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Quinazolinone linked pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) conjugates: Design, synthesis and biological evaluation as potential anticancer agents

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ABSTRACT

A series of novel quinazolinone linked pyrrolobenzodiazepine (PBD) conjugates were synthesized. These compounds **4a–f** and **5a–f** were prepared in good yields by linking C-8 of DC-81 with quinazolinone moiety through different alkane spacers. These conjugates were tested for anticancer activity against 11 human cancer cell lines and found to be very potent anticancer agents with GI_{50} values in the range of <0.1–26.2 μM. Among all the PBD conjugates, one of the conjugate **5c** was tested against a panel of 60 human cancer cells. This compound showed activity for individual cancer cell lines with GI_{50} values of <0.1 μM. The thermal denaturation studies exhibited effective DNA binding ability compared to DC-81 and these results are further supported by molecular modeling studies. The detailed biological aspects of these conjugates on A375 cell line were studied. It was observed that compounds **4b** and **5c** induced the release of cytochrome *c*, activation of caspase-3, cleavage of PARP and subsequent cell death. Further, these compounds when treated with A375 cells showed the characteristic features of apoptosis like enhancement in the levels of p53, p21 and p27 inhibition of cyclin dependent kinase-2 (CDK2) and suppression of NF-κB. Moreover, these two compounds **4b** and **5c** control the cell proliferation by regulating anti-apoptotic genes like (B-cell lymphoma 2) Bcl-2. Therefore, the data generated suggests that these PBD conjugates activate p53 and inhibit NF-κB and thereby these compounds could be promising anticancer agents with better therapeutic potential for the suppression of tumours.

1. Introduction

In recent years, combination chemotherapy with different mechanisms of action is one of the methods that are being adopted to treat cancer. Therefore, a single molecule containing more than one pharmacophore, each with different mode of action could be beneficial for the treatment of cancer. Quinazolinone is a naturally occurring alkaloid and found in a variety of bioactive natural products. In addition to a wide range of biological activities,[1–6] cytotoxicity is a frequently found property in several members of quinazolinones. Among the various classes of quinazolinones, 2-styryl substituted derivatives form an important component of pharmacologically active compounds because they are associated with inhibitory effects on tubulin polymerization.[7–9] Moreover, the quinazolinone moiety has been extensively utilized as a drug-like scaffold in medicinal chemistry, and as such, the quinazolinone skeleton is considered to be a privileged structure. Further, 2-methyl quinazolinones act as inhibitors of DNA repair enzyme poly (ADP-ribose) polymerase (PARP).[10] Therapeutic agents containing the quinazolinone core structure are in the market or are in clinical trials for the treatment of cancer.

Similarly, pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are naturally occurring DNA interactive antitumor antibiotics isolated from various *Streptomyces* species. The PBD cytotoxins exert a powerful antitumoural activity by binding in the minor groove of double stranded DNA and forming a covalent bond to the exocyclic amino group of a central guanine within a three base pair recognition site.[11–13] Some representative members of the quinazolinones, DC-81 and its conjugates are illustrated in Figure 1.

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In the past few years, several hybrid compounds, in which known antitumour agents tethered to PBD moiety, have been designed, synthesized and evaluated for their biological activity.[14–18] Recently Wang and co-workers have synthesized DC-81-indole conjugates and DC-81-enediyne conjugates as potential anticancer agents and a correlation between antitumor activity and apoptosis has been well explained in these conjugates.[19,20] For the last few years, we have been involved in the development of new synthetic strategies[21–23] for the preparation of PBD ring system and also in the design as well as synthesis of structurally modified PBDs and their conjugates.[24–29]

In continuation of these efforts it has been considered of interest to design and synthesize hybrid molecules in which the PBD ring system is linked to the quinazolinone moiety with different alkane spacers. Moreover, such a moiety could enhance the DNA-binding ability as well as improve the bioavailability upon attaching this to the PBD ring system. Initially DNA binding ability, molecular modeling studies and in vitro cytotoxicity has been carried out for these new PBD conjugates. The promising activity obtained, prompted us to investigate their role in the cell proliferation and apoptosis by using the human melanoma cell line A375. In this context some biological assays have been carried out for studying the compound induced cell cycle distribution and levels of cell cycle control proteins (p53, p27 and p21). Moreover, apoptosis associated active caspase-3, PARP, cytochrome *c* and NF-κB inhibition has also been carried out in order to understand the underlying mechanisms and pathways involved. Further, studies on anti-apoptotic genes like Bcl-2 which in turn prevents mitochondrial protein (cytochrome *c*) release have been investigated. The data obtained clearly suggests that these PBD conjugates are potential anticancer agents that follow a mitochondrial mediated intrinsic pathway involving anti-apoptotic genes like Bcl-2.

2. Results and discussion

2.1. Chemistry

The synthesis of the quinazolinone derivatives **9a–f**, **13a–c** and **14a–c** is outlined in Schemes 1 and 2. The first synthetic step involved the condensation of 5-hydroxy anthranilic acid **6** or anthranilic acid **10** with acetic anhydride to afford the desired benzoxazinones **7** and **11**, respectively in quantitative yields.[30] After evaporation of the excess of anhydride under reduced pressure, the crude product was used without any further purification. Compound **7** was coupled to substituted anilines to give compounds **8a–e**, which upon etherification by employing dibromo alkanes provided the compounds **9a–f** as shown in Scheme 1. The other types of quinazolinones were obtained by reaction of compound **11** with 4-aminophenol to afford compound **12**,[31] which upon etherification by employing dibromo alkanes provided the compounds **13a–c**. Compounds **14a–c**[32] were prepared by employing benzaldehyde and **13a–c** as shown in Scheme 2.

The synthesis of C8-linked quinazolinone-PBD conjugates (**4a–f** and **5a–f**) was carried out from the (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal **15** which was prepared by the methods reported in our earlier studies.[21–29] Compound **15** on etherification with quinazolinone precursors (**9a–f**, **13a–c** and **14a–c**) using K_2CO_3 in acetone provided the corresponding nitro thioacetals (**16a–f** and **17a–f**). Further, reduction of nitro compounds by using $SnCl_2{\cdot}2H_2O$ in methanol followed by deprotection employing $HgCl_2/CaCO_3$ afforded the desired PBD conjugates (**4a–f** and **5a–f**) as shown in Scheme 3.

2.2. Biological activity

2.2.1. DNA interaction: thermal denaturation studies

DNA-binding ability of these PBD conjugates (**4a–f** and **5a–f**) was investigated by thermal denaturation studies using calf thymus (CT) DNA. Melting studies showed that these compounds stabilize the thermal helix coil or melting stabilization for the CT-DNA duplex at pH 7.0, and incubated at 37 °C with DNA/ligand molar ratio of 5:1. The increase in the helix melting temperature (ΔT_m) for each compound was examined at 0 h and 18 h incubation at 37 °C. Interestingly, all the compounds **4a–f** and **5a–f** elevated the helix melting temperature of CT-DNA in the range of 1.0–2.3 °C. All the compounds **4a–f** and **5a–f** had shown ΔT_m ranges from 1.0 to 1.2 °C at 0 h and increased up to 2.0–2.3 °C after 18 h incubation. Moreover, the naturally occurring PBD like DC-81 (**3**) exhibited a ΔT_m of 0.7 °C under similar experimental conditions as illustrated in Table 1.

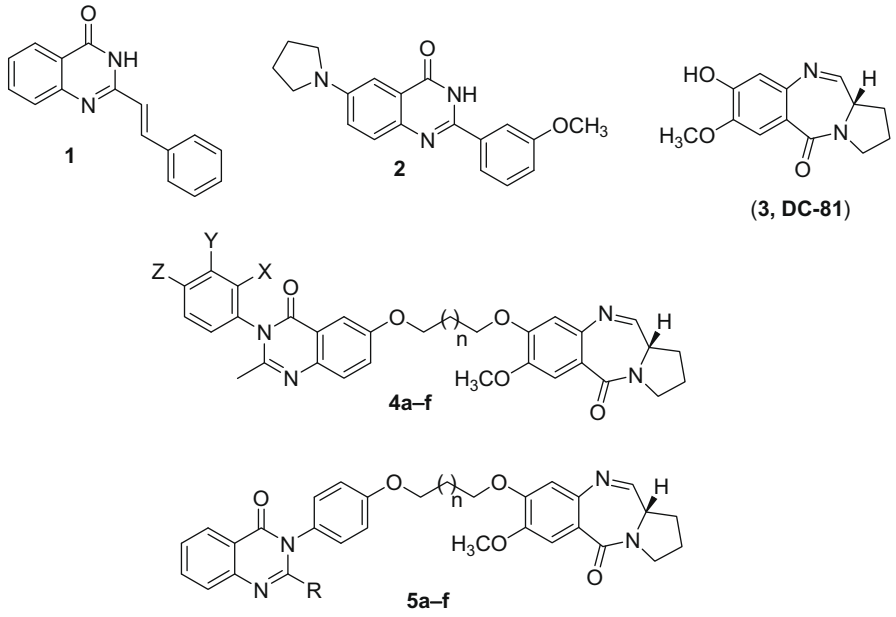
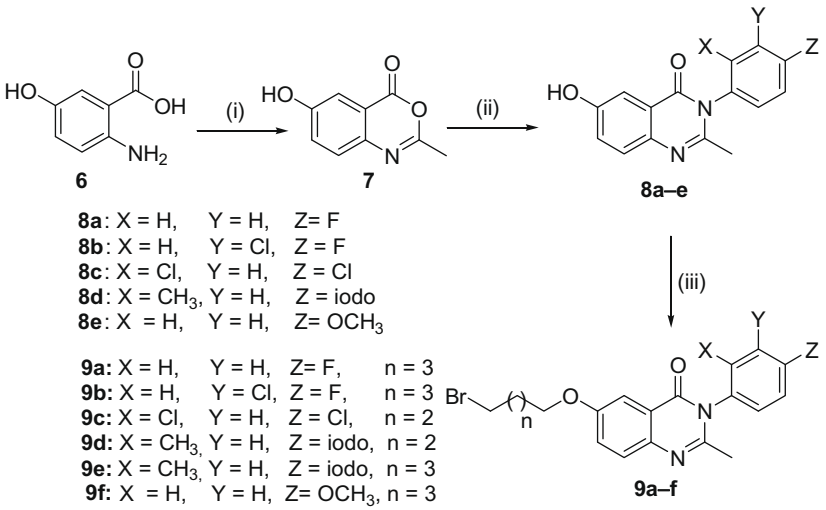
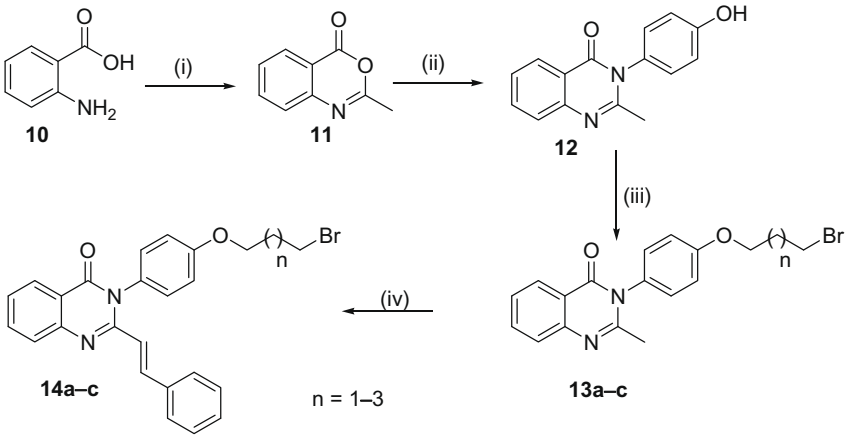


Figure 1. Structures of styryl quinzolinone (**1**), phenyl quinazolinone (**2**), DC-81 (**3**), and quinazolinone-PBD conjugates (**4a–f** and **5a–f**).



Scheme 1. Reagents and conditions: (i) $(CH_3CO)_2O$, 160–180 °C, 1 h; (ii) substituted anilines, 180 °C, 2 h, 80–86%; (iii) dibromoalkanes, K_2CO_3, acetone, reflux, 24 h, 80–88%.



