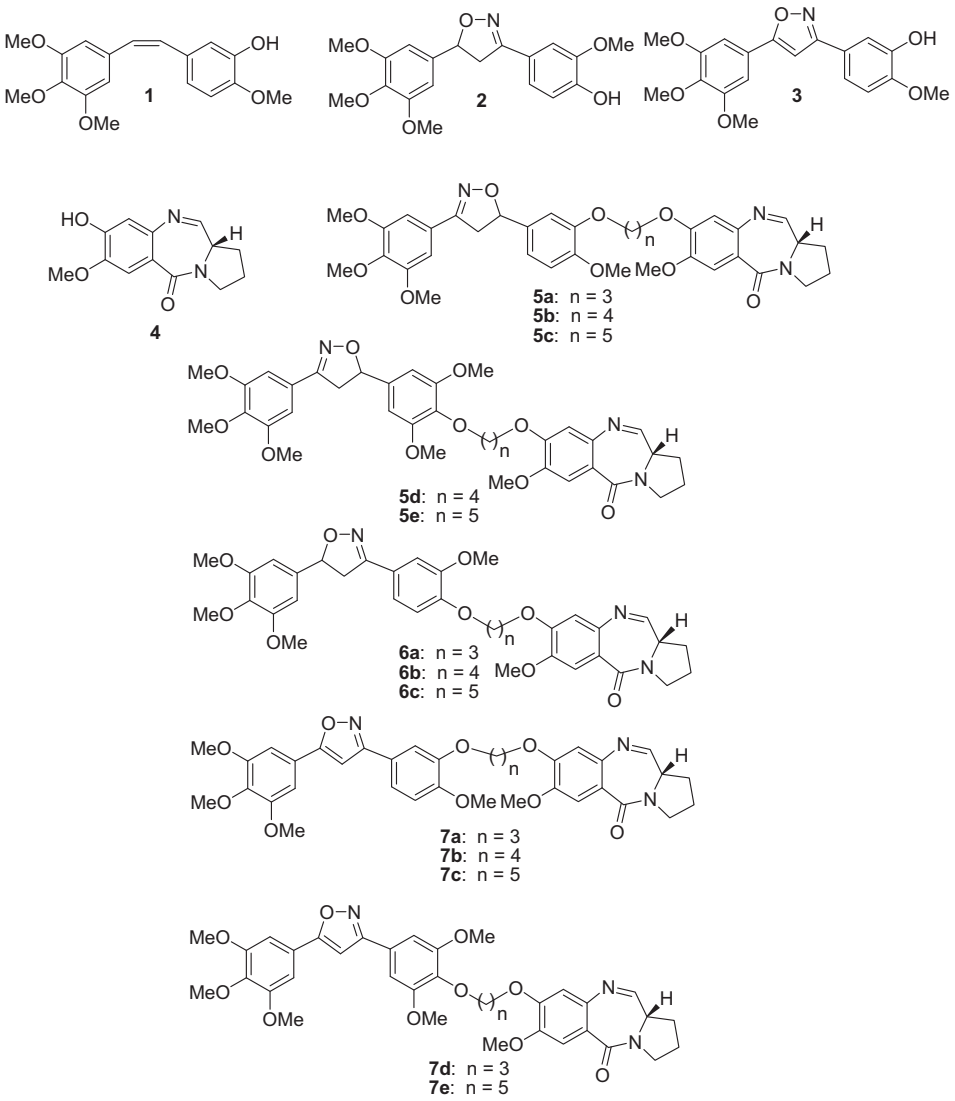


Fig. 1. Structures of combretastatin (CA-4) (**1**), 3,5-diarylisoxazoline (**2**), 3,5-diarylisoxazole (**3**), DC-81(**4**), and 3,5-diaryl-isoxazoline/isoxazole-PBD conjugates (**5a**–**e**, **6a**–**c** and **7a**–**e**).

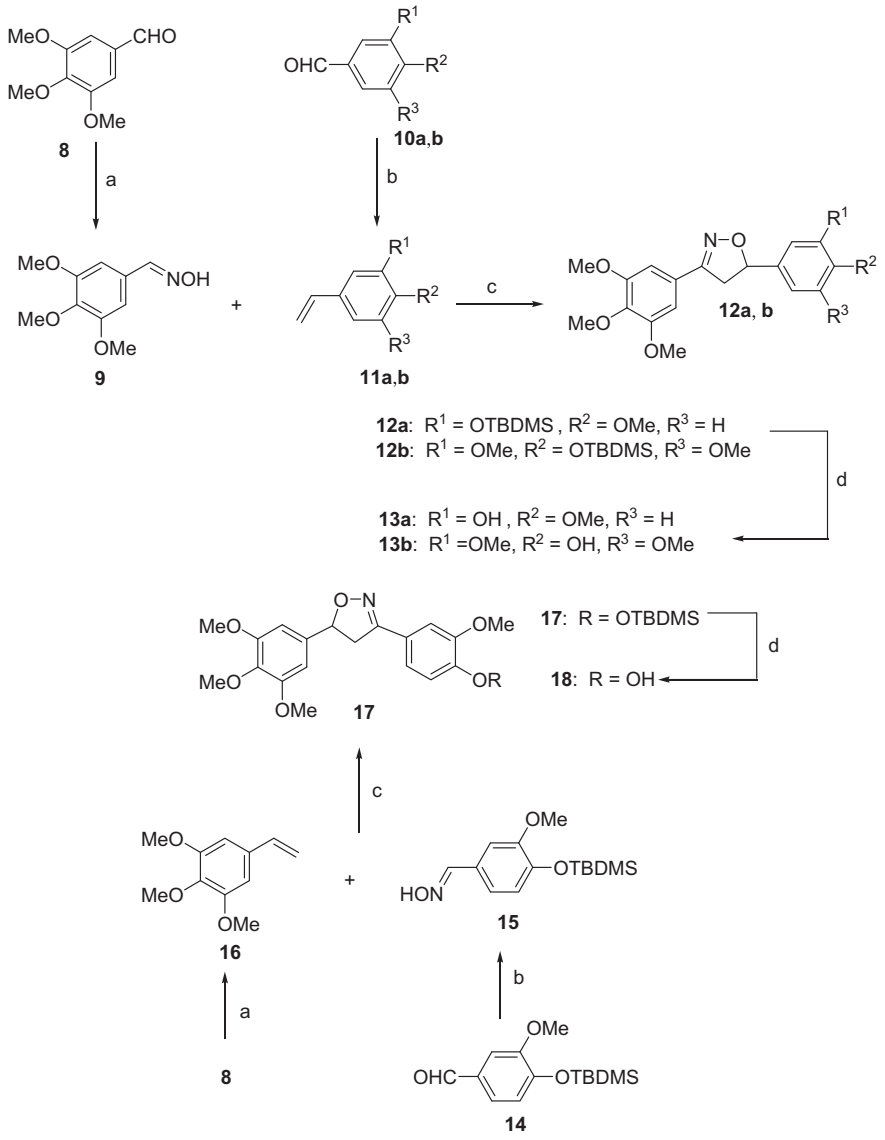
tethered to PBD, have been designed, synthesized and evaluated for their biological activity [15–18]. Recently, Wang and co-workers have synthesized indole-PBD conjugates as potential antitumour agents and a correlation between antitumour activity and apoptosis has been well explained [19]. For the last few years, we have been involved in the development of new synthetic strategies for the preparation of PBD ring system [20,21] and also in the design as well as synthesis of structurally modified PBDs and their conjugates [22–26]. More recently, we have also reported some of the PBD conjugates that demonstrated potent apoptotic activity through mitochondrial-mediated pathway [27,28].

In continuation of these efforts, we herein report the synthesis and biological evaluation of some new conjugates where in 3,5-diaryl-isoxazoline/isoxazole moieties have been linked to the pyrrolo[2,1-*c*][1,4]benzodiazepine ring system. Initially the anticancer activity has been carried out for these new PBD conjugates. In view of promising activity it has been considered of interest to investigate their role in cell proliferation and apoptosis by using the human melanoma cell line A375. The main aim of this study is to explore whether these conjugates possess better anticancer activity than DC-81 (**4**) and to substantiate these results with detailed biological studies.

2. Chemistry

The synthesis of 3,5-diaryl-isoxazoline/isoxazole derivatives (**13a**,**b**, **18** and **22a**,**b**) [10,11] was carried out from aldehydes **8**, **10a**, **b** and **14** as the starting materials. Reaction of these aldehydes with hydroxylamine in a MeOH/H_2O (3:1) solution produced the corresponding oximes **9**, **20a**,**b** and **15**. Olefins **11a**,**b** and **16** were obtained by the reaction of aldehydes **10a**,**b** and **8** with methyltriphenylphosphoniumbromide, in the presence of sodium hydride. Then these olefins **11a**,**b** and **16** were coupled to oximes **9** and **15** to provide the corresponding TBDMS protected isoxazolines **12a**, **b** and **17**. Similarly, TBDMS protected isoxazoles **21a**,**b** were prepared by employing alkyne **19** and oximes **20a**,**b**. Then, these compounds **12a**,**b**, **17** and **21a**,**b** upon deprotection with tetrabutylammonium fluoride gave the desired precursors (**13a**,**b**, **18** and **22a**,**b**) as shown in Schemes 1 and 2.

The synthesis of C8-linked 3,5-diaryl-isoxazoline/isoxazole-PBD conjugates (**5a**–**e**, **6a**–**c** and **7a**–**e**) was carried out from the (2*S*)-*N*-{4-[3-bromoalkoxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**23a**–**c**), and these were prepared by the methods reported in our earlier studies [20–28]. These upon etherification with the 3,5-diaryl-isoxazoline/isoxazole intermediates



Scheme 1. Synthesis of 3,5-diaryl-isoxazoline analogues (**13a**,**b**, **18**). *Reagents and conditions*: a) $NH_2OH.HCl$, $NaHCO_3$, CH_3OH:H_2O (3:1), 0 °C, then rt, 6 h; b) $CH_3PPh_3^+Br^-$, NaH, THF, 0 °C, then rt, 5 h; c) 13% aq NaOCl, Et_3N, CH_2Cl_2, 0 °C, then rt, 24 h; d) TBAF, THF, rt, 2 h.

using K_2CO_3 in acetone provided the corresponding nitro thioacetals (**24a**–**e**, **25a**–**c** and **26a**–**e**). Further, reduction of nitro compounds by using $SnCl_2.2H_2O$ in MeOH followed by deprotective cyclization employing $HgCl_2/CaCO_3$ afforded the desired PBD conjugates (**5a**–**e**, **6a**–**c** and **7a**–**e**) as shown in Scheme 3.

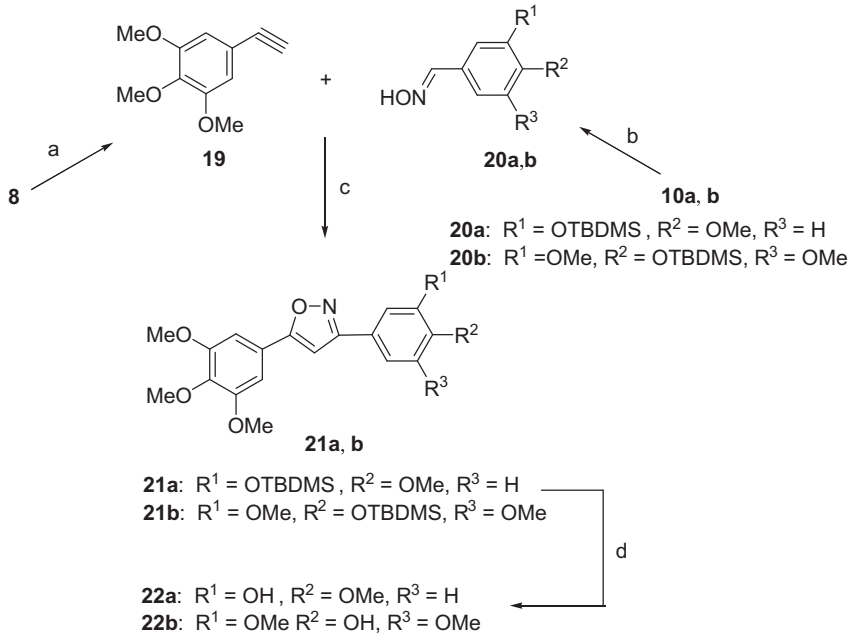
3. Results and discussion

3.1. Evaluation of biological activity

3.1.1. Anticancer activity

The anticancer activity of compounds **5b** and **6b** was evaluated against a panel of sixty human cancer cell lines derived from nine cancer cell types as shown in Table 1. The mean GI_{50} value for compound **6b** is 0.25 μM, which exhibits an interesting profile of activity for various cell lines, indicating that this compound **6b** has potent broad-spectrum anticancer activity. The promising activity shown by compounds **5b** and **6b** prompted us to evaluate the anticancer activity of the other analogues (**5a, 5c**–**e, 6a, 6c,** and **7a**–**e**) in selected human cancer cell lines of breast, cervix, lung, colon, oral, ovarian and prostate by using Sulforhodamine B (SRB) method. The compounds that exhibiting $GI_{50} \leq 10^{-5}$ μM were considered to be active on the respective cell lines. Table 2 reveals that compounds **5a, 5c**–**e, 6a, 6c,** and **7a**–**e** exhibit significant anticancer activity against some of the cell lines with GI_{50} values ranging from <0.1 to 2.15 μM. The positive control compound adriamycin demonstrated highly significant activity with the GI_{50} in the range from <0.1 to 14.0 μM and for DC-81 the GI_{50} ranged from 0.11 to 2.37 μM. Further, it was observed that among all the conjugates prepared, compounds **6a** and **6c** showed better anticancer activity against a number of cancer cell lines with GI_{50} < 0.1 μM. Similarly compounds **5a**, **5c**–**e**, and **7a**–**e** also exhibited significant activity against respective cell lines with GI_{50} values ranging from <0.1 to 2.37 μM.

