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Quinazolino linked 4β-amidopodophyllotoxin conjugates regulate angiogenic pathway and control breast cancer cell proliferation

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ABSTRACT

A series of new conjugates of quinazolino linked 4β-amidopodophyllotoxins **10aa–af** and **10ba–bf** were synthesized and evaluated for their anticancer activity against human pancreatic carcinoma (Panc-1) as well as breast cancer cell lines such as MCF-7 and MDA-MB-231 by employing MTT assay. Among these conjugates, some of them like **10bc**, **10bd**, **10be** and **10bf** exhibited high potency of cytotoxicity. Flow cytometric analysis showed that these conjugates arrested the cell cycle in the G2/M phase and caused the increase in expression of p53 and cyclin B1 protein with concomitant decrease in Cdk1 thereby suggesting the inhibitory action of these conjugates on mitosis. Interestingly, we observed a decrease in expression of proteins that control the tumor micro environment such as VEGF-A, STAT-3, ERK1/2, ERK-p, AKT-1 ser 473 phosphorylation in compounds treated breast cancer cells. Further, these effective conjugates have exhibited inhibitory action on integrin (αVβIII). Furthermore, the MCF-7 cells that were arrested and lost the proliferative capacity undergo mitochondrial mediated apoptosis by activation of caspases-9. Thus these conjugates have the potential to control breast cancer cell growth by effecting tumor angiogenesis and invasion.

1. Introduction

Angiogenesis occurs by either sprouting or non-sprouting process. Sprouting angiogensis involves branching of new capillaries from pre-existing and occurs in lung and heart. Non-sprouting angiogensis results from enlargement, splitting and fusion of pre-existing vessels produced by the proliferation of endothelial cells within the wall of a vessel. The transition between latent phase to the invasive and metastatic phase of cancer is linked to angiogenic switch. VEGF-A (VEGF) represents a critical inducer of tumour angiogenesis and is the first target of choice for anti-angiogenic therapies tested in clinical trials.[1] VEGF production is characteristic of solid tumours such as gliomas, breast and prostate cancer.[2]

Etoposide (**2**) and teniposide (**3**) are semisynthetic derivatives of podophyllotoxin (**1**), a bioactive component of *Podophyllum peltatum L*[3,4] (Fig. 1). Etoposide remains one of the most extensively used antitumor agents in clinical use for the treatment of a variety of malignancies.[5,6] In contrast to the parent podophyllotoxin, these semi-synthetic derivatives etoposide (**2**), and teniposide (**3**) interestingly differ substantially in their mechanism of action. Etoposide and other derivatives are DNA topoisomerase II inhibitors, they induce cell death by enhancing the topoisomerase II-mediated DNA cleavage through the stabilization of the transient DNA/topoisomerase II cleavage complex. In such a complex, DNA is cleaved on both strands and covalently linked to the enzyme, the topoisomerase II poison prevents it from dissociating[7] while podophyllotoxin inhibits the assembly in the microtubulin.[8] Recently, it has been suggested that etoposide–topoisomerase II interactions mediate cleavage complex stabilization, rather than etoposide–DNA, as is the case with amsacrine, another potent topoisomerase II inhibitor.[9] Despite its extensive use, etoposide is not devoid of toxic side effects, it presents several limitations such as moderate potency, poor water solubility, development of drug resistance, metabolic inactivation, and toxic effects.[10] Therefore, the structure of etoposide has been extensively modified, thus increasing the information about its structure–activity relationships. The development of etopophos (**4**) has addressed the aspect of bioavailability. The most important modification is that of the substituent in the 4β-position which has led to potent inhibitors of topoisomerase II. The replacement of C-4 sugar unit of etoposide with a heteroatom (O, N, or S) linked moieties helped in overcoming the problem of drug resistance to etoposide.[11]