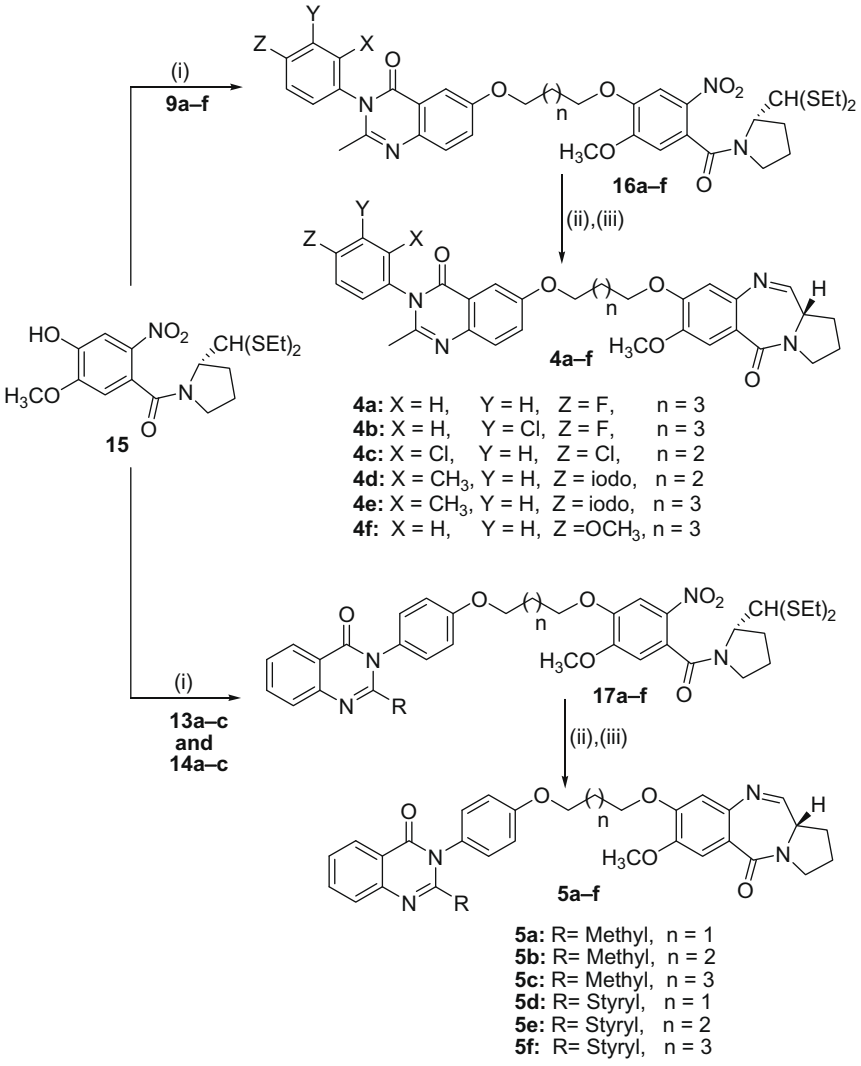
Scheme 2. Reagents and conditions: (i) $(CH_3CO)_2O$, 160–180 °C, 1 h; (ii) 4-amino phenol, 180 °C, 2 h; (iii) dibromo alkanes, K_2CO_3, acetone, 80 °C, 24 h, 85–90%; (iv) benzaldehyde, glacial acetic acid, 120 °C, 12 h, 80–82%.

2.2.2. Molecular modeling studies

The melting temperature study indicated that PBD conjugates possess DNA binding affinity, it is considered worthwhile to rationalize this data. Hence molecular modeling study was estimated by carrying out docking calculation. The dependence of docking score in the type of DNA motif employed was tested by taking about nine different DNA model sequences (A–I).[33,34] These studies are helpful to identify the suitable DNA sequence to be employed. The results of Supplementary Table S1 revealed that DNA sequence B (5′-CGCAGAAAATTTCTGCG-3′) was the best among all the sequences employed. As the ΔT_m values are very similar, we expect that the binding fitness scores are comparable. Encouragingly, all the fitnesses scores spanned in a very narrow range, which was in good agreement with the experimental observation. Sequence B was chosen as it seem to provide more reliable results in the earlier studies carried out by us. We used GOLD 3.2 program[35] for the docking studies. Although straightforward comparisons are not reliable between the docking score and experimental ΔT_m values, the docked poses provide the orientation of the ligand binding to DNA and confirm that they are indeed minor groove binders as shown in Figure 2. Further, the modeling results reveal that the nature of interaction of these compounds is non-intercalative. Thus the interaction is based on hydrogen bonding, Van der Waals contacts, hydrophobic effects and electrostatic effects.

2.2.3. Molecular dynamics simulation

Considering all the compounds with almost same ΔT_m values, we performed molecular dynamics (MD) simulation of three compounds, the ones with highest and lowest GOLD score **5c** and **5b**, respectively, and the one with highest GI_{50} value **4b**, to rigorously evaluate the dynamic nature of the complexes. MD simulation is a single-molecule atomistic in-silico technique limited by the 1–100+ ns simulation time scale and quality of the force fields. The AMBER force field provides a balanced description of base stacking and base pairing so we used AMBER 8.0[36] for the MD simulations. We used 'xmgrace' program to make graphs after MD production run. Supplementary Figure S1.Ia and Supplementary Figure S1.Ib is the graph between root mean square deviation (RMSD) versus time of DNA and ligand, respectively in MD production run. It was clear that the docked conformation of compound **5c** is stable till 2.5 ns MD simulation and after that RMSD increased, although it remains close to 3 Å till 5 ns MD simulation as shown in Supplementary Figure S1.IIIa and Supplementary Figure S1.IIIb. The compound with lowest GOLD score (**5b**) showed the highest variation in RMSDs (Supplementary Fig. S1.IIa for DNA and Supplementary Fig. S1.IIb for ligand) which is according to our expectations. In this case the DNA was also showing higher variation in RMSDs during MD simulation and RMSD of ligand is more than 4.0 Å. The snapshots of these two MD runs (Supplementary Fig. S1) showed that



Scheme 3. Reagents and conditions: (i) K_2CO_3, dry acetone, reflux, 24 h, 80–88%; (ii) $SnCl_2{\cdot}2H_2O$, CH_3OH, reflux, 6 h; (iii) $HgCl_2$, $CaCO_3$, CH_3CN–H_2O, (4:1), 12 h, 55–58%.

Table 1
Thermal denaturation data for PBD conjugates (**4a**–**f** and **5a**–**f**) with calf thymus (CT)-DNA

Compound	[PBD]:[DNA] molar ratio[b]	ΔT_m[a] (°C) after incubation at 37 °C for	
		0 h	18 h
4a	1:5	1.2	2.2
4b	1:5	1.0	2.3
4c	1:5	1.1	2.3
4d	1:5	1.0	2.0
4e	1:5	1.0	2.1
4f	1:5	1.1	2.2
5a	1:5	1.0	2.0
5b	1:5	1.0	2.1
5c	1:5	1.1	2.3
5d	1:5	1.0	2.0
5e	1:5	1.2	2.3
5f	1:5	1.1	2.0
DC-81 (**3**)	1:5	0.3	0.7

[a] For CT-DNA alone at pH 7.00 ± 0.01, ΔT_m = 68.5 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ±0.1–0.2 °C.

[b] For a 1:5 molar ratio of [PBD]/[DNA], where CT-DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01].

the part of ligand is going out from minor-groove after 2.5 ns MD simulation but in the later case it completely moved out of the groove indicating that the lowest score prediction of GOLD is valuable. The interesting results were found in the case of compound **4b**. The RMSD values of this MD run (Supplementary Fig. S1.Ia for DNA and Supplementary Fig. S1.Ib for ligand) clearly showed that this compound is strongly bound to the minor-groove of DNA and 5 ns MD simulation run did not affect the stability of docked pose. The snapshots of this MD run confirmed the strong binding ability of the compound to DNA minor-groove as shown in Figure 3. The GOLD score of this compound was also high. In principle, interaction energy is a promising approach to estimate interactions of ligands to macromolecules. The energy of interaction (E_{int}) between the DNA and the PBD molecule in a complex was calculated as a measure of stability of that complex as shown in Table 2. Poisson–Boltzmann (PB) continuum electrostatic model was used for the calculation on interaction energies in which the influence of the solvent was incorporated implicitly.[37,38] It was very clear from the negative interaction energies that all the docked complexes are favorable and stable. Further compound **4b**, which possesses experimentally highest activity, showed the highest interaction energy while the compound **5b** which had the

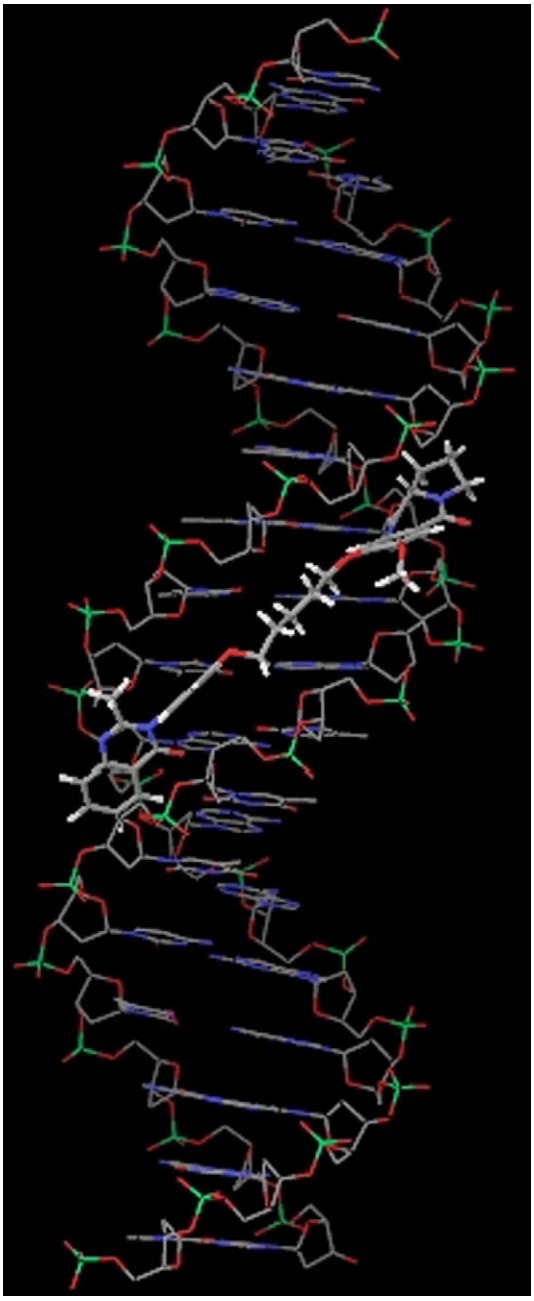


Figure 2. Docked pose of **5c** in the minor-groove of sequence-B of DNA.

lowest GOLD score also had the lowest interaction energy. Compound **5c** which had the highest GOLD score had higher interaction energy. Irrespective of the ligand and the simulation conditions this compound (**5c**) remains bound to the DNA near the preferential binding position in aqueous conditions and did not experience substantial fluctuations with respect to their initial placements in the minor groove. Docking, interaction energy calculation and variations in RMSDs obtained in the molecular dynamics simulations further validate the experimental observations apart from showing the site of interaction of the ligand with DNA.

2.2.4. In vitro cytotoxic activity

The anticancer activity of compound **5c** (NSC: 744332) was evaluated against sixty human cancer cell lines derived from nine cancer cell types at the National Cancer Institute, Bethesda as shown in Table 3. The mean GI_{50} value for the compound **5c** is 0.27 μM, which exhibited an interesting profile of activity for various cell lines, indicating that this compound **5c** had the potent broad-spectrum anticancer activity. The promising activity shown by compound **5c** prompted us to evaluate the anticancer activity of the other analogues (**4a–f**, **5a**, **5b**, and **5d–f**) in selected human cancer cell lines of breast, cervix, lung, colon, oral, ovarian and prostate by using Sulforhodamine B (SRB) method. The compounds exhibiting $GI_{50} \leqslant 10^{-5}$ μM are considered to be active on the respective cell lines. Table 4 reveals that compounds **4a–f**, **5a**, **5b**, and **5d–f** exhibited anticancer activity with GI_{50} values ranging from <0.1 to 26.2 μM. The positive control compound adriamycin demonstrated highly significant activity with the GI_{50} in the range from 0.10 to 7.25 μM and for DC-81 the GI_{50} ranged from 0.10 to 2.37 μM. SAR (structure–activity relationship) was examined for the effect of substituents on *N*-3 phenyl ring of quinazolinone moiety. Compound **4a** bearing fluorine at position 4 of *N*-3 phenyl ring showed good anticancer activity against all the 11 human cancer cell lines. Replacement of fluorine with methoxy group increased the anticancer activity in compound **4f** particularly for MCF7 and GURAV cell lines. By introducing chlorine at position 3 of *N*-3 phenyl ring, in compound **4b**, the anticancer activity increased particularly for MCF7 and GURAV cell lines with GI_{50} values of <0.1 μM. Introducing two chlorines at 2 and 4 positions in compound **4c** slightly decreased the activity. Further replacement of chlorines at positions 2 and 4 with iodine and methyl affected a slight increase in the activity in compounds **4d** and **4e**. Similarly, we observed the effect of substituents at position-2 of quinazolinone moiety. Compounds **5a–c** substituted by a methyl group at the 2 position of quinazolinone ring exhibited more activity. Replacement of 2-methyl group with a styryl group in quinazolinone ring system decreased the activity in compounds **5d–f**. Hence it is assumed that the methyl substitution at position 2 of the quinazolinone ring could play an important role in increasing the anticancer activity. Further, the PBD conjugates with odd number of alkyl spacers (n = 3 and 5) exhibited more activity than their counterpart with an even number of alkyl spacers (n = 4). The significant anticancer activity showed by the two promising compounds **4b** and **5c** prompted us to evaluate the cell viability of A375 cells, as these A375 cancerous cells are selected to carry out the further biological assays because of their resistance to radio and chemotherapy. The cell viability of these drugs is calculated as the percentage of viable drug treated cells compared with untreated cells. The cell viability of A375 cells was 58.72% and 49.01% in case of compounds **4b** and **5c**, respectively. Amongst both these series of conjugates, **5c** was found to be the most effective one as shown in Supplementary Figure S2.