MTT assay was also carried out to identify the cytotoxic effect of the most active compounds **6a**–**c** on A375 cells at 4 μM concentration. Moreover, the anticancer activity of compound **1** (CA-4), **2** (3,5-diaryl-isoxazoline) and **4** (DC-81) was examined to substantiate the potentiality of these conjugates (**6a**–**c**) as anticancer agents. Conjugates **6a** and **6b** with the exception of **6c**



Scheme 2. Synthesis of 3,5-diaryl-isoxazole analogues (**22a**,**b**). *Reagents and conditions*: a) (i) CBr_4, PPh_3, CH_2Cl_2, 0 °C, 10 min; (ii) n-BuLi, THF, −78 °C to rt; b) $NH_2OH.HCl$, $NaHCO_3$, CH_3OH:H_2O (3:1), 0 °C, then rt, 6 h; c) 13% aq NaOCl, Et_3N, CH_2Cl_2, 0 °C, then rt, 24 h; d) TBAF, THF, rt, 2 h.

showed a higher cytotoxicity than **1**, **2** and **4** on A375 cells as shown in Fig. 2.

3.1.2. Cell cycle effects

To investigate the effect of these PBD conjugates on the cell cycle progression of human cancerous cell line A375, the DNA content of the cell nuclei was measured by flow cytometric analysis (FACS analysis). Treatment of A375 cells with these compounds (**6a**–**c**) at 4 μM concentration for 24 h induced apoptosis up to 98.5%, 95.25% and 92.45% respectively of G0/G1 and there is a concomitant decrease in the G2/M phase. Whereas, DC-81 (**4**) showed 88.05% in G0/G1 phase, conjugate partner 3,5-diaryl-isoxazoline (**2**), and CA-4 (**1**) have shown 82.59% and 49.08% respectively in G0/G1 phase. Therefore, increase in cells of G0/G1 phase and decrease of G2/M phase cells clearly shows that all these conjugates cause G1 arrest and are effective in causing apoptosis in case of cancerous A375 cells. Moreover, compound **6a** could be considered as the most effective conjugate to produce cell cycle arrest (Fig. 3a and b).

3.1.3. Effect of 3,5-diaryl-isoxazoline-pyrrolobenzodiazepine conjugates on the expression of apoptotic proteins

It is well known that p53, a tumor suppressor gene, stops the formation of tumors. It exerts antiproliferation effects through its ability to function as a sequence-specific DNA-binding transcription factor. Activation of certain genes such as p53 is found to be important in the regulation of apoptotic pathway induced by various stimuli [29]. In order to understand the effect of PBD conjugates on p53 dependent apoptotic pathway, the cells were treated with these conjugate compounds and the cell lysate was used for Western blot analysis using p53 specific antibody. It is observed that the p53 levels are upregulated in case of compounds **6a**–**c** when compared to the untreated controls and interestingly, the levels of p53 are markedly high in compound **6a** as seen in Fig. 4.

Further, it was considered of interest to understand the nature of apoptotic pathway and the effect involved in regulating this activity. In this context the intrinsic apoptotic pathway was examined which is an important consequence of the mitochondrial dysfunction and cytochrome *c* release [30]. Moreover, cytosolic cytochrome *c* is known to cause activation of caspase-3 [31] and to investigate the effect on cytochrome *c*, A375 cells were treated with PBD conjugates at 4 μM concentration for 24 h. It was observed that there is an increase in cytochrome *c* protein level and this enhancement in cytochrome *c* level is more pronounced in case of compound **6a** as shown in Fig. 4. This clearly shows the involvement of mitochondria in the apoptotic pathway.

Another important consequence of apoptosis is the procaspase-3 degradation to active caspase-3 and also cleavage of DNA repair enzyme PARP (poly ADP ribose polymerase) by caspase-3 [32]. Treated and untreated cell lysates have been analyzed for the caspase-3 expression levels by fluorometric based caspase-3 assay. The order of performance of the production of active caspase-3 is **6a** > **6b** > **6c** as shown in Fig. 5.

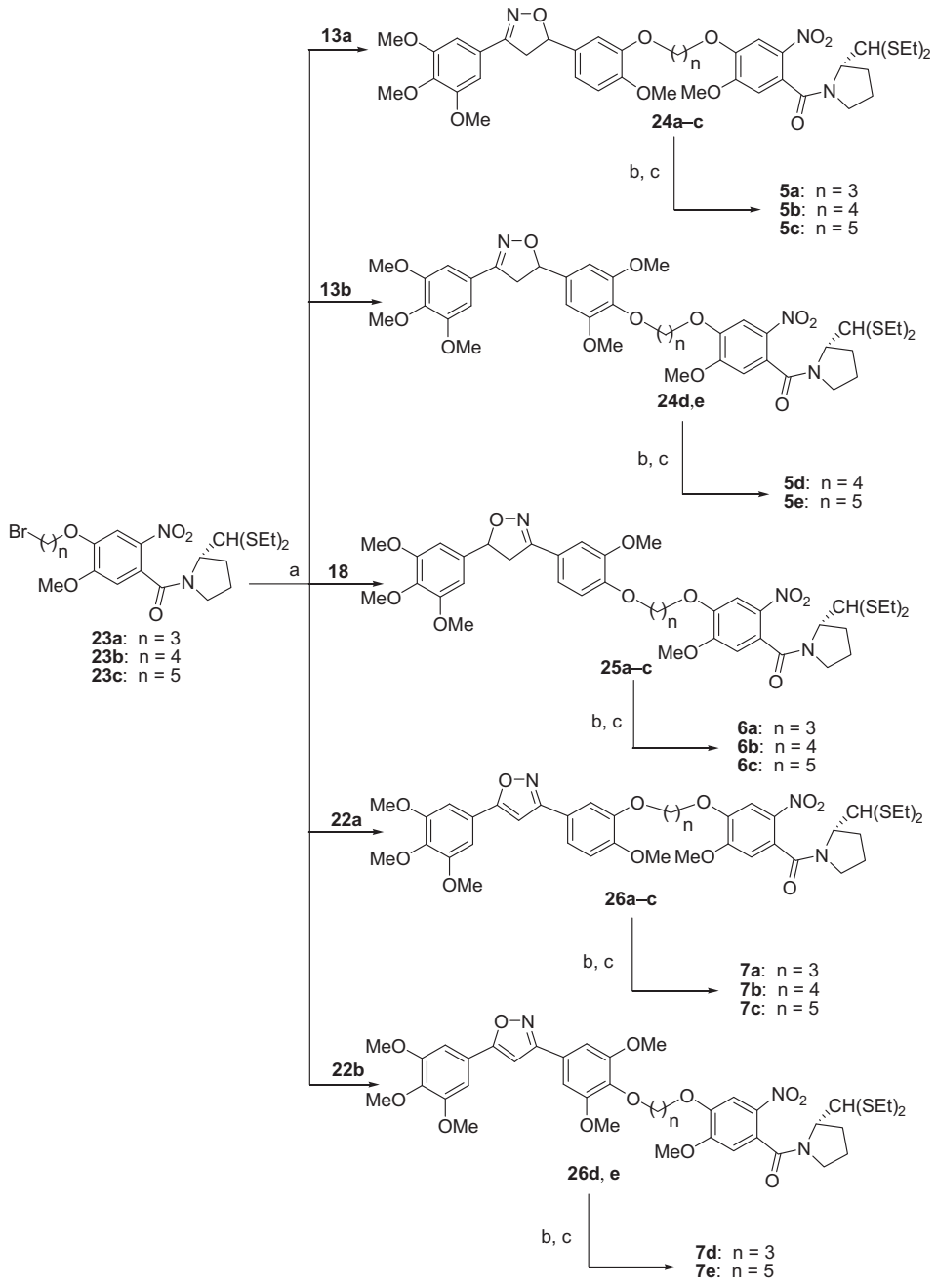
As we observed an increase in the levels of caspase-3, it is expected that there will be an enhancement in the levels of cleaved PARP [33]. An increase in the expression of cleaved PARP (85 kDa) specific to apoptosis was observed in all the conjugates (**6a**–**c**), where in compound **6a** showed the maximum increase as seen in Fig. 4.

3.1.4. Effect on cell cycle proteins

Cell cycle progression is regulated by cyclin dependent kinases (CDKs) that are activated by the binding of cyclins such as cyclin A, B, C and D and inhibited by CDK inhibitors, moreover CDK2 protein plays a pivotal role in G1–S interphase transition of cell cycle [34]. It was considered of interest to know the levels of CDK2 upon treatment with PBD conjugates (**6a**–**c**) and it was observed that there was a reduction of CDK2 levels as seen in Fig. 4. The reduction of Cdk2 protein level is slightly more in case of **6a** and **6b** than **6c.** This indicates that there is a possibility of involvement of cell cycle regulatory proteins in this event, however the intricacies involved further needs to be evaluated.

4. Conclusion

In the present study, a series of 3,5-diaryl-isoxazoline/isoxazole–pyrrolobenzodiazepine conjugates were synthesized. Compounds **5b** and **6b** that were evaluated at the National Cancer



Scheme 3. 3,5-diaryl-isoxazoline/isoxazole-pyrrolobenzodiaze conjugates (**5a**–**e**, **6a**–**c** and **7a**–**e**). *Reagents and conditions*: a) K_2CO_3, dry CH_3COCH_3, 48 h, 80–89%; b) $SnCl_2.2H_2O$, CH_3OH, reflux, 2 h; c) $HgCl_2$, $CaCO_3$, $CH_3CN–H_2O$, (4:1), 8 h, 52–59%.

Institute (NCI), Bethesda, USA exhibited potent anticancer activity against various cancer cell lines indicating that these compounds have the potential for their development as broad-spectrum anticancer agents. Further the other PBD conjugates also showed promising anticancer activity against the number of human cancer cell lines tested. Moreover, from the MTT proliferation assay it was observed that the conjugates **6a** and **6b** are more effective as antiproliferative agents than the naturally occurring PBD **4** (DC-81) in A375 cells at 4 μM concentration. The FACS analysis showed more population in G0/G1 phase indicating that all these PBD conjugates have cell cycle regulatory properties. It was also observed from the results of detailed biological assays that the p53 levels were enhanced when treated with compounds **6a**–**c**. Moreover, release of cytochrome *c* was as well observed for the conjugates (**6a**–**c**), thus indicating the involvement of mitochondrial pathway. Concomitant with this an increase in the levels of active caspase-3 and cleavage of PARP was also observed with all these conjugates, particularly it was more pronounced in case of **6a**. Further, all the conjugates (**5a**, **5c**–**e**, **6a**, **6c**, and **7a**–**e**) in which 3,5-diaryl-isoxazoline/isoxazole moiety linked to PBD ring system showed significant anticancer activity with GI_{50} values in the range of <0.1–2.15 μM in comparison to the previous prepared PBD conjugates, like C-8 linked triazolobenzothiadiazine-PBD conjugates [24] (GI_{50} values in the range of 0.22–30.30 μM), anthraquinone-PBD conjugates [23] (GI_{50} values in the range of <0.1–10 μM), 1,2,3-triazole-PBD conjugates [25] (GI_{50} values in the range of 0.13–30.50 μM), and phosphonate linked PBD conjugates [26] (GI_{50} values in the range of 0.17–30.50 μM) against 9–11 human cancer

Table 1
Anticancer activity of compounds **5b** and **6b** and in selected cancer cell lines[a]