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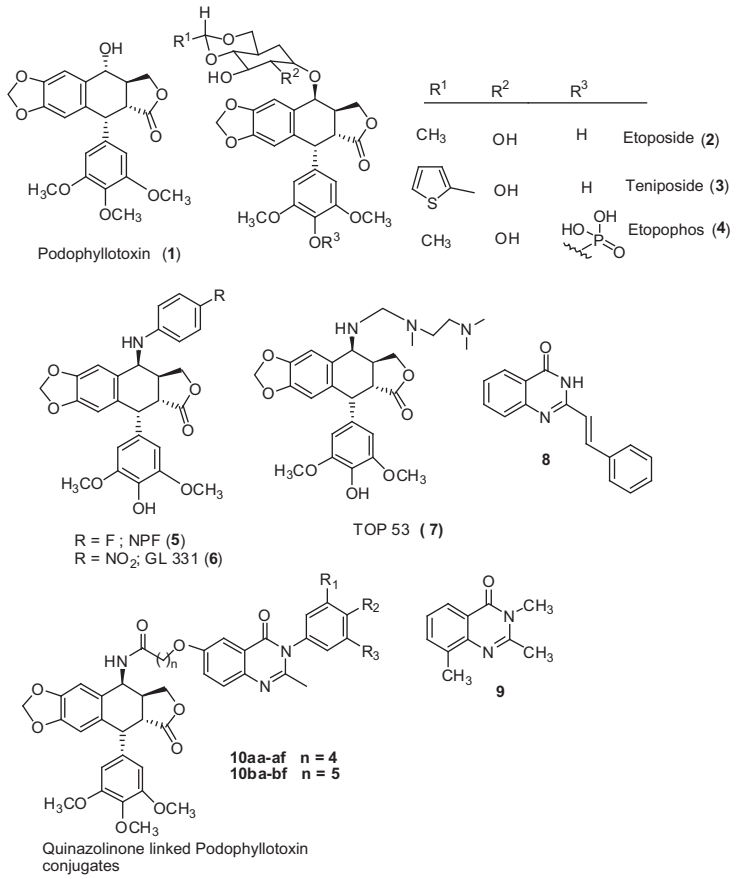


Figure 1.

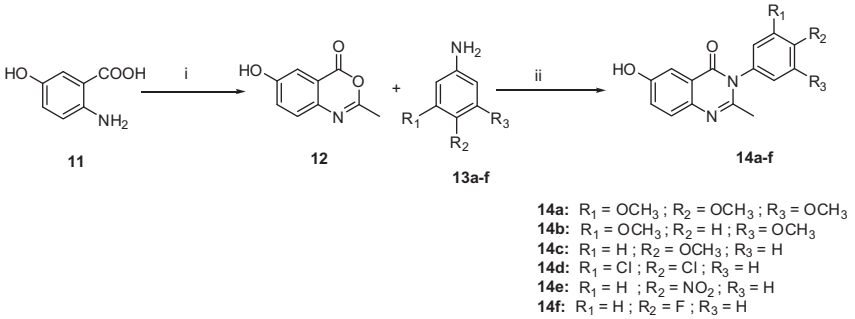
Prior molecular area-oriented structure–activity relationship (SAR) studies[12] and the composite pharmacophore model proposed by MacDonald et al.[13] designated the C-4 molecular area of **1** analogs as a variable region. The comparative molecular field analysis (CoMFA) models generated by Lee and co-workers[14,15] further demonstrated that bulky substituents at C-4 might be favorable for DNA topo-II inhibition. These postulates are compatible with the excellent activity profiles of NK 611 (5), TOP-53 (6), and GL-331.[16] In addition, both GL-331 and TOP-53 showed enhanced DNA topo-II inhibition and antitumor potential and interestingly the drug-resistance profiles were significantly different from those of **1**. This suggests the important role of different substitution at C4 with respect to the activity profiles in such analogs and the feasibility of optimizing this class of compounds through rational modifications at C4 position.[17]

Quinazolinone is a naturally occurring alkaloid and is found in a variety of bioactive natural products. The quinazolinone moiety has been utilized extensively as drug-like scaffold in medicinal chemistry and is considered to be a privileged structure[18,19] that shows broad spectrum of pharmacological activities, such as antifungal,[20] antibacterial,[21,22] antimalarial,[23] anti-inflammatory,[24] anticonvulsant,[25] antihypertensive[26] and anticancer activities.[27,28] 2-Styryl quinazolinone (**8**) derivatives also form an important component of pharmacologically active compounds because they are associated with inhibitory effects on tubulin polymerization,[28–30] as shown in Figure 1. Poly(ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme which is involved in a number of cellular processes like DNA repair and programmed cell death. 2-Methyl quinazolinone (**9**) is known to inhibit this DNA repair enzyme poly(ADP-ribose) polymerase (PARP).[31] Therapeutic agents containing the quinazolinone core structure are in the clinic and as well undergoing clinical trials for the treatment of cancer (Fig. 1).

In an ongoing effort to develop more potent anticancer agents, we have been involved in the development of new synthetic strategies[32] for the podophyllotoxin-based compounds and synthesis of new class of podophyllotoxin congeners as potential anticancer agents.[33,34] We envisaged that single molecule containing more than one pharmacophore, each with different mode of action could be beneficial for the treatment of cancer. In this connection, we have synthesized some podophyllotoxin conjugates by linking the quinazolinone moiety to the 4β-aminopodophyllotoxin scaffold through stable alkane spacers with amide bond formation. Previously it was reported that tubulin polymerization inhibitors had the potential to inhibit the angiogenesis.[35] Podophyllotoxin and its derivatives are known to inhibit tubulin polymerization.[8] Yet, not many studies have been carried out on the role of these conjugates on angiogenesis, the key signaling pathway that controls tumor micro environment. Thus here we have investigated the action of quinazolino linked podophyllotoxin conjugates on cell cycle aspect as well as angiogensis process that regulated by VEGF and its downstream protein partners.