2.2.5. Cell cycle effects

To investigate the effect of quinazolinone-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 (FACS) analysis. Drug induced hypodiploid DNA (i.e., sub-G1 phase) is the characteristic feature of apoptosis. The majority of the control cells exposed to DMSO were in the G1 phase. Cells (5.61%) were in G0, 60.65% in G1, 15.67% in S and 18.07% in G2/M phase. Treatment of A375 cells with the most promising compounds **4b** and **5c** at 4 μM concentration for 24 h induced apoptosis effects up to 24.1%, 31.52%, respectively, of sub-G1 DNA peak. Simultaneously there was a concomitant decrease in the G2/M phase in A375 cells when treated with **4b** (11.92%) and **5c** (9.15%) compounds. Increased cells of sub-G1 phase, decrease of G2/M phase cells clearly showed that all the compounds are effective in causing apoptosis in case of A375 cells as shown in Figure 4a and b.

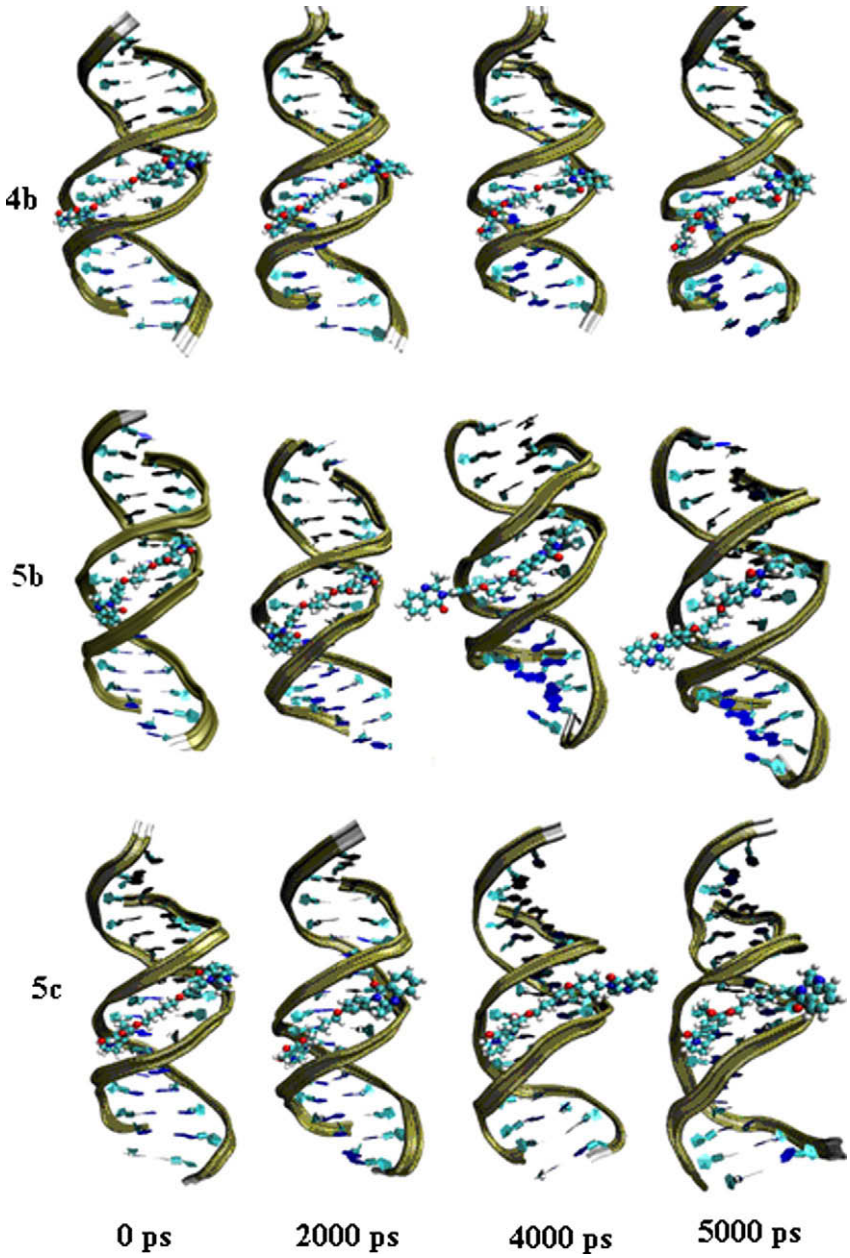


Figure 3. Snapshots of DNA–ligand complexes at different times.

Table 2
Interaction energies (kcal/mol) calculated by Poisson–Boltzmann (PB) method for DNA–ligand complexes

Complex	IE/PB	ΔT_m
DNA-**4b**	−126.35	2.3
DNA-**5b**	−48.59	2.1
DNA-**5c**	−90.03	2.3

2.2.6. Effect on p53 and p27 protein expressions

Activation of tumor suppressor genes such as p53 and p27 is found to be important in the regulation of apoptotic pathway induced by various stimuli.[39] The p53 gene, is a tumor suppressor gene, that is, its activity stops the formation of tumors. It exerts antiproliferative effect through its ability to function as a sequence-specific DNA-binding transcription factor. In order to understand the exploit of PBD conjugates on p53 and p27 dependent apoptotic pathway, the cells were treated with compounds **4b** and **5c** at a concentration of 4 μM for 24 h and the cell lysate was used for western blot analysis using p53 and p27 specific antibodies. It was observed that both the p53 and p27 levels were upregulated in the case of compounds **4b** and **5c** compared to the untreated controls. Interestingly, increase of both p53 and p27 protein levels were more prominent in compound **5c** as shown in Figure 5.

2.2.7. Effect on p21 mRNA levels

Most of the biochemical and genetic studies indicate that p21 acts as a master effector of multiple tumour suppressor pathways for anti-proliferation, which are independent of classical p53 tumour suppressor pathway.[40,41] Binding of p21 inhibits the kinase activity of cyclin dependent kinases, CDK1 and CDK2 resulting in growth arrest at specific stages of cell cycle. Hence it was considered of interest to test whether the PBD conjugates **4b** and **5c** could

Table 3
In vitro anticancer activity of compound **5c** in selected cancer cell lines[a]

Cancer panels/cell lines	GI_{50} (μM)	Cancer panels/cell lines	GI_{50} (μM)
Leukemia		*Ovarian*	
CCRF-CEM	0.16	IGROV1	0.18
HL-60(TB)	0.18	OVCAR-3	0.20
K-562	0.06	OVCAR-4	0.37
MOLT-4	0.19	OVCAR-5	0.33
RPMI-8226	0.19	OVCAR-8	0.34
SR	0.11	SK-OV-3	0.43
Non-small cell lung		*Renal*	
A549/ATCC	0.23	786-0	0.17
EKVX	0.87	A498	0.16
HOP-92	0.35	ACHN	0.29
NCI-H226	0.16	CAKI-1	0.19
NCI-H23	0.23	RXF 393	0.21
NCI-H322 M	0.26	SN12C	0.24
NCI-H460	0.20	TK-10	0.31
NCI-H522	0.12	UO-31	0.36
Colon		*Breast*	
COLO 205	0.18	MCF7	0.17
HCC-2998	0.26	NCI/ADR-RES	1.73
HCT-116	0.15	MDA-MB-231/ATCC	0.17
HCT-15	1.09	HS 578T	0.16
HT29	0.26	MDA-MB-435	0.16
KM12	0.17	BT-549	0.11
SW-620	0.16	T-47D	0.17
		MDA-MB-468	0.16
CNS		*Prostate*	
SF-268	0.26	PC-3	0.29
SF-539	0.22	DU-145	0.26
SNB-19	0.28		
SNB-75	0.38		
U251	0.16		
Melanoma		*Melanoma*	
LOX IMVI	0.15	SK-MEL-28	0.20
MALME-3M	0.23	SK-MEL-5	0.16
M14	0.17	UACC-257	0.39
SK-MEL-2	0.42	UACC-62	0.16
	Mean[b]	0.27	

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

act on p21. RNA was isolated from A375 cells treated with 4 μM of compounds **4b** and **5c** and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was carried out using p21 and GAPDH (internal or loading control) specific primers. As shown in Figure 6, an increased level of p21 transcript especially when treated with compound **5c** was observed indicating the suppression of genes that are important for cell cycle progression.

2.2.8. 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. CDK inhibitors like p21cip1, p27kip1 and p57kip2 have opposite effects on the functions mediated by CDK. p27Kip1 and p21Cip1 are thought to suppress tumor growth and prevent cell cycle progression by inhibiting Cdk2-cyclin E/A kinases. Sequestering of p27 from the cyclin E/CDK2 complex can favour the progression from G1 to S phase. The progression from G1 to S phase is mediated by cyclin E/CDK2 and is crucial for cell cycle progression. Since we observed an increased level of p21, it was considered of interest to know the levels of CDK2 upon treatment with these PBD conjugates (**4b** and **5c**) at 4 μM concentration for 24 h. The CDK2 level upon treating the A375 cells was decreased in case of **5c** as shown in Figure 7 indicating a G1 arrest.

2.2.9. Effect on NF-κB activity

Suppression of NF-κB activity is required for induction of apoptotic response, which is a prerequisite in the selection of drugs for treatment of cancer.[42,43] NF-κB is a heterodimer of two subunits (p50 and p65). To confirm whether these PBD conjugates (**4b** and **5c**) could suppress the NF-κB activity, A375 cells were treated with 4 μM concentration for 24 h. The cell lysates were collected and western blot analysis was carried out using p65 antibody. A reduction of NF-κB (p65) was observed upon treatment of A375 cells with the compounds **4b** and **5c** compared to untreated cells. The present study revealed that decrease in NF-κB expression was more pronounced in case of **5c** indicating its involvement in NF-κB pathway for activation of apoptotic response as shown in Figure 8.

2.2.10. Effect on cytochrome *c*

An important consequence of intrinsic apoptotic pathway is the mitochondrial dysfunction and cytochrome *c* release.[44] Cytosolic

Table 4
GI_{50} values (in μM) for compounds **4a–f**, **5a**, **5b** and **5d–f** in selected human cancer cell lines[a]

Compound	MCF7[b]	Zr-75–1[b]	A549[c]	HOP62[c]	SiHa[d]	Colo205[e]	KB[f]	GURAV[f]	DWD[f]	PC3[g]	A2780[h]
4a	0.15	0.16	0.13	0.18	0.19	0.15	0.15	0.13	0.15	0.17	0.14
4b	<0.1	0.15	0.13	0.14	0.17	0.13	0.15	<0.1	0.13	0.17	0.11
4c	0.14	0.17	0.18	0.18	0.18	0.17	0.16	0.17	0.15	0.18	0.15
4d	0.12	0.16	0.17	0.17	0.18	0.17	0.15	0.17	0.15	0.17	0.15
4e	0.11	0.16	0.16	0.16	0.17	0.16	0.15	0.16	0.13	0.16	0.14
4f	<0.1	0.15	0.14	0.18	0.18	0.14	0.16	<0.1	0.13	0.17	0.14
5a	1.83	0.15	1.48	0.16	0.16	0.14	0.15	0.13	0.11	0.13	0.10
5b	2.30	0.17	1.93	1.95	1.90	1.81	1.98	1.95	1.60	0.17	1.65
5d	2.03	0.16	1.88	2.15	2.00	2.12	1.78	1.85	0.14	0.16	0.14
5e	NA	0.16	2.39	2.33	2.19	2.23	2.07	1.93	0.16	1.91	0.16
5f	2.50	0.15	26.2	2.31	2.08	2.00	2.15	1.82	0.17	1.83	0.15
ADR[i]	0.17	1.79	7.25	0.14	0.17	0.14	0.17	0.17	0.10	1.81	0.16
DC-81(3)	0.17	2.37	0.16	0.15	0.17	0.11	0.17	0.16	1.49	0.20	0.14

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

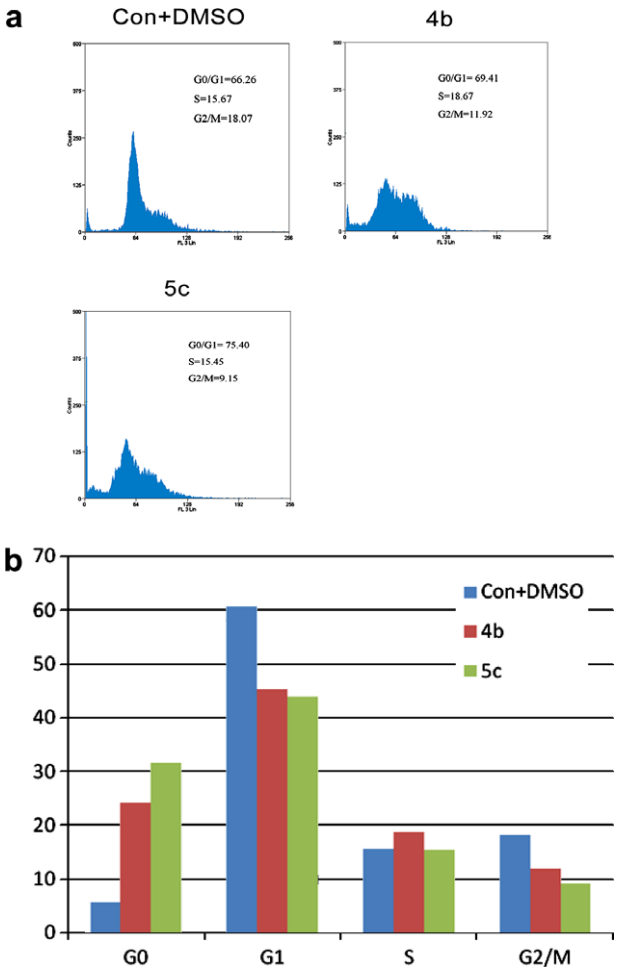


Figure 4. (a and b) Flow cytometric analysis of cell cycle distribution on A375 after exposure to PBD conjugates at 4 μM concentration for 24 h before cell cycle analysis.