Cancer panel/cell line	GI_{50} (μM)		Cancer panel/cell line	GI_{50} (μM)	
	5b	**6b**		**5b**	**6b**
Leukemia			*Ovarian*		
CCRF-CEM	1.41	0.16	IGROV1	1.57	0.24
HL-60(TB)	1.90	0.18	OVCAR-3	1.94	0.27
K-562	1.26	0.12	OVCAR-4	2.07	0.40
MOLT-4	1.85	0.20	OVCAR-5	3.13	0.42
RPMI-8226	1.43	0.24	OVCAR-8	2.55	0.37
SR	1.12	0.14	SK-OV-3	3.37	0.47
Non-smallcell lung			*Renal*		
A549/ATCC	2.33	0.26	786–0	1.57	0.28
EKVX	3.68	0.46	A498	1.72	0.18
HOP-62	1.68	0.27	ACHN	2.41	0.28
HOP-92	2.47	0.31	CAKI-1	3.09	0.25
NCI-H226	1.67	0.17	RXF 393	1.39	0.21
NCI-H23	1.83	0.27	SN12C	1.58	0.30
NCI-H322M	3.46	0.34	TK-10	3.51	0.28
NCI-H460	1.85	0.21	UO-31	2.09	0.29
NCI-H522	0.61	0.12			
Colon			*Breast*		
COLO 205	1.96	0.21	MCF7	1.75	0.20
HCC-2998	1.58	0.19	MDA-MB-	1.15	0.19
HCT-116	1.70	0.18	HS 578T	1.10	0.16
HCT-15	2.27	0.18	MDA-MB-435	3.54	0.17
HT29	2.75	0.36	BT-549	1.23	0.09
KM12	1.74	0.22	T-47D	1.31	0.28
SW-620	1.55	0.23	MDA-MB-468	1.34	0.22
CNS			*Prostate*		
SF-268	1.89	0.34	PC-3	0.32	0.58
SF-539	1.80	0.22	DU-145	0.32	2.48
SNB-19	1.96	0.34			
SNB-75	1.71	0.32			
U251	1.73	0.30			
Melanoma			*Melanoma*		
LOX IMVI	1.47	0.22	SK-MEL-28	2.07	0.20
MALME-3M	2.88	0.21	SK-MEL-5	1.47	0.16
M14	1.73	0.18	UACC-257	2.33	0.27
SK-MEL-2	2.03	0.23	UACC-62	1.52	0.16
Mean[b]	1.90(**5b**)	0.25(**6b**)			

[a] Data obtained from NCI's in vitro anticancer activity cells screen.
[b] Mean values over 60 cell lines tested.

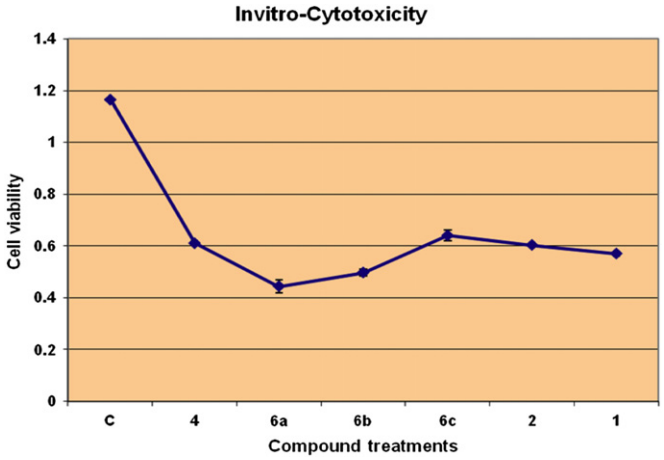


Fig. 2. Effect of PBD conjugates (**6a**–**c**) on cell viability. A375 cells were treated with 4 μM concentration of PBD conjugates as indicated for 24 h in 96-well plates seeded with 10,000 cells per well. O.D readings were taken at 420 nm wavelength which is a measure of cell viability after treatment with the respective compounds. DC-81 was used as the positive control, left side conjugate partner **(2)** and reference compound CA-4 **(1)** are also used. C: control cells (untreated cells).

cell lines in SRB assay. Hence from this data we can conclude that linking of 3,5-diaryl-isoxazoline/isoxazole moiety to the PBD ring system improved the anticancer activity. Finally, it may be concluded that one of the PBD conjugate **6a** prepared in the present investigation has the potential to be taken up for preclinical studies either alone or in combination with existing therapies.

5. Experimental protocols

All chemicals and reagents were purchased 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 silica gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. Column chromatography was performed with Merck (60–120) mesh silica gel. ^{1}H NMR spectra were

Table 2
GI_{50} values (in μM) for compounds (**5a**, **5c**–**e**, **6a**, **6c** and **7a**–**e**) in selected human cancer cell lines[a]

Compound	A549[b]	HOP62[b]	Zr-75-1[c]	MCF7[c]	A2780[d]	KB[e]	Colo205[f]	PC3[g]	SiHa[h]
5a	2.15	0.17	0.13	1.94	0.15	0.16	1.45	0.17	0.18
5c	0.15	0.18	0.12	1.85	0.14	0.16	1.32	0.16	0.16
5d	0.17	0.17	0.15	0.15	0.15	0.18	0.13	0.17	0.17
5e	0.16	0.18	0.18	0.16	0.15	0.18	0.13	0.18	0.18
6a	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
6c	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
7a	0.17	0.19	0.13	0.14	0.15	0.17	0.15	0.16	0.15
7b	0.12	0.17	0.10	0.12	0.14	0.15	0.13	0.14	0.15
7c	0.11	0.14	<0.1	<0.1	0.12	0.13	0.12	0.13	0.13
7d	0.12	0.17	0.18	<0.1	0.13	0.15	0.13	0.14	0.15
7e	0.11	0.17	0.10	0.10	0.14	0.16	0.14	0.17	0.16
DC-81(**4**)	0.16	0.15	2.37	0.17	0.14	0.17	0.11	0.20	0.17
ADR[i]	13.0	<0.1	<0.1	<0.1	<0.1	0.16	14.0	<0.1	1.9

[a] 50% growth inhibition and the values are mean of three determinations.
[b] Lung cancer.
[c] Breast cancer.
[d] Ovarian cancer.
[e] Oral cancer.
[f] Colon cancer.
[g] Prostate cancer.
[h] Cervix cancer.
[i] Adriamycin.

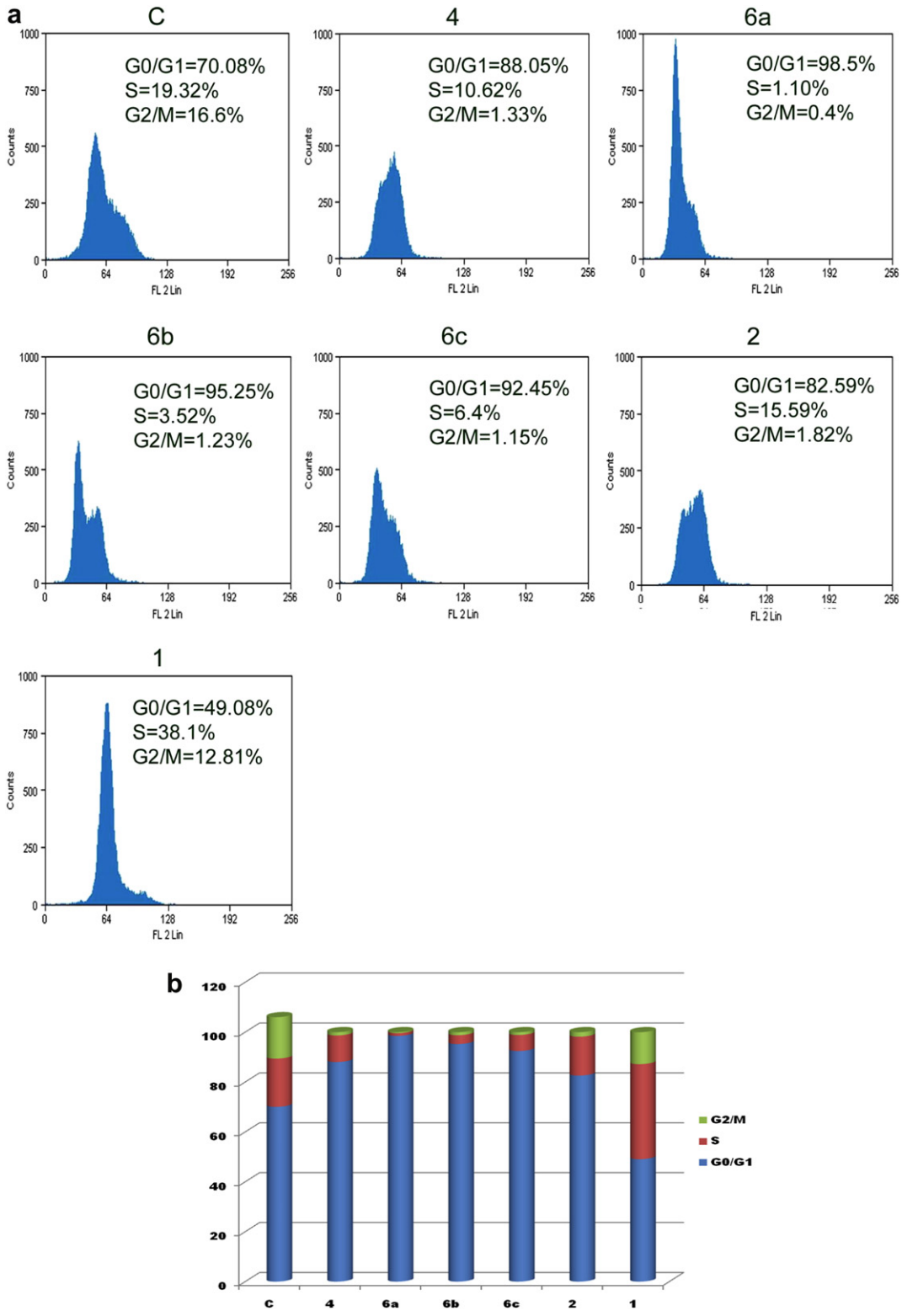


Fig. 3. (a). DNA histograms obtained by flow cytometry and the percentages of cells in G0/G1, S, and G2/M cell cycle phase, after the treatment of A375 cells with compounds **6a–c,** left side conjugate partner (**2**), reference compound CA-4 (**1**) and positive control DC-81 (**4**) at 4 μM for 24 h. (b). FACS analysis of cell cycle distribution of A375 cells after treatment with PBD conjugates (**6a–c**) for 24 h at 4 μM. DC-81 was used as the positive control, left side conjugate partner (**2**) and reference compound CA-4 (**1**) are also used. C: control cells treated with DMSO.

recorded on Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micromass, Quattro LC using ESI^+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector. High resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected.

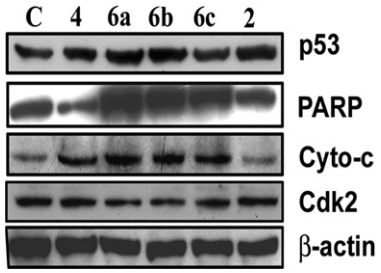


Fig. 4. Effect of 3,5-diaryl-isoxazoline/isoxazole-pyrrolobenzodiaze conjugates on the expression of apoptotic proteins. A375 cells were treated with compounds **6a**–**c**, DC-81 (**4**) and **2** at 4 μM concentrations for 24 h. The cell lysates were collected and expression levels of p53, cytochrome *c*, cleaved PARP and Cdk2 were determined by Western blot analysis. β-actin was used as loading control.

5.1. Synthesis of 3,5-diaryl-isoxazoline/isoxazole-PBD conjugates

5.1.1. Synthesis of 4-(tert-butyl-dimethyl-silyloxy)-3,5-dimethoxybenzaldehyde oxime (*20b*)

To a solution of hydroxylamine hydrochloride (12 mmol) dissolved in H_2O, $NaHCO_3$ (18 mmol) was added portion wise at 0 °C, and the mixture was stirred for 30 min at room temperature. The compound **10b** (2.96 g, 10 mmol), dissolved in MeOH, was then added to the solution, and stirring was continued for an additional 6 h. MeOH was evaporated in vacuum, and the residue extracted with diethyl ether. The organic extracts were washed with brine, dried, and evaporated under reduced pressure. This was further purified by column chromatography using ethyl acetate-hexane (8%) as eluent to afford compound **20b**. Yield 1.8 g, 60%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.98 (s, 1H, Ar**H**), 6.74 (s, 2H, Ar**H**), 3.83 (s, 6H, 2× −OC**H**$_3$), 0.99 (s, 9H, −C(C**H**$_3$)$_3$), 0.12 (s, 6H, −Si(C**H**$_3$)$_3$); MS (ESI): *m*/*z* 312 $[M + 1]^+$.