2. Chemistry

The preparation of the quinazolinone derivatives **14a–f** is depicted in Scheme 1. The first synthetic step involved the condensation of 5-hydroxy anthranilic acid (**11**) with acetic anhydride to afford the desired benzoxazinones (**12**) in quantitative yields.[36] After evaporation of the excess of anhydride under reduced



Scheme 1. Reagents and conditions: (i) $(CH_3CO)_2O$, 160–180 °C, 1 h; (ii) **13a–f**, CH_3COOH, 120 °C, reflux, 2 h.

pressure, the crude product was used without any further purification. Compound **12** was coupled to substituted anilines (**13**) to give compounds **14a–f**. Further the synthesis of quinazolinone linked 4β-amidopodophyllotoxin conjugates (**10aa–af** and **10ba–bf**) was carried out from the key intermediate, 4β-aminopodophyllotoxin (**15**).[32a,33a] 4β-Aminopodophyllotoxin (**15**) upon treatment with 5-bromopentanoylchloride and 6-bromohexanoylchloride gives **16a** and **16b**, then followed by etherification with various substituted quinazolinone precursors (**14a–f)** using K_2CO_3 in acetone provided the desired quinozolino linked 4β-amidopodophyllotoxin conjugates **10aa–af** and **10ba–bf** as outlined in Scheme 2. All the synthesized compounds were characterized by ^1H NMR, ^{13}C NMR and mass spectral data.

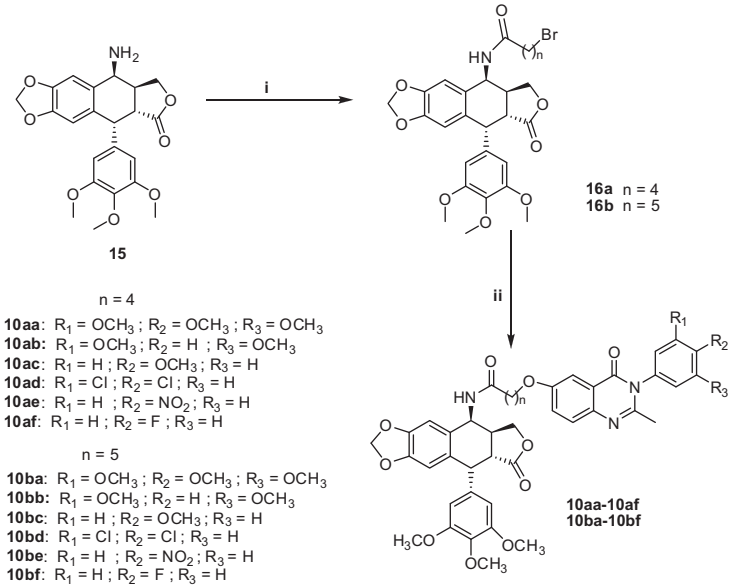
3. Biological evaluation

3.1. In vitro cytotoxicity assay

Apoptosis is the mechanism of cell death activated in mammalian cells following exposure to a wide variety of stimuli including anti-cancer agents.[37] The new series of hybrids (**10aa–10bf**) were evaluated for their cytotoxicity against three cancer cell lines such as human pancreatic carcinoma (Panc-1), human breast cancer cell lines such as MCF-7 and MDA-MB-231 by MTT assay. Amongst these conjugates, **10bc**, **10bd**, **10be** and **10bf** were found to be more potent than etoposide with regard to cytotoxicity as shown in Figure 2. MCF-7 breast cancer cells exhibit high degree of sensitivity for this series of conjugates amongst the three cancer cells.

3.2. Effect of podophyllotoxin conjugates on cell cycle

To examine the role of these conjugates in cell cycle progression of human breast cancer cells (MCF-7), the DNA content of the cell nuclei was examined by flow cytometric (FACS) analysis. MCF-7 cells were treated with 4 μM of **10aa–10bf**, using etoposide (Eto) as the positive control for 24 h. These conjugates (**10aa–10bf)** caused G2/M phase cell cycle arrest. Etoposide caused 66% cells of cells accumulated in G2/M while control DMSO treated cells have exhibited only 15% cells in G2/M phase. All the compounds were found to exhibit the G2/M phase arrest while compounds **10bc**, **10bd**, **10be** and **10bf** had shown slightly better G2/M cell cycle arrest than the other compounds tested in this series (Fig. 3). These effects were further supported by G2/M cell cycle arrest nature of many podophyllotoxin analogues such as 4′-demethyl-4-deoxy podophyllotoxin in A549 lung cancer cells,[38,39] triazole based podophyllotoxins in HepG2 cells[40] and 4DPG (4-demethyl-picropodophyllotoxin-7′-o-beta-D-glucopyranoside[4-DPG], a new podophyllotoxin isolated from rhizomes of sinopodophyllum emodi.[41] The decrease in G0/G1 percentage cells with concomitant increase in G2/M phase clearly showed G2/M cell cycle arrest.