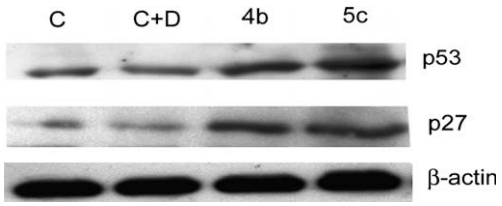


Figure 5. Effect on p53 and p27 protein expression. Cells were treated with 4 μM concentration of PBD conjugates for 24 h. The cell lysates were collected and western blot analysis was carried out using p53 and p27 specific antibody. (C) Control (untreated), (C+D) control (untreated) + DMSO. β-Actin was used as internal loading control.

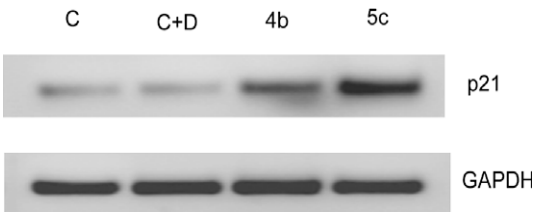


Figure 6. Expression of p21 mRNA levels in PBD conjugates treated cells by RT-PCR studies. A375 cells were exposed to 4 μM concentration of PBD conjugates for 24 h. RNA was extracted and RT-PCR was carried out using p21 specific primers and GAPDH was used as internal loading control. (C) Untreated cells, (C+D) untreated cells with DMSO compound.

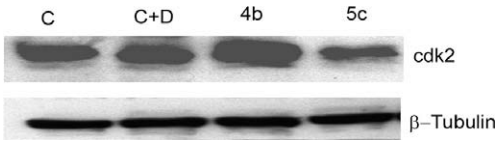


Figure 7. Effect of PBD conjugates on cyclin dependent kinase-2 (CDK2) protein levels. A375 were treated with 4 μM concentration of quinazolinones for 24 h. The cell lysates were collected and western blot analysis was carried out using CDK2 specific antibody. (C) Control (untreated), (C+D) control (untreated) + DMSO. β-Tubulin was used as internal protein loading control.

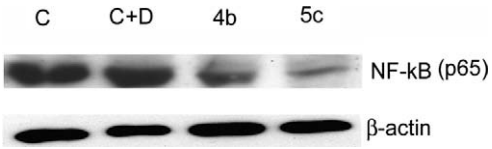


Figure 8. Effect on NF-κB activation. Cells were treated with 4 μM concentration of PBD conjugates for 24 h. The cell lysates were collected and observed for the NF-κB protein levels using NF-κB (p65) specific antibody. (C) control (untreated), (C+D) control (untreated) + DMSO. β-Actin was used as loading control.

cytochrome *c* is known to cause activation of caspase-3.[45] To investigate the effect on cytochrome *c*, A375 cells were treated with PBD conjugates (**4b** and **5c**) at 4 μM concentration for 24 h and observed that there was increase in cytochrome *c* protein levels. The enhancement in cytochrome *c* level was prominent in case of compound **5c** indicating its role in apoptosis through the involvement of mitochondrial pathway as shown in Figure 9.

2.2.11. Effect on anti-apoptotic genes (Bcl-2)

Bcl-2 family of proteins precisely regulates the protein release from mitochondria. This family is divided into two subgroups; pro-apoptotic members and anti-apoptotic members. The anti-apoptotic members like Bcl-2 prevent mitochondrial protein release.[46,47] As we observed an increase in cytosolic cytochrome *c*, it was expected a decrease in the levels of anti-apopototic factors. Hence we treated A375 cells with 4 μM of the PBD conjugates (**4b** and **5c**) for 24 h. RNA was isolated and a RT-PCR was carried out with Bcl-2 (anti-apopotitic gene) specific primers to compare the transcript levels in the treated and untreated cells. GAPDH was used as internal or loading control. As expected we observed a prominent decrease in the expression of mRNA levels of anti-apoptotic gene, Bcl-2 in both the compounds **4b** and **5c**. This study confirmed that these PBD conjugates control cell proliferation by regulating the anti-apoptotic genes as shown in Figure 10.

2.2.12. Effect on activation of caspase-3 and cleavage of PARP

One of the important characteristic features of apoptosis is the degradation of procaspase-3 to active caspase-3 and cleavage of DNA repair enzyme PARP by caspase-3.[48] Cytosolic cytochrome *c* is known to cause activation of caspase-3.[45] As we observed tremendous increase in cytosolic cytochrome *c*, it was expected

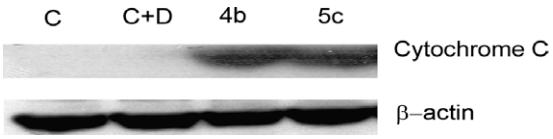


Figure 9. Effect on cytochrome *c* release from mitochondria. A375 cells were exposed to 4 μM concentration PBD conjugates for 24 h and cell lysates were collected. Western blot analysis was carried out with cytochrome *c* specific antibody. (C) Control (untreated), (C+D) control (untreated) + DMSO. β-Actin was used as internal protein loading control.

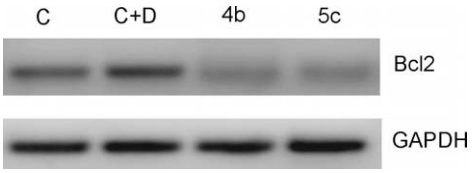


Figure 10. Expression of Bcl-2, anti-apoptotic gene, in A375 cells at mRNA level by RT-PCR analysis. A375 cells were exposed to 4 μM concentration of PBD conjugates for 24 h. RNA was extracted and RT-PCR was carried out using Bcl-2 specific primers and GAPDH specific primers. GAPDH was used as internal control. (C) Control (untreated), (C+D) control (untreated) + DMSO.

an increase in the levels of active caspase-3, which inturn cleaves the DNA repair enzyme PARP. To assess the role of PBD conjugates on the levels of active caspase-3 and cleavage of PARP, A375 cells were treated with 4 μM of the compounds **4b** and **5c** for 24 h and the expression levels of active caspase-3 was analyzed by western blot analysis. An increase in the levels of active caspase-3 was observed when the cells were treated with compounds **4b** and **5c.** An increase in the expression of cleaved PARP product (85 kDa) specific to apoptosis was observed and the levels were more pronounced in the case of compound **5c**. This data clearly indicated that these PBD conjugates induce apoptosis by caspase-mediated mechanism as shown in Figure 11a and b.

3. Conclusion

In the present study, we designed and synthesized a series of quinazolinone–pyrrolobenzodiazepine conjugates. Compound **5c** was evaluated by the National Cancer Institute, Bethesda which exhibited potent anticancer activity against various cancer cell lines indicating that this compound had the potential for its development as a broad spectrum anticancer agent. Further the other PBD analogues (**4a–f**, **5a**, **5b** and **5d–f**) also showed good anticancer activity against 11 human cancer cell lines. The thermal denaturation studies showed that these conjugates have better DNA binding ability when compared to DC-81. Moreover, these binding studies were further validated by the molecular modeling studies. In MTT proliferation assay the two promising compounds **4b** and **5c** showed potent cytotoxicity in A375 cells at 4 μM. The FACS analysis showed more population in sub-G1 phase indicating that these two PBD conjugates had apoptosis inducing ability. In order to understand the mechanism and pathways, further biological assays like western blot analysis of p53, p27, p21 and NF-κB were carried out. It was observed from the results that the p53 and p21 levels were enhanced when treated with compounds **4b** and **5c**. Moreover, the decrease in the levels of CDK2 was observed, as there is increase in the levels of p21. Further, suppression of NF-κB (p65) was observed and this was more pronounced in case of **5c**. Release of cytochrome *c* was prominent in compound **5c** indicating the involvement of mitochondrial pathway. Concomitant with this a huge decrease in the levels of anti-apoptotic gene, Bcl-2, which regulates the mitochondrial proteins, was also observed when the cells were treated with the compounds **4b** and **5c**. An increase in the levels of active caspase-3 and cleavage of PARP was also observed when cells were treated with compound **5c**. Hence in the present study out of a set of quinazolinone-PBD conjugates synthesized one of them **5c**, activated p53 pathway and also simultaneously inhibited NF-κB activity indicating its potential for activation of apoptosis in the treatment of cancer. Further, all the conjugates in which quinazolinone moiety linked to PBD ring system particularly compounds **4a–f** and **5a–c** showed significant anticancer activity with GI_{50} values in the range of <0.1–2.30 μM when compared to some of the PBD conjugates like C-8 linked 1,2,3-triazole-PBD conjugates (GI_{50} values in the range of 0.13–30.50 μM), triazolobenzothiadiazine-PBD conjugates (GI_{50} values in the range of 0.22–30.30 μM) and phosphonate linked PBD conjugates (GI_{50} values in the range of 0.17–30.50 μM) against 9–11 human cancer cell lines in SRB assay. Hence from this data we can conclude that linking of quinazolinone moiety to PBD ring system improved the anticancer activity. Further, in this investigation, antitumour proliferation mechanism of novel quinazolinone-PBD conjugates was also examined which could be helpful for future studies on such conjugates. Finally, it may be concluded that the compound **5c** has the potential to be taken up for further preclinical studies either alone or in combination with existing therapies.

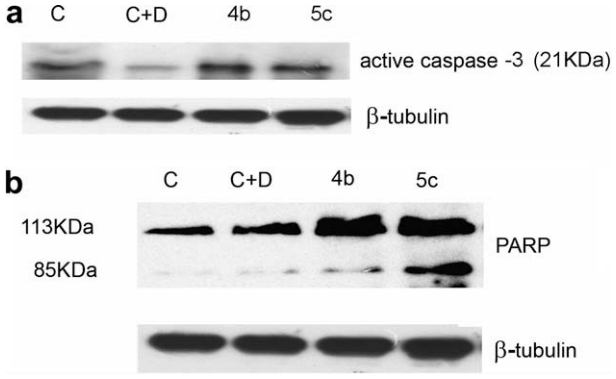


Figure 11. (a and b) Effect of PBD conjugates on active/cleaved caspase-3 and cleavage of PARP. A375 cells were treated with 4 μM concentration of PBD conjugates for 24 h. The cell lysates were collected and expression levels of active/cleaved caspase-3 (a) (antibody from Imgenex detects 21 kDa) and PARP (b) were determined by western blot analysis with specific antibodies (PARP antibody from roche detects both the 113 kDa and 85 kDa proteins). (C) Control (untreated), (C+D) control (untreated) + DMSO, in which the drug was dissolved. β-Tubulin was used as internal loading control.

4. Experimental section

4.1. General

All chemicals and reagents were obtained from Aldrich (Sigma–Aldrich, St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) or Spectrochem Pvt. Ltd (Mumbai, India) and were used without further purification. Reactions were monitored by TLC, performed on 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 spectra were recorded on Gemini Varian-VXR-unity (200 MHz) or Bruker UXNMR/XWIN-NMR (300 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from internal TMS standard. ESI spectra were recorded on Micro mass, 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.

4.2. General procedures

4.2.1. Synthesis of 3-(4-fluorophenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (8a)

Equimolar amounts **7** (354 mg, 2 mmol) and 4-flouro aniline (222 mg, 2 mmol) were heated at 120 °C for 12 h in glacial acetic acid. The reaction mixture then washed with cool sodium bicarbonate solution and then extracted with ethylacetate. The solvent was evaporated under reduced pressure to get the crude product.

This was further purified by column chromatography (15% ethyl acetate–hexane) to afford the compound **8a** as a white solid (464 mg, 86%). Mp 182–185 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.21 (s, 3H), 7.22–7.49 (m, 4H), 7.32 (d, 1H, J = 8.6 Hz), 7.54 (d, 1H, J = 8.6 Hz), 7.60 (s, 1H), 9.59 (s, OH); MS (ESI): m/z 271.1 $(M+1)^+$.

4.2.2. 3-(3-Chloro-4-fluorophenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (8b)

The compound **8b** was prepared following the method described for the preparation of the compound **8a**, employing **7** (354 mg, 2 mmol) and 3-chloro-4-flouroaniline (292 mg, 2 mmol), and the crude product was purified by column chromatography (15% ethyl acetate–hexane) to afford the compound **8b** as a white solid (518 mg, 85%); mp 144–148 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.18 (s, 3H), 7.15–7.20 (m, 1H), 7.31–7.39 (m, 3H), 7.56–7.61 (m, 2H), 9.63 (s, 1H–OH); MS (ESI): m/z 306 $(M+1)^+$.