5.1.2. Synthesis of tert-butyl-(2,6-dimethoxy-4-vinylphenoxy)-dimethyl silane (*11b*)

NaH (55%) (20 mmol), previously washed with dry hexane, was added to a stirred suspension of methyltriphenylphosphoniumbromide (10 mmol) in dry THF (15 mL) containing the appropriate aldehyde **10b** (1.96 g, 10 mmol). After the suspension was stirred for 5 h at room temperature, Et_2O (30 mL) was added, and the mixture was poured into ice water and extracted with Et_2O. The combined organic extracts were dried and evaporated, and this was further purified by column chromatography using ethyl acetate-hexane (5%) as eluent to obtain the pure product **11b** as oil. Yield 2.2 g, 75%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.60 (dd, *J* = 17.6, 10.5 Hz, 1H, olefin−**H**), 6.55 (s, 2H, Ar**H**), 5.53 (d, *J* = 17.6 Hz, 1H, olefin−**H**), 5.16 (d, *J* = 10.5 Hz, 1H, olefin−**H**), 3.82 (s, 6H, 2× −OC**H**$_3$), 1.00 (s, 9H, −C(C**H**$_3$)$_3$), 0.16 (s, 6H, −Si(C**H**$_3$)$_3$); MS (ESI): *m*/*z* 295 $[M + 1]^+$.

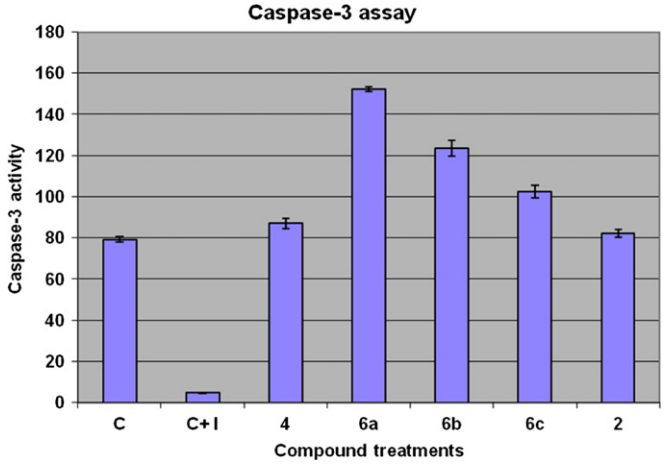


Fig. 5. Effect of PBD compounds on caspase-3 activity in A375 cells. The graph represents the increased enzymatic activity of caspase-3, during apoptotic event that occurs after the treatment of PBD conjugates (**6a**–**c**) at 4 μM concentration in A375 cells and was determined by fluorometery . Here DC-81 (**4**) is positive control and **2** is the conjugate partner. The cleavage of peptide by caspase-3 releases the fluorophore, AFC that was quantified at excitation wavelength of 400 nm and emission wavelength of 505 nm. 'I' represent the inhibitor used. C + I: Control treated with caspase-3 inhibitor. DEVD-CHO is the inhibitor here.

5.1.3. Synthesis of 5-(4-(tert-butyldimethysilyloxy)-3,5-dimethoxyphenyl)-3-(3,4,5-trimethoxy-phenyl)-4,5-dihydroisoxazole (*12b*)

To dipolarophile **11b** (10 mmol) and Et_3N (1 mmol) in CH_2Cl_2 were added, under argon atmosphere, a 13% aqueous solution of NaOCl (16 mmol) and dropwise (over a period of 1 h) at 0 °C, the appropriate oxime **9** (2.81 g, 10 mmol) in CH_2Cl_2. After being stirred at room temperature for 24 h, water was added to the reaction mixture and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with water, brine, dried (Na_2SO_4), filtered and concentrated under reduced pressure. This was further purified by column chromatography using ethyl acetate-hexane (15%) as eluent to obtain the pure product **12b** as yellow oil. Yield 3.0 g, 60%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.87 (s, 2H, Ar**H**), 6.56 (s, 2H, Ar**H**), 5.60 (dd, *J* = 10.5, 8.3 Hz, 1H, isoxazoline−**H**), 3.90 (s, 6H, 2× −OC**H**$_3$), 3.88 (s, 6H, 2× −OC**H**$_3$), 3.84 (s, 3H, −OC**H**$_3$), 3.67 (dd, *J* = 16.4, 10.5 Hz, 1H, isoxazoline−**H**), 3.24 (dd, *J* = 16.4, 8.3 Hz, 1H, isoxazoline−**H**), 1.02 (s, 9H, −C(C**H**$_3$)$_3$), 0.15 (s, 6H, −Si(C**H**$_3$)$_3$); MS (ESI): *m*/*z* 504 $[M + 1]^+$.

5.1.4. 3-(4-(tert-Butyldimethylsilyloxy)-3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydroisoxazole (*17*)

This compound was prepared according to the method described for compound **12b**, employing alkene **16** (2.94 g, 10 mmol) and oxime **15** (2.11 g, 10 mmol) to obtain the pure product **17** as yellow oil. Yield 2.5 g, 55%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.89 (d, 1H, *J* = 2.1 Hz, Ar**H**), 6.82 (d, 1H, *J* = 8.3, 2.1 Hz, Ar**H**), 6.77 (d, 1H, *J* = 8.3 Hz, Ar**H**), 6.44 (s, 2H, Ar**H**), 5.68 (dd, 1H, *J* = 10.6, 8.4 Hz, isoxazoline−**H**), 3.93 (s, 3H, −OC**H**$_3$), 3.84 (s, 6H, 2× −OC**H**$_3$), 3.65 (s, 3H, −OC**H**$_3$), 3.75 (dd, 1H, *J* = 16.6, 10.6 Hz, isoxazoline−**H**), 3.31 (dd, 1H, *J* = 16.6, 8.3 Hz, isoxazoline−**H**), 1.03 (s, 9H, −C(C**H**$_3$)$_3$), 0.17 (s, 6H, −Si(C**H**$_3$)$_3$); MS (ESI): *m*/*z* 474 $[M + 1]^+$.

5.1.5. 3-(4-(tert-Butyldimethylsilyloxy)-3,5-dimethoxyphenyl)-5-(3,4,5-trimethoxy-phenyl) isoxazole (*21b*)

This compound was prepared according to the method described for compound **12b**, employing alkyne **19** (1.92 g, 10 mmol) and oxime **20b** (2.81 g, 10 mmol). This was further purified by column chromatography using ethyl acetate-hexane (15%) as eluent to obtain the pure product **21b** as yellow oil. Yield 2.25 g, 45%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.01 (s, 4H, Ar**H**), 6.63 (s, 1H, isoxazole−**H**), 3.95 (s, 6H, 2× −OC**H**$_3$), 3.89 (s, 6H, 2× −OC**H**$_3$), 3.88 (s, 3H, −OC**H**$_3$), 1.01 (s, 9H, −C(C**H**$_3$)$_3$), 0.15 (s, 6H, −Si(C**H**$_3$)$_3$); MS (ESI): *m*/*z* 502 $[M + 1]^+$.

5.1.6. Synthesis of 2,6-dimethoxy-4-(5-(3,4,5-trimethoxyphenyl)-4,5-dihydroisoxa-zol-3-yl) phenol (*13b*)

A solution of the silyl ether **12b** (1.50 g, 3 mmol) in dry THF was treated with a 1 M solution of tetrabutylammonium fluoride in THF (3 mmol). The mixture was stirred at room temperature during 2 h, then diluted with water and extracted with ethyl acetate. The organic layers were washed with water, dried (Na_2SO_4), filtered and concentrated under reduced pressure. This was further purified by column chromatography using ethyl acetate-hexane (50%) as

eluent to obtain the pure product **13b** as a light yellow solid. Yield 0.99 g, 85%; mp 137–139 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.89 (s, 2H, ArH), 6.54 (s, 2H, ArH), 5.80 (br, 1H, –OH), 5.60 (dd, 1H, J = 10.5, 8.3 Hz, isoxazoline–H), 3.91 (s, 6H, 2× –OCH_3), 3.88 (s, 6H, 2× –OCH_3), 3.85 (s, 3H, –OCH_3), 3.68 (dd, 1H, J = 16.6, 10.5 Hz, isoxazoline–H), 3.26 (dd, 1H, J = 16.6, 8.3 Hz, isoxazoline–H); MS (ESI): m/z 390 $[M + 1]^+$.

5.1.7. 2-Methoxy-4-(5-(3,4,5-trimethoxyphenyl)-4,5-dihydroisoxazol-3-yl)phenol (**18**)

This compound was prepared according to the method described for compound **13b**, employing silyl ether **17** (1.42 g, 3 mmol). This was further purified by column chromatography using ethyl acetate-hexane (60%) as eluent to obtain the pure product **18** as light yellow solid. Yield 0.93 g, 86%; mp 130–132 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.91 (d, 1H, J = 2.1 Hz, Ar**H**), 6.85 (d, 1H, J = 8.3, 2.1 Hz, Ar**H**), 6.73 (d, 1H, J = 8.3 Hz, Ar**H**), 6.42 (s, 2H, Ar**H**), 5.85 (br, 1H, –O**H**), 5.67 (dd, 1H, J = 10.6, 8.4 Hz, isoxazoline–**H**), 3.94 (s, 3H, –OC**H**$_3$), 3.83 (s, 6H, 2× –OC**H**$_3$), 3.64 (s, 3H, –OC**H**$_3$), 3.74 (dd, 1H, J = 16.6, 10.6 Hz, isoxazoline–**H**), 3.35 (dd, 1H, J = 16.6, 8.3 Hz, isoxazoline–**H**); MS (ESI): m/z 360 $[M + 1]^+$.

5.1.8. 2,6-Dimethoxy-4-(5-(3,4,5-trimethoxyphenyl)isoxazol-3-yl) phenol (**22b**)

This compound was prepared according to the method described for compound **13b**, employing silyl ether **21b** (1.50 g, 3 mmol). This was further purified by column chromatography using ethyl acetate-hexane (60%) as eluent to obtain the pure product **22b** as light yellow solid. Yield 1.02 g, 88%; mp 195–197 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.05 (s, 2H, Ar**H**), 7.00 (s, 2H, Ar**H**), 6.64 (s, 1H, isoxazole–**H**), 5.62 (br, 1H, –O**H**), 3.98 (s, 6H, 2× –OC**H**$_3$), 3.95 (s, 6H, 2× –OC**H**$_3$), 3.87 (s, 3H, –OC**H**$_3$); MS (ESI): m/z 388 $[M + 1]^+$.

5.1.9. Synthesis of (2S)-N-{4-[3-[(2-methoxy)-5-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]propyl)oxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethyl thioacetal (**24a**)

To a solution of 2*S*-*N*-[4-(3-bromopropoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (**23a**) (521 mg, 1.0 mmol) in dry CH_3COCH_3 (20 mL) was added anhydrous K_2CO_3 (5.0 mmol) and 4-(4,5-dihydro-5-(3,4,5-trimethoxyphenyl) isoxazol-3-yl)-2-methoxyphenol of formula **13a** (1.0 mmol). The reaction mixture was refluxed in an oil bath for 24 h and the reaction was monitored by TLC using ethyl acetate-hexane (60%) as a solvent system. The $K_2CO_3$was then removed by suction filtration and the solvent was evaporated under vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate-hexane (60%) as a solvent system to obtain the pure product **24a** as a yellow solid. Yield 647 mg, 81%; mp 65–67 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.67 (s, 1H, Ar**H**), 6.90 (d, 1H, J = 2.1 Hz, Ar**H**), 6.86 (s, 2H, Ar**H**), 6.83 (d, 1H, J = 2.1 Hz, Ar**H**), 6.78 (d, 1H, J = 8.4 Hz, Ar**H**), 6.73 (s, 1H), 5.58 (dd, 1H, J = 10.5, 8.3 Hz, isoxazoline–**H**), 4.80 (d, 1H, J = 3.7 Hz, –C**H**S_2–), 4.68–4.62 (m, 1H, –NC**H**–), 4.32 (t, 4H, J = 5.2 Hz, 2× –OC**H**$_2$–), 3.90 (s, 3H, –OC**H**$_3$), 3.87 (s, 6H, 2× –OC**H**$_3$), 3.83 (s, 3H, –OC**H**$_3$), 3.82 (s, 3H, –OC**H**$_3$), 3.65 (dd, 1H, J = 16.6, 10.1 Hz, isoxazoline–**H**), 3.26 (dd, 1H, J = 16.2, 9.0 Hz, isoxazoline–**H**), 3.23–3.17 (m, 2**H**, –NC**H**$_2$–), 2.85–2.63 (m, 4H, 2× –SC**H**$_2$–), 2.39–2.32 (m, 2H, –C**H**$_2$–), 2.15–2.04 (m, 2H, –C**H**$_2$–), 1.98–1.89 (m, 2H, –C**H**$_2$–), 1.38–1.30 (m, 6H, –(C**H**$_3$)$_2$); MS (ESI): m/z 800 $[M + 1]^+$.