Scheme 2. Reagents and conditions: (i) (a) 5-bromopentanoyl chloride, (b) 6-bromohexanoyl chloride, TEA, DCM, rt, 8 h; (ii) **14a–f**, K_2CO_3, acetone, reflux, 12 h.

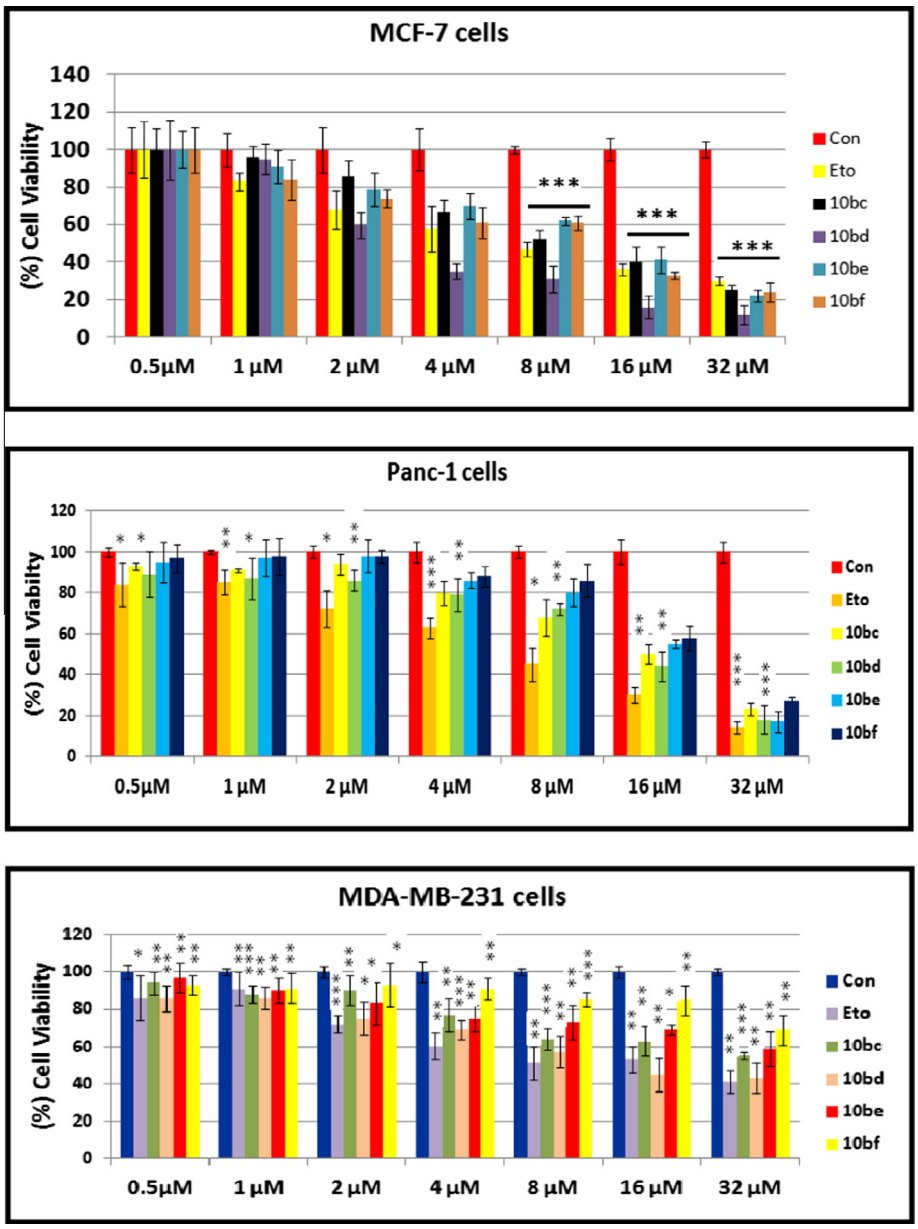


Figure 2. Effect on cell viability: MCF-7, Panc-1 and MDA-MB-231 cell viability were observed following treatment with effective conjugates (**10bc**, **10bd**, **10be** and **10bf**) for 24 h at 0.5–32 μM. 10,000 cells per well were used for this experiment. Etoposide was used as standard drug. The percentage of cell viability was plotted in the form of graph. All the experiments are carried out in triplicates. Statistical significance was assessed using student *t*-test. ***indicates $p<0.001$, **indicates $p<0.01$, *indicates $p<0.05$.