4.2.3. 3-(2,4-Dichlorophenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (8c)

The compound **8c** was prepared following the method described for the preparation of the compound **8a**, employing **7** (354 mg, 2 mmol) and 2,4-dichloroaniline (324 mg, 2 mmol), and the crude product was purified by column chromatography (16% ethyl acetate–hexane) to afford the compound **8c** as a white solid (526 mg, 82%). Mp 128–132 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.16 (s, 3H), 7.20 (d, 1H, J = 8.6 Hz), 7.25 (d, 1H, J = 7.7 Hz), 7.41 (d, 1H, J = 8.6 Hz), 7.48 (d, 2H, J = 9.4 Hz), 7.95 (d, 1H, J = 7.7 Hz), 8.93 (s, 1H–OH); MS (ESI): m/z 322 $(M+1)^+$.

4.2.4. 6-Hydroxy-3-(4-iodo-2-methylphenyl)-2-methylquinazolin-4(3*H*)-one (8d)

The compound **8d** was prepared following the method described for the preparation of the compound **8a**, employing **7** (354 mg, 2 mmol) and 4-iodo-2-methylaniline (466 mg, 2 mmol), and the crude product was purified by column chromatography (16% ethyl acetate–hexane) to afford the compound **8d** as a white solid (628 mg, 80%). Mp 119–120 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.07 (s, 3H), 2.14 (s, 3H), 6.92 (d, 1H, J = 8.0 Hz), 7.23 (d, 1H, J = 8.0 Hz), 7.53 (d, 1H, J = 8.7 Hz), 7.67 (s, 2H), 7.73 (s, 1H), 8.33 (s, 1H–OH); MS (ESI): m/z 393 $(M+1)^+$.

4.2.5. 6-Hydroxy-3-(4-methoxyphenyl)-2-methylquinazolin-4(3*H*)-one (8e)

The compound **8e** was prepared following the method described for the preparation of the compound **8a**, employing **7** (354 mg, 2 mmol) and 4-methoxyaniline (246 mg, 2 mmol), and the crude product was purified by column chromatography (14% ethyl acetate–hexane) to afford the compound **8e** as a white solid (456 mg, 81%). Mp 262–264 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.16 (s, 3H), 3.87 (s, 3H) 7.02 (d, 2H, J = 8.6 Hz), 7.16 (d, 2H, J = 8.6 Hz), 7.21 (dd, 1H, J = 8.6 Hz), 7.44 (d, 1H, J = 8.6 Hz) 7.46 (s, 1H), 9.50 (s, 1H-OH); MS (ESI): m/z 283 $(M+1)^+$.

4.2.6. Synthesis of 6-(5-bromopentyloxy)-3-(4-fluorophenyl)-2-methylquinazolin-4(3*H*)-one (9a)

To a solution of compound **8a** (270 mg, 1 mmol) in acetone (30 mL), anhydrous K_2CO_3 (553 mg, 4 mmol) and 1,5-dibromopentane (690 mg, 3 mmol) were added and the mixture was refluxed for 12 h. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography using ethyl acetate–hexane (10%) to afford the compound **9a** as a white solid (337 mg, 88%). Mp 137–140 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.52–1.75 (m, 2H), 1.77–2.05 (m, 4H), 2.24 (s, 3H), 3.46 (t, 2H, J = 6.7 Hz), 4.08 (t, 2H, J = 6.0 Hz), 7.25 (d, 4H, J = 6.0 Hz), 7.35 (d, 1H, J = 8.3 Hz), 7.58 (s, 1H), 7.61 (d, 1H, J = 9.0 Hz); MS (ESI): m/z 421.1 $(M+1)^+$.

4.2.7. 6-(5-Bromopentyloxy)-3-(3-chloro-4-fluorophenyl)-2-methylquinazolin-4(3*H*)-one (9b)

The compound **9b** was prepared following the met od described for the preparation of the compound **9a**, employing **8b** (305 mg, 1 mmol) and 1,5-dibromo pentane (690 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **9b** (384 mg, 85%). Mp 108–110 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.52–1.74 (m, 2H), 1.78–2.06 (m, 4H), 2.23 (s, 3H), 3.45 (t, 2H, J = 6.7 Hz), 4.08 (t, 2H, J = 6.0 Hz), 7.15–7.20 (m, 1H), 7.31–7.39 (m, 3H), 7.56–7.61 (m, 2H); MS (ESI): m/z 454 $(M+1)^+$.

4.2.8. 6-(4-Bromobutoxy)-3-(2,4-dichlorophenyl)-2-methylquinazolin-4(3*H*)-one (9c)

The compound **9c** was prepared following the method described for the preparation of the compound **9a**, employing **8c** (321 mg, 1 mmol) and 1,5-dibromopentane (690 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **9c** as a white solid (385 mg, 85%). Mp 110–115 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.95–2.04 (m, 2H), 2.04–2.13 (m, 2H), 2.16 (s, 3H), 3.47 (t, 2H, J = 6.5 Hz), 4.10 (t, 2H, J = 6.0 Hz), 7.26–7.33 (m, 2H), 7.44 (d, 1H, J = 8.7 Hz), 7.51 (d, 1H, J = 2.1 Hz), 7.55 (d, 1H, J = 8.7 Hz), 7.63 (s, 1H); MS (ESI): m/z 454 $(M+1)^+$.

4.2.9. 6-(4-Bromobutoxy)-3-(4-iodo-2-methylphenyl)-2-methylquinazolin-4(3*H*)-one (9d)

The compound **9d** was prepared following the method described for the preparation of the compound **9a**, employing **8d** (392 mg, 1 mmol) and 1,4-dibromobutane (648 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **9d** as a white solid (422 mg, 80%). Mp 75–77 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.92–2.01 (m, 2H), 2.02–2.07 (m, 2H), 2.09 (s, 3H), 2.20 (s, 3H), 3.45 (t, 2H, J = 6.0 Hz), 4.08 (t, 2H, J = 6.0 Hz), 6.90 (d, 1H, J = 8.3 Hz), 7.38 (d, 1H, J = 8.3 Hz), 7.61 (s, 1H), 7.63–7.76 (m, 2H), 7.79 (s, 1H); MS (ESI): m/z 528 $(M+1)^+$.

4.2.10. 6-(5-Bromopentyloxy)-3-(4-iodo-2-methylphenyl)-2-methylquinazolin-4(3*H*)-one (9e)

The compound **9e** was prepared following the method described for the preparation of the compound **9a**, employing **8d** (392 mg, 1 mmol) and 1,5-dibromopentane (690 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **9e** as a white solid (432 mg, 80%). Mp 85–90 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.60–1.71 (m, 2H), 1.80–2.02 (m, 4H), 2.08 (s, 3H), 2.19 (s, 3H), 3.45 (t, 2H, J = 6.0 Hz), 4.08 (t, 2H, J = 6.0 Hz), 6.90 (d, 1H, J = 8.3 Hz), 7.38 (d, 1H, J = 8.3 Hz), 7.60 (s, 1H), 7.63–7.76 (m, 2H), 7.79 (s, 1H); MS (ESI): m/z 542 $(M+1)^+$.

4.2.11. 6-(5-Bromopentyloxy)-3-(4-methoxyphenyl)-2-methylquinazolin-4(3*H*)-one (9f)

The compound **9f** was prepared following the method described for the preparation of the compound **9a**, employing **8e** (282 mg, 1 mmol) and 1,5-dibromopentane (690 mg, 3 mmol), and the crude product was purified by column chromatography (10% EtOAc–hexane) to afford the compound **9f** as a white solid (366 mg, 85%). Mp 81–83 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.59–1.72 (m, 2H), 1.79–2.02 (m, 4H), 2.24 (s, 3H), 3.45 (t, 2H, J = 6.0 Hz), 3.88 (s, 3H), 4.07 (t, 2H, J = 6.0 Hz), 7.05 (d, 2H, J = 7.6 Hz), 7.17 (d, 2H, J = 7.6 Hz), 7.35 (d, 1H, J = 7.6 Hz), 7.57–7.63 (m, 2H); MS (ESI): m/z 432 $(M+1)^+$.

4.2.12. Synthesis of 3-(4-hydroxyphenyl)-2-methylquinazolin-4(3*H*)-one (12)

Equimolar amounts (1.61 g, 10 mmol) of 2-methyl-4*H*-benzo[*d*][1,3]oxazin-4-one (**11**) and 4-amino phenol (1.30 g, 12 mmol) were heated at 160–180 °C for 2 h in oil bath. Upon cooling, the solid reaction material was crystallized from ethanol to give pure 3-(4-hydroxyphenyl)-2-methylquinazolin-4(3*H*)-one **12**, which can be used for the next step without any further purification.

4.2.13. Synthesis of 3-(4-(3-bromopropoxy)phenyl)-2-methylquinazolin-4(3*H*)-one (13a)

The compound **13a** was prepared following the method described for the preparation of the compound **9a,** employing **12** (252 mg, 1 mmol) and 1,3-dibromopropane (606 mg, 3 mmol) and the crude product was purified by column chromatography using ethylacetate-hexane (10%) to afford the compound **13a** as a light yellow solid (335 mg, 90% yield). Mp 122–124 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.23 (s, 3H), 2.31–2.41 (m, 2H), 3.61 (t, 2H, J = 6.0 Hz), 4.17 (t, 2H, J = 6.0 Hz), 7.03 (d, 2H, J = 9.0 Hz), 7.15 (d, 2H, J = 9.0 Hz), 7.39–7.46 (m, 1H), 7.62 (d, 1H, J = 7.5 Hz), 7.68–7.76 (m, 1H), 8.23 (dd, 1H, J = 1.5, 8.3 Hz); MS (ESI): *m/z* 373 $(M+1)^+$.

4.2.14. 3-(4-(4-Bromobutoxy)phenyl)-2-methylquinazolin-4(3*H*)-one (13b)

The compound **13b** was prepared following the method described for the preparation of the compound **9a,** employing **12** (252 mg, 1 mmol) and 1,4-dibromobutane (648 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **9b** as a light yellow solid (328 mg, 85% yield). Mp 132–133 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.95–2.05 (m, 2H), 2.06–2.15 (m, 2H), 2.26 (s, 3H), 3.51 (t, 2H, J = 6.0 Hz), 4.06 (t, 2H, J = 6.0 Hz), 7.03 (d, 2H, J = 9.0 Hz), 7.16 (d, 2H, J = 9.0 Hz), 7.43–7.49 (m, 1H), 7.66 (d, 1H, J = 7.5 Hz) 7.72–7.79 (m, 1H) 8.25 (dd, 1H, J = 1.5, 8.3 Hz); MS (ESI): *m/z* 387 $(M+1)^+$.

4.2.15. 3-(4-(5-Bromopentyloxy)phenyl)-2-methylquinazolin-4(3*H*)-one (13c)

The compound **13c** was prepared following the method described for the preparation of the compound **9a**, employing **12** (252 mg, 1 mmol) and 1,5-dibromopentane (690 mg, 3 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **13c** as a light yellow solid (353 mg, 88%). Mp 119–121 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.59–1.72 (m, 2H), 1.81–2.01 (m, 4H), 2.26 (s, 3H), 3.51 (t, 2H, J = 6.0 Hz), 4.06 (t, 2H, J = 6.0 Hz), 7.03 (d, 2H, J = 9.0 Hz), 7.16 (d, 2H, J = 9.0 Hz), 7.43–7.49 (m, 1H), 7.66 (d, 1H, J = 7.5 Hz), 7.72–7.79 (m, 1H), 8.25 (dd, 1H, J = 1.5, 8.3 Hz); MS (ESI): *m/z* 401 $(M+1)^+$.

4.2.16. Synthesis of (*E*)-3-(4-(3-bromopropoxy)phenyl)-2-styrylquinazolin-4(3*H*)-one (14a)

To a solution of compound **13a** (372 mg, 1 mmol) in acetic acid (10 mL), benzaldehyde (127 mg, 1.2 mmol) was added and the mixture was refluxed for 12 h. The reaction mixture then washed with cool sodium bicarbonate solution and then extracted with ethylacetate. The solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **14a** as a creamish white solid (378 mg, 82%). Mp 178–181 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.33–2.44 (m, 2H), 3.66 (t, 2H, J = 6.0 Hz), 4.21 (t, 2H, J = 6.0 Hz), 6.47 (d, 1H, J = 15.2 Hz), 7.09 (d, 2H, J = 8.4 Hz), 7.21 (d, 2H, J = 8.4 Hz), 7.28–7.41 (m, 5H), 7.75–7.85 (m, 3H), 7.98 (d, 1H, J = 15.2 Hz), 8.30 (dd, 1H, J = 1.5, 8.3 Hz); MS (ESI): *m/z* 462 $(M+1)^+$.

4.2.17. (*E*)-3-(4-(4-Bromobutoxy)phenyl)-2-styrylquinazolin-4(3*H*)-one (14b)

The compound **14b** was prepared following the method described for the preparation of the compound **14a**, employing **13b** (386 mg, 1 mmol) and benzaldehyde (127 mg, 1.2 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **14b** as a creamish white solid (380 mg, 80%). Mp 158–161 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.96–2.05 (m, 2H), 2.06–2.15 (m, 2H), 3.43 (t, 2H, J = 6.5 Hz), 4.05 (t, 2H, J = 6.5 Hz), 6.43 (d, 1H, J = 15.5 Hz), 7.03 (d, 2H, J = 8.1 Hz), 7.20 (d, 2H, J = 9.0 Hz), 7.25–7.35 (m, 5H), 7.72–7.78 (m, 3H), 7.95 (d, 1H, J = 15.5 Hz), 8.26 (dd, 1H, J = 1.5, 8.1 Hz); MS (ESI): *m/z* 476 $(M+1)^+$.