5.1.10. (2S)-N-{4-[4-[(2-Methoxy-5-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]butyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**24b**)

This compound was prepared according to the method described for compound **24a**, employing compound **23b** (535 mg, 1 mmol) and compound **13a** (1 mmol) to obtain the pure product **24b** as a yellow solid. Yield 666 mg, 82%; mp 64–66 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.62 (s, 1H, Ar**H**), 6.86 (s, 2H, Ar**H**), 6.84 (dd, 1H, J = 8.4, 2.0 Hz, Ar**H**), 6.82 (d, 1H, J = 2.0 Hz, Ar**H**) 6.79 (d, 1H, J = 8.1 Hz, Ar**H**), 6.73 (s, 1H, Ar**H**), 5.60 (dd, 1H, J = 10.1, 9.4 Hz, isoxazoline–**H**), 4.82 (d, 1H, J = 3.7 Hz, –C**H**S_2–), 4.70–4.61 (m, 1H, –NC**H**–), 4.10 (t, 4H, J = 6.4 Hz, 2× –OC**H**$_2$–), 3.90 (s, 3H, –OC**H**$_3$), 3.87 (s, 6H, 2× –OC**H**$_3$), 3.83 (s, 3H, –OC**H**$_3$), 3.82 (s, 3H, –OC**H**$_3$), 3.66 (dd, 1H, J = 16.6, 10.1 Hz, isoxazoline–**H**), 3.25 (dd, 1H, J = 16.2, 9.4 Hz, isoxazoline–**H**), 3.23–3.17 (m, 2H, –NC**H**$_2$–), 2.85–2.65 (m, 4H, 2× –SC**H**$_2$–), 2.15–1.99 (m, 2H, –C**H**$_2$–), 1.96–1.91 (m, 2H, –C**H**$_2$–), 1.85–1.73 (m, 4H, 2× –C**H**$_2$–), 1.40–1.29 (m, 6H, –(C**H**$_3$)$_2$); MS (ESI): m/z 814 $[M + 1]^+$.

5.1.11. (2S)-N-{4-[5-[(2-Methoxy-5-(3-[2,3,4-triMethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**24c**)

This compound was prepared according to the method described for compound **24a**, employing compound **23c** (549 mg, 1 mmol) and compound **13a** (1 mmol) to obtain the pure product **24c** as a yellow solid. Yield 736 mg, 89%; mp 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.66 (s, 1H, Ar**H**), 6.93 (d, 1H, J = 3.0 Hz, Ar**H**), 6.92 (s, 2H, Ar**H**), 6.91 (d, 1H, J = 8.3, 3.0 Hz, Ar**H**), 6.86 (d, 1H, J = 8.3 Hz, Ar**H**), 6.81 (s, 1H, Ar**H**), 5.68 (dd, 1H, J = 10.5, 9.0 Hz, isoxazoline–**H**), 4.88 (d, 1H, J = 3.7 Hz, –C**H**S_2–), 4.74–4.67 (m, 1H, –NC**H**–), 4.44–4.04 (m, 4H, 2× –OC**H**$_2$–), 3.93 (s, 3H, –OC**H**$_3$), 3.88 (s, 9H, 3× –OC**H**$_3$), 3.86 (s, 3H, –OC**H**$_3$), 3.73 (dd, 1H, J = 16.4, 10.5 Hz, isoxazoline–**H**), 3.32 (dd, 1H, J = 16.4, 9.0 Hz, isoxazoline–**H**), 3.26–3.18 (m, 2H, –NC**H**$_2$–), 2.86–2.67 (m, 4H, 2× –SC**H**$_2$–), 2.00–1.76 (m, 4H, 2× –C**H**$_2$–), 1.73–1.62 (m, 4H, 2× –C**H**$_2$–), 1.39–1.30 (m, 8H, –(C**H**$_3$)$_2$, –C**H**$_2$–); MS (ESI): m/z 828 $[M + 1]^+$.

5.1.12. (2S)-N-{4-[4-[(2,6-Dimethoxy-4-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]butyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**24d**)

This compound was prepared according to the method described for compound **24a**, employing compound **23b** (535 mg, 1 mmol) and compound **13b** (1.0 mmol) to obtain the pure product **24d** as a yellow solid. Yield 676 mg, 80%; mp 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.70 (s, 1H, Ar**H**), 6.94 (s, 2H, Ar**H**), 6.82 (s, 1H, Ar**H**), 6.60 (s, 2H, Ar**H**), 5.68 (dd, 1H, J = 10.7, 8.6 Hz, isoxazoline–**H**), 4.85 (d, 1H, J = 3.7 Hz, –C**H**S_2–), 4.76–4.64 (m, 1H, –NC**H**–), 4.01 (t, 4H, J = 6.2 Hz, 2× –OC**H**$_2$–), 3.94 (s, 3H, –OC**H**$_3$), 3.89 (s, 6H, 2× –OC**H**$_3$), 3.88 (s, 6H, 2× –OC**H**$_3$), 3.84 (s, 3H, –OCH_3), 3.76 (dd, 1H, J = 16.6, 10.9 Hz, isoxazoline–**H**), 3.34 (dd, 1H, J = 16.6, 8.8 Hz, isoxazoline–**H**), 3.28–3.18 (m, 2H, –NC**H**$_2$–), 2.85–2.68 (m, 4H, 2× –SC**H**$_2$–), 2.14–2.07 (m, 4H, 2× –C**H**$_2$–), 1.99–1.90 (m, 4H, 2× –C**H**$_2$–), 1.38–1.31 (m, 6H, –(C**H**$_3$)$_2$); MS (ESI): m/z 844 $[M + 1]^+$.

5.1.13. (2S)-N-{4-[5-[(2,6-Dimethoxy-4-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**24e**)

This compound was prepared according to the method described for compound **24a**, employing compound **23c** (549 mg, 1 mmol) and compound **13b** (1.0 mmol) to obtain the pure product **24e**. Yield 686 mg, 80%; mp 67–69 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.62 (s, 1H, Ar**H**), 6.87 (s, 2H, Ar**H**), 6.76 (s, 1H, Ar**H**), 6.53 (s, 2H, Ar**H**), 5.61 (dd, 1H, J = 10.9, 8.3 Hz, isoxazoline–**H**), 4.82 (d, 1H, J = 3.7 Hz, –C**H**S_2–), 4.71–4.61 (m, 1H, –NC**H**–), 4.10 (t, 4H, J = 6.9 Hz, 2× –OC**H**$_2$–), 3.92 (s, 3H, –OC**H**$_3$), 3.88 (s, 9H, 3× –OC**H**$_3$), 3.84 (s, 3H, –OC**H**$_3$), 3.83 (s, 3H, –OC**H**$_3$), 3.70 (dd, 1H, J = 16.4, 10.9 Hz, isoxazoline–**H**), 3.27 (dd, 1H, J = 16.4, 8.3 Hz, isoxazoline–**H**), 3.24–3.20 (m, 2H, –NC**H**$_2$–), 2.83–2.65 (m, 4H, 2× –SC**H**$_2$–), 2.15–2.04 (m, 2H, –C**H**$_2$–),

2.00–1.87 (m, 4H, 2× $-CH_2-$), 1.87–1.66 (m, 4H, 2× $-CH_2-$), 1.39–1.36 (m, 6H, $-(CH_3)_2$); MS (ESI): *m*/*z* 858 $[M+1]^+$.

*5.1.14. (2S)-N-{4-[3-[(2-Methoxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]propyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**25a**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23a** (521 mg, 1.0 mmol) and compound **18** (1 mmol) to obtain the pure product **25a** as a yellow solid. Yield 639 mg, 80%; mp 65–67 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.63 (s, 1H, Ar**H**), 7.38 (d, 1H, J = 2.0 Hz, Ar**H**), 7.06 (dd, 1H, J = 8.2, 2.0 Hz, Ar**H**), 6.87 (d, 1H, J = 8.2 Hz, Ar**H**), 6.83 (s, 1H, Ar**H**), 6.63 (s, 2H, Ar**H**), 5.58 (dd, 1H, J = 10.5, 8.3 Hz, isoxazoline–**H**), 4.81 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.68–4.61 (m, 1H, $-NCH-$), 4.32 (t, 4H, J = 5.2 Hz, 2× $-OCH_2-$), 3.89 (s, 3H, $-OCH_3$), 3.87 (s, 6H, 2× $-OCH_3$), 3.83 (s, 6H, 2× $-OCH_3$), 3.70 (dd, 1H, J = 16.6, 10.5 Hz, isoxazoline–**H**) 3.27 (dd, 1H, J = 16.6, 8.3 Hz, isoxazoline–**H**) 3.24–3.14 (m, 2H, $-NCH_2-$), 2.84–2.63 (m, 4H, 2× $-SCH_2-$), 2.39–2.29 (m, 2H, $-CH_2-$), 2.16–2.05 (m, 2H, $-CH_2-$), 1.83–1.71 (m, 2H, $-CH_2-$), 1.38–1.29 (m, 6H, $-(CH_3)_2$); MS (ESI): *m*/*z* 800 $[M+1]^+$.

*5.1.15. (2S)-N-{4-[4-[(2-Metloxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]butyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**25b**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23b** (535 mg, 1 mmol) and compound **18** (1 mmol) to obtain the pure product **25b** as a yellow solid. Yield 683 mg, 84%; mp 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.69 (s, 1H, Ar**H**), 7.41 (d, 1H, J = 2.2 Hz, Ar**H**), 7.04 (dd, 1H, J = 8.3, 2.2 Hz, Ar**H**), 6.87 (d, 1H, J = 8.3 Hz, Ar**H**), 6.81 (d, 1H, J = 2.2 Hz, Ar**H**), 6.61 (s, 2H, Ar**H**), 5.67 (dd, 1H, J = 10.5, 9.0 Hz, isoxazoline–**H**), 4.88 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.74–4.69 (m, 1H, $-NCH-$), 4.14 (t, 4H, J = 3.7 Hz, 2× $-OCH_2-$), 3.92 (s, 3H, $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 3.87 (s, 6H, 2× $-OCH_3$), 3.84 (s, 3H, $-OCH_3$), 3.75 (dd, 1H, J = 16.6, 10.5 Hz, I isoxazoline–**H**) 3.32 (dd, 1H, J = 16.6, 9.0 Hz, isoxazoline–**H**) 3.28–3.19 (m, 2H, $-NCH_2-$), 2.85–2.69 (m, 4H, 2× $-SCH_2-$), 2.14–2.06 (m, 4H, 2× $-CH_2-$), 1.86–1.73 (m, 4H, 2× $-CH_2-$), 1.39–1.30 (m, 6H, $-(CH_3)_2$); MS (ESI): *m*/*z* 814 $[M+1]^+$.

*5.1.16. (2S)-N-{4-[5-[(2-Methoxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**25c**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23c** (549 mg, 1 mmol) and compound **18** (1 mmol) to obtain the pure product **25c** as a yellow solid. Yield 719 mg, 87%; mp 69–71 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.70 (s, 1H, Ar**H**), 7.38 (d, 1H, J = 2.3 Hz, Ar**H**), 7.14 (dd, 1H, J = 8.4, 2.3 Hz, Ar**H**), 6.87 (d, 1H, J = 8.4 Hz, Ar**H**), 6.83 (d, 1H, J = 2.2 Hz, Ar**H**), 6.61 (s, 2H, Ar**H**), 5.61 (dd, 1H, J = 10.6, 8.6 Hz, isoxazoline–**H**), 4.81 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.69–4.62 (m, 1H, $-NCH-$), 4.10 (t, 4H, J = 3.7 Hz, 2× $-OCH_2-$), 3.93 (s, 3H, $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 3.85 (s, 6H, 2× $-OCH_3$), 3.82 (s, 3H, $-OCH_3$), 3.71 (dd, 1H, J = 16.4, 10.6 Hz, isoxazoline–**H**) 3.29 (dd, 1H, J = 16.6, 8.6 Hz, I isoxazoline–**H**) 3.24–3.17 (m, 2H, $-NCH_2-$), 2.84–2.66 (m, 4H, 2× $-SCH_2-$), 2.36–2.19 (m, 4H, 2× $-CH_2-$), 1.94–1.60 (m, 4H, 2× $-CH_2-$), 1.39–1.28 (m, 8H, $-(CH_3)_2$, $-CH_2-$); MS (ESI): *m*/*z* 828 $[M+1]^+$.