3.3. Effect of conjugates on angiogenic signalling

Vascular endothelial growth factor also known as vascular permeability factor has been identified as a key mediator of tumour angiogenesis.[50] In this regard we have examined the effect of conjugates on angiogenesis. Here human umbelical endothelial vein cell (HUVEC) were seeded on matrigel having all growth factors including VEGF-A and allowed to form tube formation and were treated with conjugates (etoposide, **10bc**, **10bd**, **10be** and **10bf**) for 6 h. We observed the inhibition tube formation with these conjugates with **10bd** and **10bf** being the most effective (Fig. 4a). We have also tested the effect on cell viability in HUVEC cells by these molecules at 1 μM concentration for 6 h. Interestingly conjugates did not cause any considerable change in cell viability (Fig. 4b). Constitutive and elevated levels of STAT-3 were observed in more than 80% of human pancreatic cancer cell lines. Moreover, STAT-3 activation correlated with the VEGF expresssion levels.[2] The transcription factors STAT1 and STAT3 appear to play opposite roles in tumorigenesis. Recent studies have emphasized the involvement of ERK (extracellular regulated kinase) and AKT-1, STAT-3 and their role in endothelial cell proliferation and are the most important downstream targets of angiogenesis.[48] Hence the conjugates **10bc**, **10bd**, **10be** and **10bf** were treated with MCF-7 cell lines at 4 μM for 24 h. We observed the decrease in protein levels of VEGF-A, ERK1/2, ERK1/2-phospho, STAT-3, AKT-1 ser 473 phosphorylation and the increased level of STAT-1. No detectable change was observed in AKT-1 protein level indicating the active form of AKT-1 was affected moderately. These conjugates affected the VEGF and its related proteins and thus these conjugates might have potential to decrease the tumor vasculature in breast cancer