4.2.18. (*E*)-3-(4-(5-Bromopentyloxy)phenyl)-2-styrylquinazolin-4(3*H*)-one (14c)

The compound **14c** was prepared following the method described for the preparation of the compound **14a,** employing **13c** (400 mg, 1 mmol) and benzaldehyde (127 mg, 1.2 mmol), and the crude product was purified by column chromatography (10% ethyl acetate–hexane) to afford the compound **14c** as a creamish white solid (396 mg, 81%). Mp 128–130 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.65–1.71 (m, 2H), 1.84–1.92 (m, 2H), 1.93–2.03 (m, 2H), 3.43 (t, 2H, J = 6.5 Hz), 4.06 (t, 2H, J = 6.5 Hz), 6.43 (d, 1H, J = 15.5 Hz), 7.03 (d, 2H, J = 8.1 Hz), 7.20 (d, 2H, J = 9.0 Hz), 7.25–7.35 (m, 5H), 7.72–7.78 (m, 3H), 7.95 (d, 1H, J = 15.5 Hz), 8.27 (dd, 1H, J = 1.5, 8.1 Hz); MS (ESI): *m/z* 490 $(M+1)^+$.

4.2.19. Synthesis of (2*S*)-*N*-{4-{5-[3-(4-fluorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (16a)

To a solution of (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl)-pyrrolidine-2-carboxaldehyde diethyl thioacetal **15** (400 mg, 1 mmol) in acetone (10 mL) anhydrous K_2CO_3 (552 mg, 4 mmol) and the compound **9a** (419 mg, 1 mmol) were added. The reaction mixture was heated to reflux for 4–6 h. After completion of the reaction as indicated by TLC, potassium carbonate was removed by suction filtration and the solvent was removed under vacuum. The crude product thus obtained was purified by column chromatography (50% ethyl acetate–hexane) to afford the pure compound **16a** as a yellow solid (590 mg, 80%). Mp 120–122 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31–1.39 (m, 6H), 1.68–1.78 (m, 2H), 1.79–2.16 (m, 8H), 2.13 (s, 3H), 2.69–2.86 (m, 4H), 3.19–3.32 (m, 2H), 3.87 (s, 3H), 4.00–4.10 (m, 4H), 4.54–4.66 (m, 1H), 4.77 (d, 1H, J = 3.6 Hz), 6.72 (s, 1H), 7.17 (d, 4H, J = 6.7 Hz), 7.25 (dd, 1H, J = 2.6, 9.2 Hz), 7.45–7.53 (m, 1H), 7.56 (s, 1H), 7.92 (s, 1H); MS (ESI): *m/z* 740 $(M+1)^+$.

4.2.20. (2*S*)-*N*-{4-{5-[3-(3-chloro-4-fluorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2 carboxaldehyde diethyl thioacetal (16b)

This compound was prepared according to the method described for compound **16a** employing compound **15** (400 mg, 1 mmol) and compound **9b** (454 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **16b** as a yellow solid (633 mg, 82%). Mp 93–95 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31–1.39 (m, 6H), 1.68–1.78 (m, 2H), 1.79–2.16 (m, 8H), 2.24 (s, 3H), 2.67–2.86 (m, 4H), 3.19–3.32 (m, 2H), 3.94 (s, 3H), 4.09–4.16 (m, 4H), 4.67–4.73 (m, 1H), 4.86 (d, 1H, J = 3.6 Hz), 6.81 (s, 1H), 7.15–7.20

(m, 1H), 7.31–7.39 (m, 3H), 7.56–7.61 (m, 2H), 7.65 (s, 1H); MS (ESI): *m/z* 774 $(M+1)^+$.

4.2.21. (2*S*)-*N*-{4-{4-[3-(2,4-Dichlorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazoli nyl]oxybutyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (16c)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **9c** (456 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **16c** as a yellow solid (620 mg, 80%). Mp 99–101 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.67–1.78 (m, 2H), 1.79–2.13 (m, 6H), 2.17 (s, 3H), 2.65–2.86 (m, 4H), 3.18–3.26 (m, 2H), 3.92 (s, 3H), 4.13–4.24 (m, 4H), 4.62–4.71 (m, 1H), 4.83 (d, 1H, *J* = 3.7 Hz), 6.75 (s, 1H), 7.27 (d, 1H, *J* = 8.4 Hz), 7.32 (dd, 1H, *J* = 2.6, 8.6 Hz), 7.45 (dd, 1H, *J* = 2.2, 8.4 Hz), 7.54–7.59 (m, 2H), 7.62 (s, 1H), 7.64 (d, 1H, *J* = 2.2 Hz); MS (ESI): *m/z* 776 $(M+1)^+$.

4.2.22. (2*S*)-*N*-{4-{4-[3-(4-Iodo-2-methylphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxybutyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthio acetal (16d)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **9d** (527 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **16d** as a yellow solid (677 mg, 80%). Mp 88–90 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.79–2.16 (m, 8H), 2.15 (s, 3H), 2.08 (s, 3H), 2.67–2.86 (m, 4H), 3.18–3.30 (m, 2H), 3.93 (s, 3H), 4.14–4.22 (m, 4H), 4.65–4.72 (m, 1H), 4.85 (d, 1H, *J* = 3.7 Hz), 6.75 (s, 1H), 6.89 (d, 1H, *J* = 8.1 Hz), 7.32 (dd, 1H, *J* = 2.1, 8.4 Hz), 7.51–7.65 (m, 3H), 7.70 (d, 1H, *J* = 8.1 Hz), 7.76 (s, 1H); MS (ESI): *m/z* 847 $(M+1)^+$.

4.2.23. (2*S*)-*N*-{4-{5-[3-(4-Iodo-2-methylphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthio acetal (16e)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **9e** (541 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **16e** as a yellow solid (696 mg, 81%). Mp 93–95 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.67–1.78 (m, 2H), 1.79–2.16 (m, 8H), 2.08 (s, 3H), 2.15 (s, 3H), 2.67–2.86 (m, 4H), 3.18–3.30 (m, 2H), 3.93 (s, 3H), 4.14–4.22 (m, 4H), 4.65–4.72 (m, 1H), 4.85 (d, 1H, *J* = 3.7 Hz), 6.75 (s, 1H), 6.89 (d, 1H, *J* = 8.1 Hz), 7.32 (dd, 1H, *J* = 2.1, 8.4 Hz), 7.51–7.65 (m, 3H), 7.70 (d, 1H, *J* = 8.1 Hz), 7.76 (s, 1H); MS (ESI): *m/z* 861 $(M+1)^+$.

4.2.24. (2*S*)-*N*-{4-{5-[3-(4-Methoxyphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolin yl]oxypentyloxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (16f)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **9f** (431 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **16f** as a yellow solid (638 mg, 85%). Mp 75–78 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31–1.39 (m, 6H), 1.67–1.77 (m, 2H), 1.89–2.16 (m, 8H), 2.23 (s, 3H), 2.67–2.86 (m, 4H), 3.20–3.32 (m, 2H), 3.88 (s, 3H), 3.94 (s, 3H), 4.08–4.16 (m, 4H), 4.67–4.73 (m, 1H), 4.87 (d, 1H, *J* = 3.7 Hz), 6.81 (s, 1H), 7.05 (d, 2H, *J* = 8.8 Hz), 7.16 (d, 2H, *J* = 8.8 Hz), 7.34 (dd, 1H, *J* = 3.3, 8.8 Hz), 7.58 (d, 1H, *J* = 8.8 Hz), 7.60 (s, 1H), 7.66 (s, 1H); MS (ESI): *m/z* 752 $(M+1)^+$.

4.2.25. Synthesis of (2*S*)-*N*-{4-[3-[4-(2-methyl-4-oxo-3,4-dihydro-3-quinazolinyl) phenoxy]propoxy}-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehyde diethylthioacetal (17a)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and the compound **13a** (373 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the pure compound **17a** as a yellow solid (567 mg, 82%). Mp 72–74 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.72–1.79 (m, 2H), 1.88–2.03 (m, 4H), 2.27 (s, 3H), 2.67–2.90 (m, 4H), 3.21–3.31 (m, 2H), 3.96 (s, 3H), 4.03–4.18 (m, 4H), 4.67–4.76 (m, 1H), 4.89 (d, 1H, *J* = 3.7 Hz), 6.84 (s, 1H), 7.04 (d, 2H, *J* = 8.3 Hz), 7.17 (d, 2H, *J* = 7.5 Hz), 7.39–7.49 (m, 1H), 7.62 (d, 1H, *J* = 8.30 Hz), 7.65 (s, 1H), 7.69–7.78 (m, 1H), 8.22 (d, 1H, *J* = 7.5 Hz); MS (ESI): *m/z* 693.91 $(M+1)^+$.

4.2.26. (2*S*)-*N*-{4-[4-[4-(2-Methyl-4-oxo-3,4-dihydro-3-quinazolin-yl)phenoxy]butyl oxy}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehydediethylthioacetal (17b)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **13b** (387 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **17b** as a yellow solid (564 mg, 80%). Mp 76–79 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.66–2.16 (m, 8H), 2.27 (s, 3H), 2.67–2.90 (m, 4H), 3.21–3.31 (m, 2H), 3.96 (s, 3H), 4.03–4.18 (m, 4H), 4.67–4.76 (m, 1H), 4.89 (d, 1H, *J* = 3.7 Hz), 6.84 (s, 1H), 7.04 (d, 2H, *J* = 8.3 Hz), 7.17 (d, 2H, *J* = 7.5 Hz), 7.39–7.49 (m, 1H), 7.62 (d, 1H, *J* = 8.30 Hz), 7.65 (s, 1H), 7.69–7.78 (m, 1H), 8.22 (d, 1H, *J* = 7.5 Hz); MS (ESI): *m/z* 707.91 $(M+1)^+$.

4.2.27. (2*S*)-*N*-{4-[5-[4-(2-Methyl-4-oxo-3,4-dihydro-3-quinazolin-yl)phenoxy] pentyl oxy}-5-methoxy-2-nitrobenzoyl}-pyrrolidine-2-carboxaldehyde diethythioacetal (17c)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **13c** (401 mg, 1 mmol). The crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **17c** as a yellow solid (612 mg, 85%). Mp 79–82 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.67–1.78 (m, 2H), 1.79–2.16 (m, 8H), 2.27 (s, 3H), 2.67–2.90 (m, 4H), 3.21–3.31 (m, 2H), 3.96 (s, 3H), 4.03–4.18 (m, 4H), 4.67–4.76 (m, 1H), 4.89 (d, 1H, *J* = 3.7 Hz), 6.84 (s, 1H), 7.05 (d, 2H, *J* = 9.0 Hz), 7.17 (d, 2H, *J* = 9.0 Hz), 7.43–7.50 (m, 1H), 7.66 (s, 1H), 7.68 (d, 1H, *J* = 8.3 Hz), 7.73–7.80 (m, 1H), 8.27 (d, 1H, *J* = 7.5 Hz); MS (ESI): *m/z* 721.91 $(M+1)^+$.

4.2.28. (2*S*)-*N*-{4-{3-[(4–4-Oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazoliny l)phenyl)oxy]propoxy}}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (17d)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **14a** (461 mg, 1 mmol). The crude product was purified by column chromatography (40% ethyl acetate–hexane) to afford the compound **17d** as a yellow solid (640 mg, 82%). Mp 100–102 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.72–1.79 (m, 2H), 1.88–2.03 (m, 4H), 2.65–2.86 (m, 4H), 3.18–3.29 (m, 2H), 3.92 (s, 3H), 4.09–4.25 (m, 4H), 4.62–4.71 (m, 1H), 4.83 (d, 1H, *J* = 3.7 Hz), 6.43 (d, 1H, *J* = 15.1 Hz), 6.78 (s, 1H), 7.05 (d, 2H, *J* = 9.0 Hz), 7.20 (d, 2H, *J* = 8.3 Hz), 7.27–7.33 (m, 5H), 7.40–7.47 (m, 1H), 7.64 (s, 1H), 772–7.77 (m, 2H), 7.95 (d, 1H, *J* = 15.1 Hz), 8.26 (d, 1H, *J* = 7.5 Hz); MS (ESI): *m/z* 782 $(M+1)^+$.

4.2.29. 2*S*)-*N*-{4-{4-[(4-4-Oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazolinyl)phenyl)oxy]butyloxy}}-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (17e)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **14b** (475 mg, 1 mmol). The crude product was purified by column chromatography (40% ethyl acetate–hexane) to afford the compound **17e** as a yellow solid (637 mg, 80%). Mp 102–104 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.66–2.16 (m, 8H), 2.65–2.86 (m, 4H), 3.18–3.29 (m, 2H), 3.94 (s, 3H), 4.06–4.19 (m, 4H), 4.62–4.71 (m, 1H), 4.83 (d, 1H, J = 3.7 Hz), 6.43 (d, 1H, J = 15.10 Hz), 6.78 (s, 1H), 7.05 (d, 2H, J = 9.0 Hz), 7.20 (d, 2H, J = 8.3 Hz), 7.27–7.33 (m, 5H), 7.40–7.47 (m, 1H), 7.64 (s, 1H), 7.72–7.77 (m, 2H), 7.95 (d, 1H, J = 15.1 Hz), 8.26 (d, 1H, J = 7.5 Hz); MS (ESI): *m/z* 796 $(M+1)^+$.