*5.1.17. (2S)-N-{4-[3-[(2-Methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolyl-phenyl)oxy]propyl)oxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethyl thioacetal (**26a**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23a** (521 mg, 1.0 mmol) and compound **22a** (1.0 mmol) to obtain the pure product **26a** as a yellow solid. Yield 693 mg, 87%; mp 66–68 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.70 (s, 1H, Ar**H**), 7.43 (d, 1H, J = 2.0 Hz, Ar**H**), 7.30 (dd, 1H, J = 8.3, 2.0 Hz, Ar**H**), 7.00 (s, 2H, Ar**H**), 6.86 (d, 1H, J = 8.3 Hz, Ar**H**), 6.74 (s, 1H, Ar**H**), 6.63 (s, 1H, isoxazole–**H**), 4.81 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.68–4.61 (m, 1H, $-NCH-$), 4.38–4.30 (m, 4H, 2× $-OCH_2-$), 3.95 (s, 6H, 2× $-OCH_3$), 3.92 (s, 3H, $-OCH_3$), 3.91 (s, 3H, $-OCH_3$), 3.86 (s, 3H, $-OCH_3$), 3.26–3.12 (m, 2H, $-NCH_2-$), 2.78–2.65 (m, 4H, 2× $-SCH_2-$), 2.45–2.27 (m, 4H, 2× $-CH_2-$), 1.89–1.67 (m, 2H, $-CH_2-$), 1.42–1.22 (m, 6H, $-(CH_3)_2$); MS (ESI) : *m*/*z* 798 $[M+1]^+$.

*5.1.18. (2S)-N-{4-[4-[(2-Methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolyl-phenyl)oxy]butyl)oxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethyl thioacetal (**26b**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23b** (535 mg, 1.0 mmol) and compound **22a** (1.0 mmol) to obtain the pure product **26b** as a yellow solid. Yield 648 mg, 80%; mp 65–67 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.69 (s, 1H, Ar**H**), 7.45 (d, 1H, J = 1.8 Hz, Ar**H**), 7.29 (dd, 1H, J = 8.3, 2.0 Hz, Ar**H**), 7.00 (s, 2H, Ar**H**), 6.88 (d, 1H, J = 8.4 Hz, Ar**H**), 6.73 (s, 1H, Ar**H**), 6.65 (s, 1H, isoxazole–**H**), 4.80 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.69–4.61 (m, 1H, $-NCH-$), 4.49–4.29 (m, 4H, 2× $-OCH_2-$), 3.95 (s, 6H, 2× $-OCH_3$), 3.91 (s, 3H, $-OCH_3$), 3.90 (s, 3H, $-OCH_3$), 3.87 (s, 3H, $-OCH_3$), 3.24–3.15 (m, 2H, $-NCH_2-$), 2.79–2.68 (m, 4H, 2× $-SCH_2-$), 2.33–2.24 (m, 2H, $-CH_2-$), 1.92–1.83 (m, 2H, $-CH_2-$), 1.80–1.69 (m, 4H, 2× $-CH_2-$), 1.39–1.28 (m, 6H, $-(CH_3)_2$); MS (ESI) : *m*/*z* 812 $[M+1]^+$.

*5.1.19. (2S)-N-{4-[5-[(2-Methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolyl- phenyl)oxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl} pyrrolidine-2-carboxaldehyde diethyl thioacetal (**26c**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23c** (549 mg, 1.0 mmol) and compound **22a** (1.0 mmol) to obtain the pure product **26c** as yellow solid. Yield 701 mg, 85%; mp 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.65 (s, 1H, Ar**H**), 7.40 (d, 1H, J = 1.8 Hz, Ar**H**), 7.31 (dd, 1H, J = 8.3, 2.0 Hz, Ar**H**), 7.00 (s, 2H, Ar**H**), 6.90 (d, 1H, J = 8.4 Hz, Ar**H**), 6.85 (s, 1H, Ar**H**), 6.70 (s, 1H, isoxazole–**H**), 4.88 (d, 1H, J = 3.7 Hz, $-CHS_2-$), 4.74–4.66 (m, 1H, $-NCH-$), 4.16–4.02 (m, 4H, 2× $-OCH_2-$), 3.95 (s, 3H, $-OCH_3$), 3.89 (s, 6H, 2× $-OCH_3$), 3.88 (s, 3H, $-OCH_3$), 3.86 (s, 3H, $-OCH_3$), 3.29–3.20 (m, 2H, $-NCH_2-$), 2.86–2.67 (m, 4H, 2× $-SCH_2-$), 2.00–1.89 (m, 4H, 2× $-CH_2-$), 1.75–1.60 (m, 4H, 2× $-CH_2-$), 1.40–1.22 (m, 8H, $-(CH_3)_2$, $-CH_2-$); MS (ESI): *m*/*z* 826 $[M+1]^+$.

*5.1.20. (2S)-N-{4-[3-[(2,6-Dimethoxy-4-(5-[3,4,5-trimethoxyphenyl]-5-isoxa-zolylphenyl)oxy]propyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**26d**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23a** (521 mg, 1.0 mmol) and compound **22b** (1.0 mmol) to obtain the pure product **26d** as a yellow solid. Yield 694 mg, 84%; mp 78–80 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.68 (s, 1H, Ar**H**), 7.09 (s, 2H, Ar**H**), 7.06 (s, 2H, Ar**H**), 6.82 (s, 1H, Ar**H**), 6.72 (s, 1H, isoxazole–**H**), 4.88 (d, 1H, J = 4.5 Hz, $-CHS_2-$), 4.75–4.67 (m, 1H, $-NCH-$), 4.07 (t, 4H, J = 6.0 Hz, 2× $-OCH_2-$), 3.96 (s, 6H, 2× $-OCH_3$), 3.94 (s, 9H, 3× $-OCH_3$), 3.91 (s, 3H, $-OCH_3$), 3.33–3.20 (m, 2H, $-NCH_2-$), 2.85–2.67 (m, 4H, 2× $-SCH_2-$), 2.15–2.06 (m, 2H, $-CH_2-$), 2.01–1.83 (m, 2H, $-CH_2-$), 1.81–1.55 (m, 2H, $-CH_2-$), 1.39–1.28 (m, 6H, $-(CH_3)_2$); MS (ESI): *m*/*z* 828 $[M+1]^+$.

*5.1.21. (2S)-N-{4-[5-[(2,6-Dimethoxy-4-(5-[3,4,5-trimethoxyphenyl]-5-isoxa-zolylphenyl)oxy]pentyl)oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (**26e**)*

This compound was prepared according to the method described for compound **24a**, employing compound **23c** (549 mg, 1.0 mmol) and compound **22b** (1.0 mmol) to obtain the pure product **26e** as a yellow

solid. Yield 710 mg, 83%; mp 79–81 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.69 (s, 1H, Ar**H**), 7.09 (s, 2H, Ar**H**), 7.05 (s, 2H, Ar**H**), 6.83 (s, 1H, Ar**H**), 6.72 (s, 1H, isoxazole–**H**), 4.88 (d, 1H, J = 4.5 Hz, –C**H**S_2–), 4.73–4.67 (m, 1H, –NC**H**–), 4.16–4.04 (m, 4H, 2× –OC**H**$_2$–), 3.95 (s, 6H, 2× –OC**H**$_3$), 3.94 (s, 9H, 3× –OC**H**$_3$), 3.90 (s, 3H, –OC**H**$_3$), 3.32–3.15 (m, 2H, –NC**H**$_2$–), 2.85–2.65 (m, 4H, 2× –SC**H**$_2$–), 2.35–2.21 (m, 2H, –C**H**$_2$–), 2.01–1.92 (m, 2H, –C**H**$_2$–), 1.88–1.61 (m, 4H, 2× –C**H**$_2$–), 1.39–1.30 (m, 8H, –(C**H**$_3$)$_2$, –CH$_2$–); MS (ESI): m/z 856 [M + 1]$^+$.

5.1.22. Synthesis of 7-methoxy-8-{3-[(2-methoxy-5-(3-[3,4,5-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]propyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**5a**)

To a solution of compound **24a** (799 mg, 1 mmol) in MeOH (20 mL), $SnCl_2.2H_2O$ (5 mmol) was added and refluxed for 1–2 h. The MeOH was evaporated in vacuum and the aqueous layer was then carefully adjusted to pH 8 with 10% $NaHCO_3$ solution and then extracted with ethyl acetate (20–30 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated under vacuum to afford the amino diethyl thioacetal, which due to potential stability problems proceeded for the next step. A solution of amino diethyl thioacetal (1 mmol), $HgCl_2$ (2.26 mmol) and $CaCO_3$ (2.46 mmol) in CH_3CN/H_2O (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material (12 h). The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a celite bed. The clear yellow organic supernatant was washed with saturated 5% $NaHCO_3$ (20 mL) and brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated in vacuum and purified by column chromatography (MeOH/$CHCl_3$, 2%) to obtain the pure product **5a** as a white solid. Yield 335 mg, 52%; mp 59–61 °C; $[\alpha]_D^{25}$ + 143.5° (c0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.65 (d, 1H, J = 4.3 Hz, imine–**H**), 7.50 (s, 1H, Ar**H**), 6.95 (d, 1H, J = 8.4 Hz, Ar**H**), 6.93 (s, 3H, Ar**H**), 6.86 (d, 1H, J = 2.6 Hz, Ar**H**), 6.85 (s, 1H, Ar**H**), 5.65 (dd, 1H, J = 10.4, 8.3 Hz isoxazoline–**H**), 4.32–4.19 (m, 4H, 2× –OC**H**$_2$–), 3.89 (s, 6H, 2× –OC**H**$_3$), 3.88 (s, 6H, 2× –OC**H**$_3$), 3.84 (s, 3H, –OC**H**$_3$), 3.69 (dd, 1H, J = 16.3, 10.4 Hz, isoxazoline–**H**), 3.61–3.52 (m, 3H, –NC**H**$_2$–, –NC**H**–), 3.31 (dd, 1H, J = 8.6, 7.3 Hz, isoxazoline–**H**), 2.42–2.27 (m, 2H, –C**H**$_2$–), 2.11–2.01 (m, 2H, –C**H**$_2$–), 1.81–1.68 (m, 2H, –C**H**$_2$–); IR (KBr) (U_{max}/cm^{-1}): 3380, 2925, 1599, 1511, 1461, 1425, 1371, 1260, 1176, 1126, 1023, 896, 816, 761, 631; MS (ESI): m/z 646 [M + 1]$^+$; HRMS (ESI m/z) for $C_{35}H_{40}N_3O_9$, calcd 646.2764, found 646.2745 [M + 1]$^+$.

5.1.23. 7-Methoxy-8-{4-[(2-methoxy-5-(3-[3,4,5-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]butyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**5b**)

The compound **5b** was prepared according to the method described for the compound **5a**, employing the compound **24b** (813 mg, 1 mmol) to obtain the pure product **5b** as a white solid. Yield 382 mg, 58%; mp 72–74 °C; $[\alpha]_D^{25}$ + 148.3° (c0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.67 (d, 1H, J = 4.3 Hz, imine–H), 7.50 (s, 1H, ArH), 7.02 (d, 1H, J = 8.6 Hz, ArH), 6.87 (d, 1H, J = 2.2 Hz, ArH), 6.84 (d, 1H, J = 8.6 Hz, ArH), 6.82 (s, 1H, ArH), 6.61 (s, 2H, ArH), 5.71 (dd, 1H, J = 11.2, 8.6 Hz, isoxazoline–H), 4.18–4.08 (m, 4H, 2× –OCH$_2$–), 3.92 (s, 3H, –OCH$_3$), 3.89 (s, 3H, –OCH$_3$), 3.86 (s, 6H, 2× –OCH$_3$), 3.84 (s, 3H, –OCH$_3$), 3.71 (dd, 1H, J = 16.5, 11.2 Hz, isoxazoline–H), 3.61–3.51 (m, 3H, –NCH$_2$–, –NCH–), 3.38–3.25 (dd, 1H, J = 16.5, 8.6 Hz, isoxazoline–H), 2.38–2.25 (m, 2H, –CH$_2$–), 2.11–2.01 (m, 2H, –CH$_2$–), 1.71–1.61 (m, 4H, 2× –CH$_2$–); IR (KBr) (U_{max}/cm^{-1}): 3418, 2924, 2853, 1602, 1511, 1459, 1372, 1258, 1173, 1127, 1021, 903, 818, 766, 637; MS (ESI): m/z 660 [M + 1]$^+$; HRMS (ESI m/z) for $C_{36}H_{42}N_3O_9$, calcd 660.2921, found 660.2898 [M + 1]$^+$.