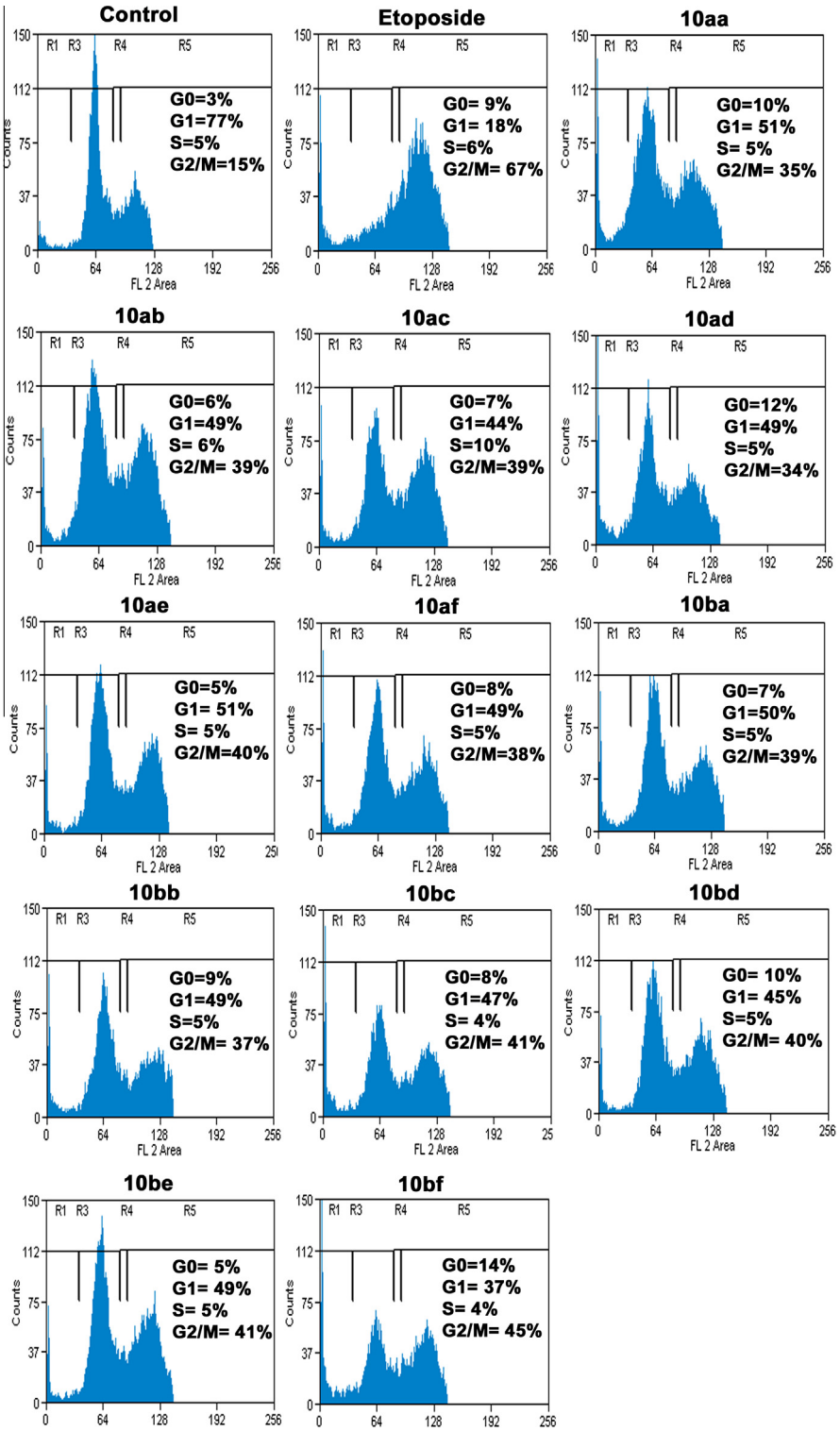


Figure 3. Effect of quinazolinone podophyllotoxin conjugates on cell cycle. MCF-7 cells were treated with compounds **10aa**–**10af** and **10ba**–**10bf** at 4 μM concentration for 24 h. Etoposide (Eto) as used as positive control. Conjugates treatment caused large accumulation of cells at G2/M phase of cell cycle with concomitant decrease of G1 phase cells.

as shown in Figure 4c.

3.4. Effect of conjugates on cell cycle regulators and apoptotic proteins

In general p53 mediates either apoptosis or cell cycle arrest in response to DNA damage, thus acting as a molecular guardian of the genome.[42–44] In addition p53 blockage of cells at the G2 checkpoint involves the inhibition of cdc2, the cyclin dependent kinases required for entry into mitosis. Cdc2 is inhibited by three transcriptional targets such as p53, Gadd45, p21 and 14-3-3 sigma. Moreover central regulators of progression from G2 to mitosis are B-type cyclins complexed with Cdc2 (i.e, Cdk1). The binding of cdc2 to cyclin B1 is required for cyclin B1 activity.[45,46] Treatment