4.2.30. (2*S*)-*N*-{4-{5-[(4-4-Oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazoliny l) phenyl)oxy]pentyloxy}}-5-methoxy -2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethylthioacetal (17f)

This compound was prepared according to the method described for compound **16a**, employing compound **15** (400 mg, 1 mmol) and compound **14c** (489 mg, 1 mmol). The crude product was purified by column chromatography (40% ethyl acetate–hexane) to afford the compound **17f** as a yellow solid (711 mg, 88%). Mp 106–108 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.30–1.40 (m, 6H), 1.67–1.78 (m, 2H), 1.79–2.16 (m, 8H), 2.65–2.86 (m, 4H), 3.18–3.29 (m, 2H), 3.94 (s, 3H), 4.06–4.19 (m, 4H), 4.62–4.71 (m, 1H), 4.83 (d, 1H, J = 3.7 Hz), 6.43 (d, 1H, J = 15.1 Hz), 6.78 (s, 1H), 7.05 (d, 2H, J = 9.0 Hz), 7.20 (d, 2H, J = 8.3 Hz), 7.27–7.33 (m, 5H), 7.40–7.47 (m, 1H), 7.64 (s, 1H), 7.72–7.77 (m, 2H), 7.95 (d, 1H, J = 15.1 Hz), 8.26 (d, 1H, J = 7.5 Hz); MS (ESI): *m/z* 810 $(M+1)^+$.

4.2.31. 7-Methoxy-8-{5-[3-(4-fluorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (4a)

To a solution of compound **16a** (739 mg, 1 mmol) in methanol (20 mL), $SnCl_2{\cdot}2H_2O$ (5 mmol) was added and refluxed for 1–2 h. The methanol 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–water (4:1) was stirred slowly at room temperature until TLC indicated complete loss of starting material (12 h). The reaction mixture was diluted with ethylacetate (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 give the final product **4a** as a white solid (390 mg, 58% yield). Mp 96–98 °C; $[\alpha]_D^{27}$ +118.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.53–1.75 (m, 4H), 1.76–2.11 (m, 6H), 2.20 (s, 3H), 3.50–3.87 (m, 3H), 3.92 (s, 3H), 4.00–4.20 (m, 4H), 6.81 (s, 1H), 7.17–7.40 (m, 6H), 7.46–7.70 (m, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.5, 24.0, 28.6, 28.7, 29.5, 46.6, 53.6, 56.1, 68.2, 68.7, 107.1, 110.3, 110.5, 116.9, 117.1, 120.0, 121.2, 125.0, 128.3, 129.8, 129.8, 133.7, 140.5, 141.7, 147.7, 150.7, 151.5, 157.7, 161.4, 162.1, 162.3, 163.9, 164.6; MS (ESI): *m/z* 585 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{33}H_{33}FN_4O_5$ calcd 585.2513, found 585.2498 $(M+1)^+$; Anal. Calcd for $C_{33}H_{33}FN_4O_5$: C, 67.79; H, 5.69; N, 9.58. Found: C, 67.56; H, 5.40; N, 9.34.

4.2.32. 7-Methoxy-8-{5-[3-(3-chloro-4-fluorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepine-5-one (4b)

The compound **4b** was prepared according to the method described for the compound **4a**, employing the compound **16b** (773 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **4b** as a white solid (346 mg, 56% yield). mp 95–97 °C; $[\alpha]_D^{27}$ +159.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.59–1.74 (m, 4H), 1.84–2.11 (m, 6H), 2.25 (s, 3H), 3.53–3.63 (m, 1H), 3.68–3.88 (m, 2H), 3.94 (s, 3H), 4.02–4.16 (m, 4H), 6.81 (s, 1H), 7.13–7.23 (m, 1H), 7.30–7.41 (m, 3H), 7.51 (s, 1H), 7.56–7.63 (m, 2H), 7.67 (d, 1H, J = 3.3 Hz): MS (ESI): *m/z* 619 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{33}H_{32}FClN_4O_5$ calcd 619.2123, found 619.2123 $(M+1)^+$; Anal. Calcd for $C_{33}H_{32}FClN_4O_5$: C, 64.02; H, 5.21; N, 9.05. Found: C, 63.79; H, 5.28; N, 9.22.

4.2.33. 7-Methoxy-8-{4-[3-(2,4-dichlorophenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxybutyloxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepine-5-one (4c)

The compound **4c** was prepared according to the method described for the compound **4a**, employing the compound **16c** (775 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **4c** as a white solid (341 mg, 55% yield). Mp 114–116 °C; $[\alpha]_D^{27}$ +147.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.64–1.80 (m, 2H), 1.96–2.13 (m, 6H), 2.20 (s, 3H), 3.53–3.86 (m, 3H), 3.92 (s, 3H), 4.07–4.24 (m, 4H), 6.82 (s, 1H), 7.26–7.32 (m, 1H), 7.37 (d, 1H, J = 7.8 Hz), 7.46 (d, 1H, J = 6.8 Hz), 7.51 (s, 1H), 7.61 (s, 1H), 7.62–7.68 (m, 3H); MS (ESI): *m/z* 621 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{32}H_{30}Cl_2N_4O_5$ calcd 621.1671, found 621.1650 $(M+1)^+$; Anal. Calcd for $C_{32}H_{30}Cl_2N_4O_5$: C, 61.84; H, 4.87; N, 9.01. Found: C, 61.69; H, 4.47; N, 8.85.

4.2.34. 7-Methoxy-8-{4-[3-(4-iodo-2-methylphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxybutyloxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepine-5-one (4d)

The compound **4d** was prepared according to the method described for the compound **4a**, employing the compound **16d** (846 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **4d** as a white solid (402 mg, 58% yield). Mp 98–100 °C; $[\alpha]_D^{27}$ +112.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.57–1.75 (m, 4H), 1.85–2.10 (m, 4H), 2.08 (s, 3H), 2.16 (s, 3H), 3.51–3.88 (m, 3H), 3.94 (s, 3H), 4.02–4.16 (m, 4H), 6.81 (s, 1H), 6.90 (d, 1H, J = 8.3 Hz), 7.37 (d, 1H, J = 2.4, 9.1 Hz), 7.51 (s, 1H), 7.57–7.81 (m, 5H); MS (ESI): *m/z* 693 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{33}H_{33}IN_4O_5$ calcd 693.1573, found 693.1569 $(M+1)^+$; Anal. Calcd for $C_{33}H_{33}IN_4O_5$: C, 57.23; H, 4.80; N, 8.09. Found: C, 57.39; H, 4.57; N, 7.95.

4.2.35. 7-Methoxy-8-{5-[3-(4-iodo-2-methylphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4] benzodiazepine-5-one (4e)

The compound **4e** was prepared according to the method described for the compound **4a**, employing the compound **16e** (860 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **4e** as a white solid (395 mg, 56% yield). Mp 104–106 °C; $[\alpha]_D^{27}$ +123.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.59–1.75 (m, 4H), 1.84–2.02 (m, 6H), 2.08 (s, 3H), 2.16 (s, 3H), 3.51–3.88 (m, 3H), 3.94 (s, 3H), 4.02–4.16 (m, 4H), 6.81 (s, 1H), 6.90 (d, 1H, J = 8.3 Hz), 7.37 (d, 1H, J = 2.4, 9.1 Hz), 7.51 (s, 1H), 7.57–7.81 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 17.0, 22.5, 23.5, 24.1, 28.5,

28.7, 29.5, 46.5, 53.6, 56.1, 68.2, 68.6, 95.2, 107.1, 110.3, 111.4, 120.0, 121.1, 125.1, 128.3, 129.6, 136.7, 136.8, 137.8, 140.3, 140.5, 141.9, 147.7, 150.7, 151.1, 157.7, 161.3, 162.3, 164.5; MS (ESI): *m/z* 707 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{34}H_{35}IN_4O_5$ calcd 707.1730, found 707.1720 $(M+1)^+$; Anal. Calcd for $C_{34}H_{35}IN_4O_5$: C, 57.80; H, 4.99; N, 7.93. Found: C, 57.79; H, 4.69; N, 7.36.

4.2.36. 7-Methoxy-8-{5-[3-(4-methoxyphenyl)-2-methyl-4-oxo-3,4-dihydro-6-quinazolinyl]oxypentyloxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (4f)

The compound **4f** was prepared according to the method described for the compound **4a**, employing the compound **16f** (751 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **4f** as a white solid (390 mg, 58% yield). Mp 100–102 °C; $[\alpha]_D^{27}$ +152.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.59–1.75 (m, 4H), 1.84–2.02 (m, 6H), 2.08 (s, 3H), 2.16 (s, 3H), 3.51–3.88 (m, 3H), 3.94 (s, 3H), 4.02–4.16 (m, 4H), 6.81 (s, 1H), 7.07 (d, 2H, *J* = 8.8 Hz), 7.19 (d, 2H, *J* = 8.8 Hz), 7.35 (dd, 1H, *J* = 2.4, 8.8 Hz), 7.47 (s, 1H), 7.55 (s, 1H), 7.56–7.61 (m, 1H), 7.69 (d, 1H, *J* = 4.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.6, 24.1, 28.6, 28.7, 29.5, 46.6, 53.6, 55.5, 56.1, 68.1, 68.7, 107.1, 110.3, 111.5, 115.1, 120.0, 121.4, 124.8, 128.2, 128.9, 130.4, 140.5, 141.9, 147.7, 150.7, 152.2, 157.5, 159.8, 162.3, 162.3, 164.6; MS (ESI): *m/z* 597 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{34}H_{36}N_4O_6$ calcd 597.2713; found 597.2700 $(M+1)^+$; Anal. Calcd for $C_{34}H_{36}N_4O_6$: C, 68.44; H, 6.08; N, 9.39. Found: C, 68.12; H, 6.22; N, 9.16.

4.2.37. Synthesis of 7-methoxy-8{-3-[4-(2-methyl-4-oxo-3,4-dihydro-3-quinazolinyl) phenoxy]propoxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzo diazepine-5-one (5a)

The compound **5a** was prepared according to the method described for the compound **4a**, employing the compound **17a** (693 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5a** as a white solid (312 mg, 58%). Mp 76–78 °C; $[\alpha]_D^{27}$ +135.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.84–2.09 (m, 6H), 2.26 (s, 3H), 3.70–3.87 (m, 3H), 3.95 (s, 3H), 4.01–4.10 (m, 4H), 6.82 (s, 1H), 7.03 (d, 2H, *J* = 8.8 Hz), 7.15 (d, 2H, *J* = 8.8 Hz), 7.44–7.49 (m, 1H), 7.52 (s, 1H), 7.67 (d, 2H, *J* = 6.3 Hz), 7.74–7.79 (m, 1H), 8.27 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 24.0, 24.2, 28.8, 29.4, 46.5, 53.6, 56.0, 64.4, 65.2, 110.5, 111.5, 115.3, 115.9, 120.2, 120.6, 126.4, 126.6, 126.9, 128.8, 130.1, 134.4, 140.4, 147.3, 147.7, 150.5, 154.7, 159.1, 162.4, 164.5; MS (ESI): *m/z* 539 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{31}H_{30}N_4O_5$ calcd 539.2294, found 539.2290 $(M+1)^+$; Anal. Calcd for $C_{31}H_{30}N_4O_5$: C, 69.13; H, 5.61; N, 10.40. Found: C, 68.96; H, 5.44; N, 9.93.

4.2.38. 7-Methoxy-8{-4-[4-(2-methyl-4-oxo-3,4-dihydro-3-quinazolinyl)phenoxy] butyloxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (5b)

The compound **5b** was prepared according to the method described for the compound 4a, employing the compound **17b** (706 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5b** as a white solid (303 mg, 55% yield). Mp 79–80 °C; $[\alpha]_D^{27}$ +139.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.57–1.75 (m, 4H), 1.85–2.10 (m, 4H), 2.26 (s, 3H), 3.70–3.87 (m, 3H), 3.95 (s, 3H), 4.01–4.10 (m, 4H), 6.82 (s, 1H), 7.03 (d, 2H, *J* = 8.8 Hz), 7.15 (d, 2H, *J* = 8.8 Hz), 7.44–7.49 (m, 1H), 7.52 (s, 1H), 7.67 (d, 2H, *J* = 6.3 Hz), 7.74–7.79 (m, 1H), 8.27 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 24.0, 24.2, 25.5, 25.8, 29.4, 46.5, 53.5, 55.9, 67.5, 68.3, 110.2, 111.3, 115.4, 115.5, 120.0, 120.5, 126.3, 126.5, 126.8, 128.8, 129.9, 134.3, 140.3, 147.2, 147.6, 150.5, 154.6, 159.1, 162.3, 164.4; MS (ESI): *m/z* 553 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{32}H_{32}N_4O_5$ calcd 539.2294, found 539.2290 $(M+1)^+$; Anal. Calcd for $C_{32}H_{32}N_4O_5$: C, 69.55; H, 5.84; N, 10.14. Found: C, 69.23; H, 5.62; N, 9.89.