5.1.24. 7-Methoxy-8-{5-[(2-methoxy-5-(3-[3,4,5-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxy]pentyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**5c**)

The compound **5c** was prepared according to the method described for the compound **5a**, employing the compound **24c** (827 mg, 1 mmol) to obtain the pure product **5c** as a white solid. Yield 397 mg, 59%; mp 65–67 °C; $[\alpha]_D^{25}$ + 140.0° (c0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.65 (d, 1H, J = 4.3 Hz, imine–H), 7.50 (s, 1H, ArH), 6.93 (s, 3H, ArH), 6.90 (d, 1H, J = 2.3 Hz, ArH), 6.85 (d, 1H, J = 8.7 Hz, ArH), 6.79 (s, 1H, ArH), 5.67 (dd, 1H, J = 10.2, 8.7 Hz, isoxazoline–H), 4.04 (t, 4H, J = 6.5 Hz, 2× –OCH$_2$–), 3.92 (s, 3H, –OCH$_3$), 3.87 (s, 9H, 3× –OCH$_3$), 3.85 (s, 3H, –OCH$_3$), 3.70 (dd, 1H, J = 16.2, 10.2 Hz, isoxazoline–H), 3.61–3.54 (m, 3H, –NCH$_2$–, –NCH–), 3.32 (dd, 1H, J = 16.4, 8.7 Hz, isoxazoline–H), 2.33–2.28 (m, 2H, –CH$_2$–), 2.08–2.02 (m, 2H, –CH$_2$–), 1.96–1.87 (m, 2H, –CH$_2$–), 1.69–1.62 (m, 4H, 2× –CH$_2$–); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.4, 162.2, 155.9, 153.1, 150.6, 149.3, 148.6, 147.6, 140.4, 139.7, 132.9, 124.7, 119.9, 118.4, 111.5, 111.4, 110.6, 110.2, 103.9, 82.6, 68.6, 60.7, 56.1, 55.9, 55.9, 53.5, 46.4, 43.0, 29.4, 28.7, 23.9, 22.3; IR (KBr) (U_{max}/cm^{-1}): 3381, 2933, 1601, 1511, 1458, 1424, 1371, 1258, 1172, 1127, 1020, 898, 817, 764, 636; MS (ESI): m/z 674 [M + 1]$^+$; HRMS (ESI m/z) for $C_{37}H_{44}N_3O_9$, calcd 674.3077, found 674.3063 [M + 1]$^+$.

5.1.25. 7-Methoxy-8-{4-[(2,6-dimethoxy-4-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxybutyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4] benzodiazepine-5-one (**5d**)

The compound **5d** was prepared according to the method described for the compound **5a**, employing the compound **24d** (844 mg, 1.0 mmol) to obtain the pure product **5d** as a white solid. Yield 392 mg, 57%; mp 65–67 °C; $[\alpha]_D^{25}$ + 152.4° (c0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.65 (d, 1H, J = 4.3 Hz, imine–H), 7.51 (s, 1H, ArH), 6.93 (s, 2H, ArH) 6.82 (s, 1H, ArH), 6.60 (s, 2H, ArH), 5.67 (dd, 1H, J = 10.9, 8.7 Hz, isoxazoline–H), 4.01 (t, 4H, J = 6.5 Hz, 2× –OCH$_2$–), 3.92 (s, 3H, –OCH$_3$), 3.88 (s, 9H, 3× –OCH$_3$), 3.83 (s, 6H, 2× –OCH$_3$), 3.73 (dd, 1H, J = 16.8, 10.9 Hz, isoxazoline–H), 3.61–3.54 (m, 3H, –NCH$_2$–, –NCH–), 3.33 (dd, 1H, J = 16.9, 8.7 Hz, isoxazoline–H), 2.36–2.28 (m, 2H, –CH$_2$–), 2.13–1.90 (m, 2H, –CH$_2$–), 1.69–1.59 (m, 4H, 2× –CH$_2$–); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.2, 155.9, 153.6, 153.2, 150.8, 147.6, 140.5, 139.7, 136.7, 136.2, 124.6, 119.9, 111.3, 110.3, 103.9, 102.5, 82.74, 68.5, 60.8, 56.1, 56.0, 53.6, 46.5, 43.3, 30.7, 29.5, 24.0; IR (KBr) (U_{max}/cm^{-1}): 3380, 2930, 1597, 1507, 1460, 1419, 1371, 1237, 1125, 1002, 895, 831, 763, 635; MS (ESI): m/z 690 [M + 1]$^+$; HRMS (ESI m/z) for $C_{37}H_{44}N_3O_{10}$, calcd 690.3026, found 690.3016 [M + 1]$^+$.

5.1.26. 7-Methoxy-8-{5-[(2,6-dimethoxy-4-(3-[2,3,4-trimethoxyphenyl]-4,5-dihydro-5-isoxazolylphenyl)oxypentyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4] benzodiazepine-5-one (**5e**)

The compound **5e** was prepared according to the method described for the compound **5a**, employing the compound **24e** (858 mg, 1.0 mmol) to afford the compound **5e** as a white solid. Yield 407 mg, 58%; mp 62–64 °C; $[\alpha]_D^{25}$ + 154.6° (c0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.67 (d, 1H, J = 4.3 Hz, imine–H), 7.50 (s, 1H, ArH), 6.93 (s, 2H, ArH) 6.80 (s, 1H, ArH), 6.61 (s, 2H, ArH), 5.68 (t, 1H, J = 10.1, 8.7 Hz, isoxazoline–H), 4.13–3.98 (m, 4H, 2× –OCH$_2$–), 3.94 (s, 3H, –OCH$_3$), 3.89 (s, 9H, 3× –OCH$_3$), 3.85 (s, 6H, 2× –OCH$_3$), 3.74 (dd, 1H, J = 16.8, 10.1 isoxazoline–H), 3.61–3.49 (m, 3H, –NCH$_2$–, –NCH–), 3.33 (dd, 1H, J = 16.8, 8.7 Hz, isoxazoline–H), 2.39–2.25 (m, 2H, –CH$_2$–), 2.13–1.76 (m, 4H, 2× –CH$_2$–), 1.74–1.58 (m, 4H, 2× –CH$_2$–); IR (KBr) (U_{max}/cm^{-1}): 3417, 2925, 2855, 1599, 1507, 1460, 1420, 1371, 1238, 1124, 1019, 894, 829, 764, 632; MS

(ESI): *m*/*z* 704 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{38}H_{46}N_3O_{10}$, calcd 704.3183, found 704.3185 $[M + 1]^+$.

*5.1.27. 7-Methoxy-8-{3-[(2-methoxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]propyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**6a**)*

The compound **6a** was prepared according to the method described for the compound **5a**, employing the compound **25a** (799 mg, 1.0 mmol) to afford the compound **6a** as a white solid. Yield 374 mg, 58%; mp 63–65 °C; $[\alpha]_D^{25}$ + 144.5° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, 1H, J = 4.3 Hz, imine–H), 7.51 (s, 1H, ArH), 7.00 (d, 1H, J = 8.0 Hz, ArH), 6.93 (d, 1H, J = 2.1 Hz, ArH), 6.88 (d, 1H, J = 8.0 Hz, ArH), 6.85 (s, 1H, ArH), 6.61 (s, 2H, ArH), 5.65 (dd, 1H, J = 10.3, 9.3 Hz, isoxazoline–H), 4.26 (t, 4H, J = 5.8 Hz, 2× $-OCH_2-$), 3.92 (s, 3H, $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 3.86 (s, 6H, 2× $-OCH_3$), 3.83 (s, 3H, $-OCH_3$), 3.72 (dd, 1H, J = 16.4, 10.3 Hz, isoxazoline–H), 3.61–3.53 (m, 3H, $-NCH_2-$, $-NCH-$), 3.31 (dd, 1H, J = 16.4, 9.3 Hz, isoxazoline–H), 2.42–2.26 (m, 2H, $-CH_2-$), 2.08–2.01 (m, 2H, $-CH_2-$), 1.84–1.73 (m, 2H, $-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.5, 162.3, 155.9, 153.4, 150.5, 150.1, 149.5, 147.7, 140.3, 137.6, 136.5, 122.2, 120.2, 114.5, 112.3, 111.5, 110.6, 109.1, 102.6, 82.4, 65.3, 60.7, 56.1, 56.0, 55.9, 53.6, 46.5, 43.4, 29.5, 28.9, 24.0; IR (KBr) (U_{max}/cm^{-1}): 3378, 2928, 1597, 1511, 1461, 1425, 1367, 1335, 1241, 1123, 1019, 893, 814, 628; MS (ESI): *m*/*z* 646 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{35}H_{40}N_3O_9$, calcd 646.2764, found 646.2748 $[M + 1]^+$.

*5.1.28. 7-Methoxy-8-{4-[(2-methoxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]butyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**6b**)*

The compound **6b** was prepared according to the method described for the compound **5a**, employing the compound **25b** (813 mg, 1 mmol) to obtain the pure product **6b** as a white solid. Yield 362 mg, 55%; mp 72–74 °C; $[\alpha]_D^{25}$ + 145.6° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, 1H, J = 4.3 Hz, imine–H), 7.50 (s, 1H, ArH), 6.90–6.97 (m, 4H, ArH) 6.87 (s, 1H, ArH), 6.81 (s, 1H, ArH), 5.67 (dd, 1H, J = 10.1, 9.3 Hz, isoxazoline–H), 4.10 (t, 4H, J = 5.4 Hz, 2× $-OCH_2-$), 3.92 (s, 3H, $-OCH_3$), 3.88 (s, 6H, $-OCH_3$), 3.85 (s, 6H, 2× $-OCH_3$), 3.72 (dd, 1H, J = 16.4, 10.1 Hz, isoxazoline–H), 3.61–3.51 (m, 3H, $-NCH_2-$, $-NCH-$), 3.32 (dd, 1H, J = 16.4, 9.3 Hz, isoxazoline–H), 2.36–2.24 (m, 2H, $-CH_2-$), 2.11–1.99 (m, 2H, $-CH_2-$), 1.71–1.56 (m, 4H, 2× $-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 164.3, 162.1, 155.8, 153.2, 150.4, 150.1, 149.2, 147.4, 140.3, 136.4, 137.4, 121.8, 120.1, 119.9, 111.7, 111.3, 110.2, 108.8, 102.5, 82.4, 68.4, 60.7, 56.1, 56.0, 55.9, 53.6, 46.5, 43.4, 29.5, 25.8, 24.1; IR (KBr) (U_{max}/cm^{-1}): 3388, 2924, 2854, 1602, 1511, 1459, 1371, 1257, 1172, 1125, 1018, 900, 816,763, 634; MS (ESI): *m*/*z* 660 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{36}H_{42}N_3O_9$, calcd 660.2921, found 660.2914 $[M + 1]^+$.

*5.1.29. 7-Methoxy-8-{5-[(2-methoxy-4-(5-[3,4,5-trimethoxyphenyl]-4,5-dihydro-3-isoxazolylphenyl)oxy]pentyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**6c**)*

The compound **6c** was prepared according to the method described for the compound **5a**, employing the compound **25c** (828 mg, 1 mmol) to obtain the pure product **6c** as a white solid. Yield 376 mg, 56%; mp 66–68 °C; $[\alpha]_D^{25}$ + 138.2° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.65 (d, 1H, J = 4.3 Hz, imine–H), 7.51 (s, 1H, ArH),7.05 (d, 1H, J = 8.0 Hz, ArH), 6.90 (d, 1H, J = 2.4 Hz, ArH), 6.86 (d, 1H, J = 8.1 Hz, ArH) 6.84 (s, 1H, ArH), 6.62 (s, 2H, ArH), 5.63 (dd, 1H, J = 10.5, 9.5 Hz, isoxazoline–H), 4.13 (t, 4H, J = 5.8 Hz, 2× $-OCH_2-$), 3.93 (s, 3H, 2× $-OCH_3$), 3.87 (s, 3H, 2× $-OCH_3$), 3.84 (s, 6H, 2× $-OCH_3$), 3.82 (s, 3H, 2× $-OCH_3$), 3.74 (dd, 1H, J = 7.3, 5.8 Hz, isoxazoline–H), 3.62–3.51 (m, 3H, $-NCH_2-$, $-NCH-$), 3.33 (dd, 1H, J = 8.7, 8.0 Hz, isoxazoline–H), 2.43–2.23 (m, 2H, $-CH_2-$), 2.10–2.01 (m, 4H, 2× $-CH_2-$), 1.78–1.64 (m, 4H, 2× $-CH_2-$); IR (KBr) (U_{max}/cm^{-1}): 3380, 2928, 1599, 1511, 1461, 1426, 1370, 1241, 1176, 1125, 1022, 896, 816, 759, 624; MS (ESI): *m*/*z* 674 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{37}H_{44}N_3O_9$, calcd 674.3077, found 674.3066 $[M + 1]^+$.