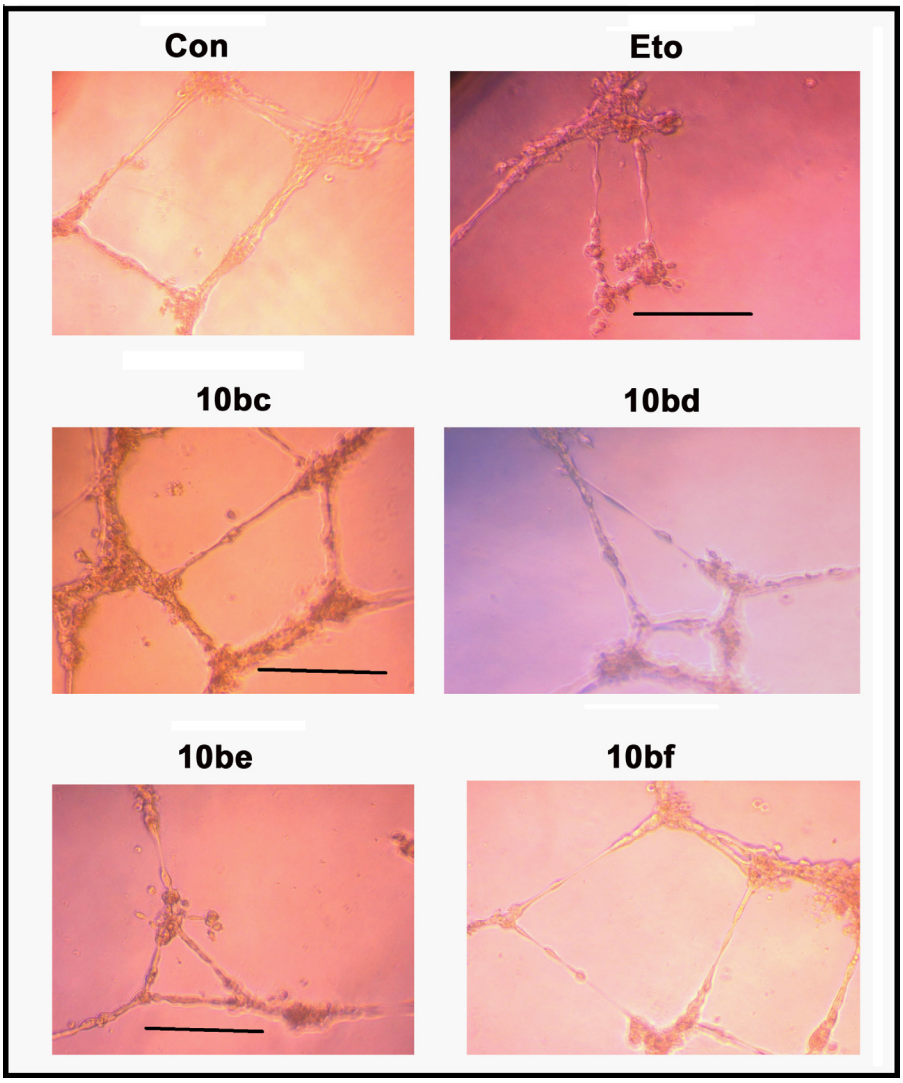


Figure 4a. The HUVEC cells were spread on matrigel and allowed to form tube like structure formation (tubulogenesis) for 6 h and were followed by compound treatment (Etoposide, **10bc**, **10bd**, **10be** and **10bf** at 1 μM for another 6 h. The effect of conjugates on endothelial cell tube formation were examined by microscopy and bar indicates 20 μM.

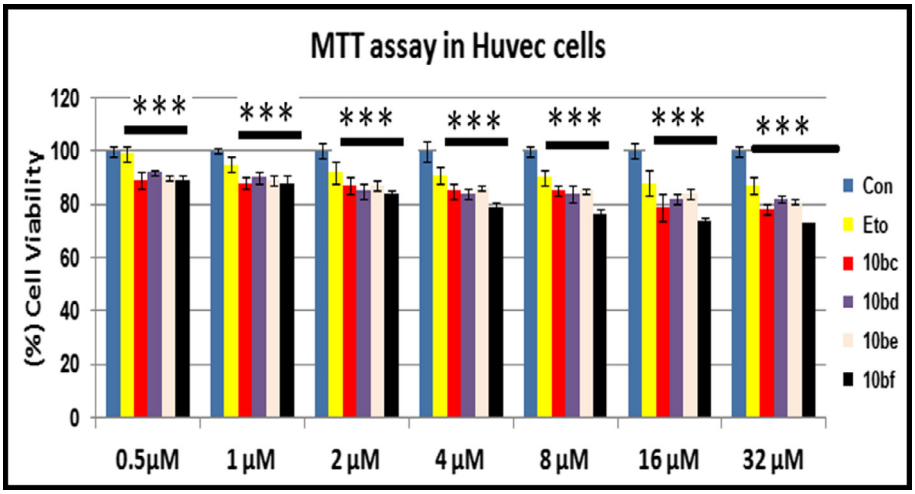


Figure 4b. Effect on cell viability: HUVEC cell viability was examined following treatment with effective conjugates (**10bc**, **10bd**, **10be** and **10bf**) for 6 h at 0.5–32 μM. 10,000 cells per well were used for this experiment. Etoposide was used as standard drug. The percentage of cell viability was plotted in the form of graph. All the experiments are carried out in triplicates. Statistical significance was assessed using student *t*-test. ***Indicates $p<0.001$.

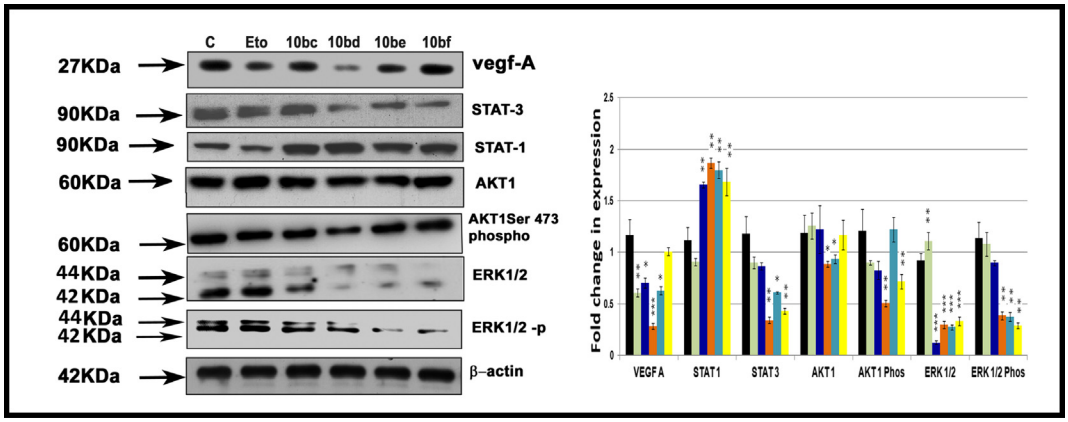


Figure 4c. Effect of conjugates on angiogenic signalling: MCF-7 cells were treated with effective compounds (**10bc**, **10bd**, **10be** and **10bf**) and Etoposide (Eto) at 4 μM concentration for 24 h. The lysates were subjected to Western blot for VEGF-A, STAT-3, STAT-1, AKT1, AKT-1 ser 473 phosphorylation, ERK1/2, ERK 1/2 phosphorylation. Here β-actin was used as loading control. All the experiments was carried out in triplicates. Statistical significance was assessed using student *t*-test. ***indicates $p<0.001$, **indicates $p<0.01$, *indicates $p<0.05$.