4.2.39. 7-Methoxy-8{-5-[4-(2-methyl-4-oxo-3,4-dihydro-3-quinazolinyl)phenoxy] pentyloxy}-(11a*S*)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (5c)

The compound **5c** was prepared according to the method described for the compound **4a**, employing the compound **17c** (720 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5c** as a white solid (328 mg, 58% yield). Mp 83–85 °C; $[\alpha]_D^{27}$ +142.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.57–1.75 (m, 4H), 1.86–2.11 (m, 6H), 2.26 (s, 3H), 3.70–3.87 (m, 3H), 3.95 (s, 3H), 4.01–4.10 (m, 4H), 6.82 (s, 1H), 7.03 (d, 2H, *J* = 8.8 Hz), 7.15 (d, 2H, *J* = 8.8 Hz), 7.44–7.49 (m, 1H), 7.52 (s, 1H), 7.67 (d, 2H, *J* = 6.3 Hz), 7.74–7.79 (m, 1H), 8.27 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.5, 24.1, 24.3, 28.6, 28.8, 29.5, 46.6, 53.6, 56.1, 67.9, 68.7, 110.4, 111.5, 115.6, 120.0, 120.7, 126.4, 126.6, 127.0, 128.9, 130.0, 134.4, 140.5, 147.3, 147.7, 150.7, 154.7, 159.3, 162.3, 164.5; MS (ESI): *m/z* 567 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{33}H_{34}N_4O_5$ calcd 567.2607, found 567.2600 $(M+1)^+$; Anal. Calcd for $C_{33}H_{34}N_4O_5$: C, 69.95; H, 6.05; N, 9.89. Found: C, 69.76; H, 6.00; N, 9.52.

4.2.40. 7-Methoxy-8-{3-[(4–4-oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazolinylphenyl)oxy]propoxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzo diazepine-5-one (5d)

The compound **5d** was prepared according to the method described for the compound **4a**, employing the compound **17d** (781 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5d** as a white solid (344 mg, 55% yield). Mp 74–76 °C; $[\alpha]_D^{27}$ +158.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.87–2.14 (m, 6H), 3.70–3.87 (m, 3H), 3.96 (s, 3H), 4.04–4.20 (m, 4H), 6.47 (d, 1H, *J* = 15.3 Hz), 6.89 (s, 1H), 7.09 (d, 2H, *J* = 8.0 Hz), 7.21 (d, 2H, *J* = 8.0 Hz), 7.28–7.38 (m, 5H), 7.46 (d, 1H, *J* = 8.0 Hz), 7.53 (s, 1H), 7.67 (d, 1H, *J* = 5.1 Hz), 7.78 (d, 2H, *J* = 3.6 Hz), 7.97 (d, 1H, *J* = 15.3 Hz), 8.28 (d, 1H, *J* = 6.8 Hz); MS (ESI): *m/z* 627 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{38}H_{34}N_4O_5$ calcd 627.2607, found 627.2600 $(M+1)^+$; Anal. Calcd for $C_{38}H_{34}N_4O_5$: C, 72.83; H, 5.47; N, 8.94. Found: C, 72.57; H, 5.19; N, 8.78.

4.2.41. 7-Methoxy-8-{4-[(4–4-oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazolinylphenyl)oxy]butyloxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (5e)

The compound **5e** was prepared according to the method described for the compound **4a**, employing the compound **17e** (795 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5e** as a white solid (358 mg, 56% yield). Mp 76–78 °C; $[\alpha]_D^{27}$ +171.5 (*c* = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.57–1.75 (m, 4H), 1.85–2.10 (m, 4H), 3.70–3.87 (m, 3H), 3.95 (s, 3H), 4.04–4.20 (m, 4H), 6.47 (d, 1H, *J* = 15.3 Hz), 6.89 (s, 1H), 7.09 (d, 2H, *J* = 8.0 Hz), 7.21 (d, 2H, *J* = 8.0 Hz), 7.28–7.38 (m, 5H), 7.47 (d, 1H, *J* = 8.0 Hz), 7.53 (s, 1H), 7.67 (d, 1H, *J* = 5.1 Hz), 7.78 (d, 2H, *J* = 3.6 Hz), 7.99 (d, 1H, *J* = 15.3 Hz), 8.30 (d, 1H, *J* = 6.8 Hz); MS (ESI): *m/z* 641 $(M+1)^+$; HRMS (ESI *m/z*) for $C_{39}H_{36}N_4O_5$ calcd 641.2742, found 641.2734 $(M+1)^+$; Anal. Calcd for $C_{39}H_{36}N_4O_5$: C, 73.11; H, 5.66; N, 8.74. Found: C, 72.89; H, 5.39; N, 8.51.

4.2.42. 7-Methoxy-8-{5-[(4-4-oxo-2-[(*E*)-2-phenyl-1-ethenyl]-3,4-dihydro-3-quinazolinylphenyl)oxy]pentyloxy}-(11a*S*)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepine-5-one (5f)

The compound **5f** was prepared according to the method described for the compound **4a**, employing the compound **17f**

(809 mg, 1.0 mmol). The crude product was purified by column chromatography (MeOH/$CHCl_3$, 2%) to afford the compound **5f** as a white solid (372 mg, 57% yield). Mp 78–80 °C; $[\alpha]_D^{27}$ +162.5 (c = 0.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.61–1.82 (m, 4H), 1.87–2.14 (m, 6H), 3.70–3.87 (m, 3H), 3.96 (s, 3H) 4.04–4.20 (m, 4H), 6.47 (d, 1H, J = 15.3 Hz), 6.89 (s, 1H), 7.08 (d, 2H, J = 8.7 Hz), 7.22 (d, 2H, J = 8.0 Hz), 7.30–7.42 (m, 5H), 7.48 (dd, 1H, J = 3.6, 8.0 Hz), 7.53 (s, 1H), 7.69 (d, 1H, J = 5.1 Hz), 7.80 (d, 2H, J = 3.6 Hz), 7.98 (d, 1H, J = 15.3 Hz), 8.31 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 22.5, 24.0, 28.5, 28.8, 29.4, 46.5, 53.5, 56.0, 67.9, 68.6, 110.3, 111.4, 115.4, 115.5, 119.8, 120.0, 120.7, 126.3, 126.9, 127.1, 127.6, 128.6, 129.1, 129.4, 134.3, 135.2, 139.6, 140.4, 147.6, 150.6, 151.9, 159.2, 162.2, 162.4, 164.4; MS (ESI): m/z 655 $(M+1)^+$; HRMS (ESI m/z) for $C_{40}H_{38}N_4O_5$ calcd 655.2920, found 655.2899 $(M+1)^+$; Anal. Calcd for $C_{40}H_{38}N_4O_5$: C, 73.38; H, 5.85; N, 8.56. Found: C, 73.53; H, 6.08; N, 8.72.

4.3. Thermal denaturation studies

The compounds **4a–f** and **5a–f** were subjected to DNA thermal melting (denaturation) studies using duplex form calf thymus DNA (CT-DNA) using modification reported procedure.[49] Working solutions were produced by appropriate dilution in aqueous buffer (10 mM NaH_2PO_4/NaH_2PO_4, 1 mM Na_2EDTA, pH 7.00+0.01) containing CT-DNA, (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in methanol to obtain a fixed [PBD]/[DNA] molar ratio of 1:5 The DNA–PBD solutions were incubated at 37 °C for 0 h prior to analysis sample were monitored a 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller. Heating was applied at a rate of 1 °C min^{-1} in the 40–90 °C range. DNA helix-coil transition temperatures (T_m) were determined from the maxima in the d (A260)/dT derivative plots. Results for each compound are shown as mean ± standard derivation from the least three determinations and are corrected for the effects of methanol co solvent using a linear correction term. Ligand-induced alteration in DNA melting behavior are given by $\Delta T_m = T_m$ (DNA + PBD) − T_m (DNA alone), where the T_m value for the PBD free CT-DNA is 68.5 ± 0.001 the fixed [PBD]/[DNA] ratio used did not result in binding saturation of the host DNA duplex for any compound examined.

4.4. Molecular modeling studies

SYBYL 6.9.2 program (Tripos Inc., St. Louis, MO) was used for drawing the quinazolinone derivatives. All the compounds were minimized to 0.001 k cal mol^{-1}/Å root-mean-square gradient by using Gasteiger–Hückel partial atomic charges and Tripos force field. Radius (30 Å) from the middle atom of DNA was used as a central atom to scan the entire DNA sequences in molecular docking. Our ongoing studies indicate that the GOLD docking on DNA sequences is the best docking protocol and can reproduce the crystallographic poses of DNA–ligand complex. Thus we used GOLD 3.2 program for docking of this series of compounds. The default parameter in GOLD 3.2 (number of islands 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1, and the van der Waals and hydrogen bonding were set to 4.0 and 2.5, respectively) was used. The 10 best conformations were generated for each compound. The final docked conformation of DNA (5′-CGCAGAAAATTTCTGCG3′)–ligand complexes from the GOLD docking were opted as an input for MD simulations. The 'leaprc.gaff' (generalized AMBER force filed) was used to prepare the ligands while 'leaprc.ff03' was used for DNA. The final input files were created by merging DNA and ligand in a complex as docked pose. The 'addions' command implemented in 'xleap' of AMBER 8.0 was used to add the 32 Na^+ ions explicitly to neutralize the system. The 'solvateoct' command was used to solvate the complex in a 10 Å water box with TIP3P water. Equilibration of the solvated complex was done by carrying out a short minimization (500 steps of each steepest descent and conjugate gradient method), 50 ps of heating and 50 ps of density equilibration with weak restraints on the complex followed by 500 ps of constant pressure equilibration at 300 K. The final production run was performed for the 5 ns and the coordinates were recorded in every 10 ps. Before submitting for the MM-PBSA production run we verified that the system has equilibrated. We extracted 500 snapshots from production runs by using 'extract_coords.mmpbsa' script and calculated the interaction energies by using 'binding_energy.mmpbsa' script. Both the scripts are available on AMBER web-site. The final reported binding energies are the average of all the 500 snapshots.

4.5. In vitro evaluation of cytotoxic activity

The synthesized compounds **4a–f** and **5a–f** 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, DWD, GURAV), 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 mM L-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.

4.6. 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 Penicillin 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.

4.7. MTT cell proliferation assay

A375 cell lines were seeded in a 96-well plate at a cell density of 10,000 cells/well. After overnight (O/N) incubation, the PBD conjugates (**4b** and **5c**) were added to the culture media and incubated for 24 h. The cytotoxicity was assayed by 3-(4,5-dimethyl-2-thiozolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) dye uptake. The

cell lines (A375) were incubated at 37 °C for 2 h with MTT at 10% (i.e., 30 μL) of the culture volume. After incubation period, cultures were removed from incubator and dissolved the resulting formazan by adding 100 μL of extraction buffer (20% SDS, 50% dimethyl formamide). After overnight incubation at 37 °C, the absorbance was measured at 570 nm using Multimode VarioskanFlash (Thermo Fisher Scientifics) with medium as blank.

4.8. Cell cycle analysis

5×10^5 Cells each of A375 cell lines were seeded in 60 mm dish and were allowed to grow for 24 h. Various concentrations (0–4 μM) of **4b** and **5c** were added to the culture media, and the cells were incubated for an additional 24 h. Cells were harvested 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 flowcytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

4.9. Protein extraction and western blot analysis

Total cell lysates from cultured A375 cells were obtained by lysing the cells in ice-cold RIPA buffer (1XPBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and containing 100 μg/mL PMSF, 5 μg/mL Aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin and 100 μg/mL NaF. After centrifugation at 12,000 rpm for 10 min, the protein in supernatant was quantified by Bradford method (BIO-RAD) using Multimode varioskan instrument (Thermo-Fischer Scientifics). Thirty micrograms of protein per lane was applied in 12% SDS–polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked at room temperature for 2 h in TBS + 0.1% Tween20 (TBST) containing 5% blocking powder (Santacruz). The membrane was washed with TBST for 5 min, and primary antibody was added and incubated at 4 °C overnight (O/N). Mouse monoclonal antibodies against p53 (1:100); Goat polyclonal β-tubulin (1:100), Rabbit p27 (c-19) (1:500) and rabbit polyclonal cdk-2 (M2) (1:500) were purchased from Santacruz, CA. NF-κB (1:500), cytochrome c (1:500), rabbit polyclonal β-tubulin (1:500), cleaved/active caspase-3 (1:200) and β-actin (1:500) antibodies were purchased from Imgenex, USA. Rabbit polyclonal PARP (1:2000) was purchased from Roche Scientifics. 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.

4.10. RNA isolation and quantitative RT-PCR

RNA isolation was carried out by trizol method (Invitrogen) according to the manufacturer's recommendation. C DNA synthesis was carried out using Invitrogen Super Script II (first strand synthesis kit). Second strand synthesis was done by carrying out PCR with gene specific primers and GAPDH was used as internal control. Bcl-2 primer sequence FP-5′CTGTGGATGACTGAGTACCT3′ and RP-5′GAGACAGCCAGGAGA AATCA3′ p21 primer sequence FP-5′ CACCGAGACACCACTGGAGG3′ and RP-5′GAGAAGATCAGCCGGC GTTT3′. The amplified PCR product was visualized on a 1% agarose gel stained with ethidium bromide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.015.

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