*5.1.30. 7-Methyloxy-8-{3-[(2-methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolylphenyl)oxy]propyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**7a**)*

The compound **7a** was prepared according to the method described for the compound **5a**, employing the compound **26a** (797 mg, 1.0 mmol) to obtain the pure product **7a** as a white solid. Yield 360 mg, 56%; mp 59–61 °C; $[\alpha]_D^{25}$ + 147.6° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, 1H, J = 4.4 Hz, imine–H), 7.50 (s, 1H, ArH), 7.47 (d, 1H, J = 2.2 Hz), 7.39 (dd, 1H, J = 8.0, 2.2 Hz, ArH), 7.05 (s, 2H, ArH), 6.94 (d, 1H, J = 8.0 Hz, ArH), 6.87 (s, 1H, ArH), 6.72 (s, 1H, isoxazole–H), 4.32 (t, 4H, J = 5.8 Hz, 2× $-OCH_2-$), 3.95 (s, 6H, 2× $-OCH_3$), 3.92 (s, 6H, 2× $-OCH_3$), 3.91 (s, 3H, 2× $-OCH_3$), 3.83–3.50 (m, 3H, $-NCH_2-$, $-NCH-$), 2.50–2.25 (m, 2H, $-CH_2-$), 2.12–1.98 (m, 2H, $-CH_2-$), 1.69–1.59 (m, 2H, $-CH_2-$); IR (KBr) (U_{max}/cm^{-1}): 3385, 2923, 2852, 1600, 1575, 1504, 1459, 1428, 1379, 1334, 1260, 1177, 1127, 1022, 870, 765, 612; MS (ESI): *m*/*z* 644 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{35}H_{38}N_3O_9$, calcd 644.2608, found 644.2638 $[M + 1]^+$.

*5.1.31. 7-Methyloxy-8-{4-[(2-methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolylphenyl)oxy]butyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**7b**)*

The compound **7b** was prepared according to the method described for the compound **5a**, employing the compound **26b** (811 mg, 1.0 mmol) to obtain the pure product **7b** as a white solid. Yield 381 mg, 58%; mp 65–67 °C; $[\alpha]_D^{25}$ + 149.4° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, 1H, J = 4.3 Hz, imine–H), 7.50 (s, 1H, ArH), 7.45 (d, 1H, J = 2.2 Hz, ArH), 7.37 (d, 1H, J = 8.0, 2.2 Hz, ArH), 7.04 (s, 2H, ArH), 6.93 (d, 1H, J = 8.0 Hz, ArH), 6.82 (s, 1H, ArH), 6.72 (s, 1H, isoxazole–H), 4.24–4.18 (m, 4H, 2× $-OCH_2-$), 3.94 (s, 9H, 3× $-OCH_3$), 3.90 (s, 6H, 2× $-OCH_3$), 3.85–3.68 (m, 1H, $-NCH-$), 3.62–3.53 (m, 2H, $-NCH_2-$), 2.34–2.28 (m, 2H, $-CH_2-$), 2.13–2.02 (m, 2H, $-CH_2-$), 1.75–1.67 (m, 4H, 2× $-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.8, 164.5, 162.6, 162.2, 153.4, 150.8, 150.6, 148.5, 147.6, 140.4, 139.6, 122.7, 121.4, 119.9, 119.8, 111.3, 111.2, 110.7, 110.2, 102.9, 96.9, 68.4, 60.8, 56.1, 53.4, 46.5, 29.4, 25.6, 24.0; IR (KBr) (U_{max}/cm^{-1}): 3383, 2933, 1606, 1505, 1458, 1378, 1333, 1258, 1176, 1127, 1018, 865, 793, 626; MS (ESI): *m*/*z* 658 $[M + 1]^+$; HRMS (ESI *m*/*z*) for $C_{36}H_{40}N_3O_9$, calcd 658.2764, found 658.2740 $[M + 1]^+$.

*5.1.32. 7-Methyloxy-8-{5-[(2-methoxy-5-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolylphenyl)oxy]pentyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**7c**)*

The compound **7c** was prepared according to the method described for the compound **5a**, employing the compound **26c** (825 mg, 1.0 mmol) to obtain the pure product **7c** as a white solid. Yield 382 mg, 57%; mp 70–72 °C; $[\alpha]_D^{25}$ + 150.5° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.64 (d, 1H, J = 4.3 Hz, imine–H), 7.49 (s, 1H, ArH), 7.43 (d, 1H, J = 2.1 Hz, ArH), 7.34 (dd, 1H, J = 8.7, 2.1 Hz, ArH), 7.03 (s, 2H, ArH), 6.93 (d, 1H, J = 8.0 Hz, ArH), 6.78 (s, 1H, ArH), 6.69 (s, 1H, isoxazole–H), 4.12 (t, 4H, J = 5.1 Hz, 2× $-OCH_2-$), 3.93 (s, 6H, 2× $-OCH_3$), 3.91 (s, 6H, 2× $-OCH_3$), 3.89 (s, 3H, $-OCH_3$), 3.83–3.67 (m, 1H, $-NCH-$), 3.60–3.51 (m, 2H, $-NCH_2-$), 2.32–2.25 (m, 2H, $-CH_2-$), 2.03–1.89 (m, 2H, $-CH_2-$), 1.72–1.62 (m, 6H, 3×

$-CH_2-$); IR (KBr) (U_{max}/cm^{-1}): 3378, 2937, 1601, 1505, 1458, 1426, 1380, 1334, 1260, 1128, 1019, 867, 772, 621; MS (ESI): *m/z* 672 $[M + 1]^+$; HRMS (ESI *m/z*) for $C_{37}H_{42}N_3O_9$, calcd 672.2921, found 672.2904 $[M + 1]^+$.

5.1.33. 7-Methoxy-8-{3-[(2,6-dimethoxy-4-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolylphenyl)oxy]propyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**7d**)

The compound **7d** was prepared according to the method described for the compound **5a**, employing the compound **26d** (827 mg, 1.0 mmol) to obtain the pure product **7d** as a white solid. Yield 370 mg, 55%; mp 76–78 °C; $[\alpha]_D^{25}$ + 154.5° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.67 (d, 1H, J = 4.3 Hz, imine–H), 7.51 (s, 1H, ArH), 7.06 (s, 2H, ArH), 7.04 (s, 2H, ArH), 6.90 (s, 1H, ArH), 6.71 (s, 1H, isoxazole–H), 4.26 (t, 4H, J = 6.7 Hz, 2× $-OCH_2-$), 3.95 (s, 9H, 3× $-OCH_3$), 3.91 (s, 6H, 2× $-OCH_3$), 3.85 (s, 3H, $-OCH_3$), 3.76–3.69 (m, 1H, –NCH–), 3.63–3.52 (m, 2H, $-NCH_2-$), 2.34–2.27 (m, 2H, $-CH_2-$), 2.10–2.01 (m, 2H, $-CH_2-$), 1.73–1.64 (m, 2H, $-CH_2-$); IR (KBr) (U_{max}/cm^{-1}): 3383, 2927, 1600, 1503, 1463, 1426, 1382, 1332, 1240, 1126, 1002, 847, 783; MS (ESI): *m/z* 674 $[M + 1]^+$; HRMS (ESI *m/z*) for $C_{36}H_{40}N_3O_{10}$, calcd 674.2713, found 674.2740 $[M + 1]^+$.

5.1.34. 7-Methoxy-8-{5-[(2,6-dimethoxy-4-(5-[3,4,5-trimethoxyphenyl]-3-isoxazolylphenyl)oxy]pentyloxy}-(11aS)-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepine-5-one (**7e**)

The compound **7e** was prepared according to the method described for the compound **5a**, employing the compound **26e** (856 mg, 1.0 mmol) to obtain the pure product **7e** as a white solid. Yield 406 mg, 58%; mp 66–68 °C; $[\alpha]_D^{25}$ + 157.5° (c0.1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ 7.66 (d, 1H, J = 4.3 Hz, imine–H), 7.51 (s, 1H, ArH), 7.08 (s, 2H, ArH), 7.04 (s, 2H, ArH), 6.81 (s, 1H, ArH), 6.72 (s, 1H, isoxazole–H), 4.06 (t, 4H, J = 4.9 Hz, 2× $-OCH_2-$), 3.94 (s, 6H, 2× $-OCH_3$), 3.92 (s, 9H, 3× $-OCH_3$), 3.90 (s, 3H, $-OCH_3$), 3.83–3.69 (m, 1H, –NCH–), 3.65–3.52 (m, 2H, $-NCH_2-$), 2.26–2.37 (m, 2H, $-CH_2-$), 2.10–1.79 (m, 2H, $-CH_2-$), 1.66–1.59 (m, 6H, 3× $-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.2, 164.5, 162.8, 162.2, 153.7, 153.4, 150.7, 147.6, 140.4, 139.4, 139.0, 124.0, 122.4, 119.9, 111.3, 110.2, 103.9, 103.0, 97.0, 68.8, 60.8, 56.1, 56.0, 53.6, 46.5, 29.5, 28.5, 24.0, 22.2; IR (KBr) (U_{max}/cm^{-1}): 3418, 2934, 1601, 1503, 1464, 1429, 1384, 1333, 1242, 1127, 850, 792, 615; MS (ESI): *m/z* 702 $[M + 1]^+$; HRMS (ESI *m/z*) for $C_{38}H_{44}N_3O_{10}$, calcd 702.3026, found 702.3035 $[M + 1]^+$.

5.2. Anticancer activity screening

The synthesized compounds (**5a**, **5c**–**e**, **6a**, **6c** and **7a**–**e**) were evaluated for their in vitro cytotoxicity in selected human cancer cell lines of breast (Zr-75-1, MCF7), lung (A-549, HOP62), colon (Colo205), oral (KB), prostate (PC-3), ovarian (A2780) and cervix (SiHa) origin. A protocol of 48 h continuous drug exposure was used and a sulforhodamine B (SRB) protein assay was used to estimate cell viability or growth. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mML-glutamine, and were inoculated into 96-well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations. Each compound was evaluated for four concentrations (0.1, 1, 10 and 100 μM) and each was done in triplicate wells. Plates were incubated further for 48 h, and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and plates were again incubated for 60 min at 4 °C. The plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelengths. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. The above determinations were repeated three times.

5.3. Cell culture

The human melanoma cell line A375 purchased from American Type culture collection was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum and 100 U/mL Pencillin and 100 μg/mL streptomycin sulfate (Sigma). These cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

5.4. MTT cell proliferation assay

Cell viability was assessed by the MTT based assay using WST-1 (premix WST-1 cell proliferation Assay system, Takara), is more sensitive than MTT. Briefly, A375 cells were seeded in a 96-well plate (TPP) at a cell density of 10,000 cells/well. After overnight incubation, the cells were treated with compounds **6a**–**c**, DC-81 (**4**), **2** and CA-4 (**1**) incubated for 24 h. The medium was then discarded and replaced with fresh 100 μL media followed by addition of 10 μL of WST-1 dye. Plates were incubated at 37 °C for 30 min. Optical density (O.D.) was read at 420 nm using Multimode Varioskan Flash (Thermo scientifics).

5.5. Cell cycle analysis

5×10^5 A375 Cells were seeded in 60 mm dish and were allowed to grow for 24 h. Compounds **6a**–**c**, DC-81 (**4**), **2** and CA-4 (**1**) at 4 μM concentration were added to the culture media and the cells were incubated for an additional 24 h. Harvesting of cells was done with Trypsin-EDTA, fixed with ice-cold 70% ethanol at 4 °C for 30 min, washed with PBS and incubated with 1 mg/mL RNaseA solution (Sigma) at 37 °C for 30 min. Cells were collected by centrifugation at 2000 rpm for 5 min and further stained with 250 μL of DNA staining solution [10 mg of Propidium Iodide (PI), 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of sterile MilliQ water at room temperature for 30 min in the dark]. The DNA contents of 20,000 events were measured by flow cytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

5.6. Protein extraction and Western blot analysis

Total cell lysates were isolated from cultured A375 cells after compound treatments as mentioned earlier were obtained by lysing the cells in ice-cold RIPA buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS containing protease inhibitors). After centrifugation at 12,000 rpm for 10 min, the protein in supernatant was quantified by Bradford method (BIO-RAD) using Multimode varioskan Flash instrument (Thermo Scientifics). Thirty micrograms of protein per lane was applied in 12% SDS polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidinedifluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in

TBS + 0.1% Tween 20 (TBST) containing 5% blocking powder (Santa Cruz). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight (O/N). The primary antibodies p53, Cdk2 were purchased from Santa Cruz and cytochrome *c* and PARP antibodies from Imgenex. After three TBST washes, the membrane was incubated with corresponding horseradish peroxidase-labeled secondary antibody (1:2000) (Santa Cruz) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min and the protein blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd.). The X-ray films were developed with developer and fixed with fixer solution.

Acknowledgments

We thank the National Cancer Institute, Bethesda, for in vitro anticancer assay in human cell lines. J.S.N.R., D.D.G., E.V.B., M.A.A., and F. S. are thankful to CSIR, New Delhi, for the award of research fellowships. We are also thankful to the Department of Biotechnology (BT/PR/7037/Med/14/933/2006), New Delhi; for financial assistance.

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