of MCF-7 cells with effective compounds (**10bc**, **10bd**, **10be** and **10bf**) for 24 h at 4 μM concentration lead to increase in expression of p53 and cyclin B1 protein with concomitant decrease in Cdk1. The change in protein expression was observed. These results revealed the inhibitory action of these conjugates on mitosis (Fig. 5). Apoptotic signalling pathways are induced by caspases which then cleave many other protein substrates resulting in cell death. Among all caspases, caspase-3, -6 and -7 function as effectors of cell death, whereas caspase-2, -8 and -9 act as initiator of apoptotic pathways.[47] As MCF-7 cells do not contain caspase-3, the role of caspase-9 has been examined as a function of apoptosis. An increase in caspase-9 level was observed in these conjugates wherein etoposide was employed as standard (Fig. 5). Further it is also reported that etoposide predominantly targets the nucleus of the cancer cell and stimulates the release of caspase-sensitive factors that interact with mitochondria to trigger cytochrome-c release in Jurkat-T-lymphocyte.[48] Therefore the possible role of these conjugates towards the release of cytochrome-c from mitochondria in to cytosol has been examined. After the extraction of cytosolic as well as mitochondrial fraction the cytosolic fraction was analysed by Western blot analysis. As expected, an increase of cytochrome-c expression in the cytosol was observed that reveal the induction of apoptosis by these conjugates as shown in Figure 5.

3.5. Effect of conjugates on mitochondrial membrane potential

Mitochondria is a vital organelle in the propagation and dissipation of mitochondrial electrochemical potential gradient.[49] To investigate the effect of these quinazolino linked podophyllotoxin on MMP in breast cancer cells, we have performed a functional assay to estimate MMP using JC-1 dye. In healthy cells with high mitochondrial $\Delta\psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low $\Delta\psi m$, JC-1 remains in the monomeric form, which shows only green fluorescence. Here control cells have exhibited high red fluorescence intensity whereas in cells treated with (Eto, **10bd** and **10bf**) conjugates decrease in red fluorescence intensity (J-aggregates) with concomitant increase in green fluorescence intensity (J-monomers) was

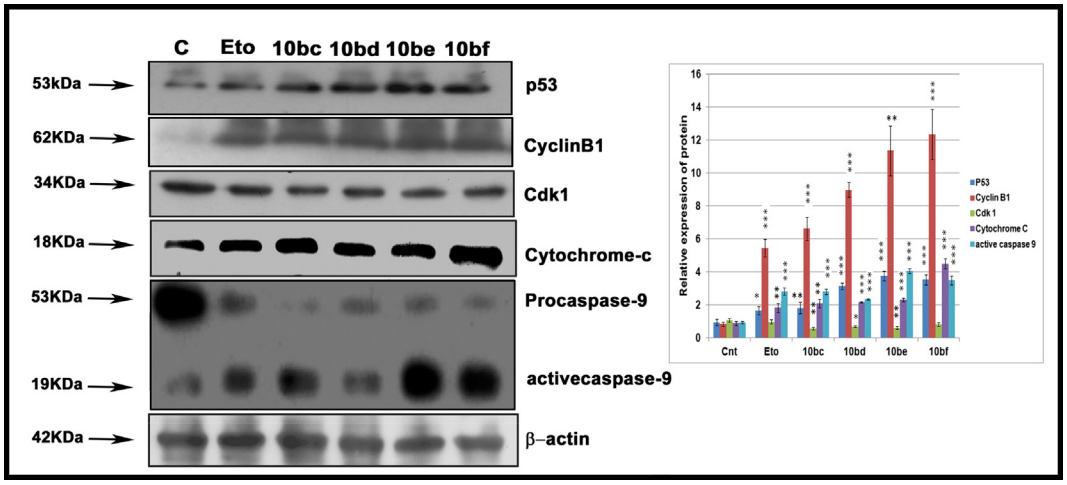


Figure 5. Effect of quinazolino linked podophyllotoxin conjugates on proteins that cause G2/M cell cycle arrest and apoptosis. MCF-7 cells were treated with conjugates **10bc**, **10bd**, **10be** and **10bf** for 24 h and cell lysates were subjected to Western blot using p53, cyclin B1, Cdk1, cytosolic cytochrome-c and pro, active caspase-9. Etoposide (Eto) was used as positive control. β-actin was used as loading control. All the experiments was carried out in triplicates. Statistical significance was assessed using student *t*-test. ***indicates $p<0.001$, **indicates $p<0.01$, *indicates $p<0.05$.

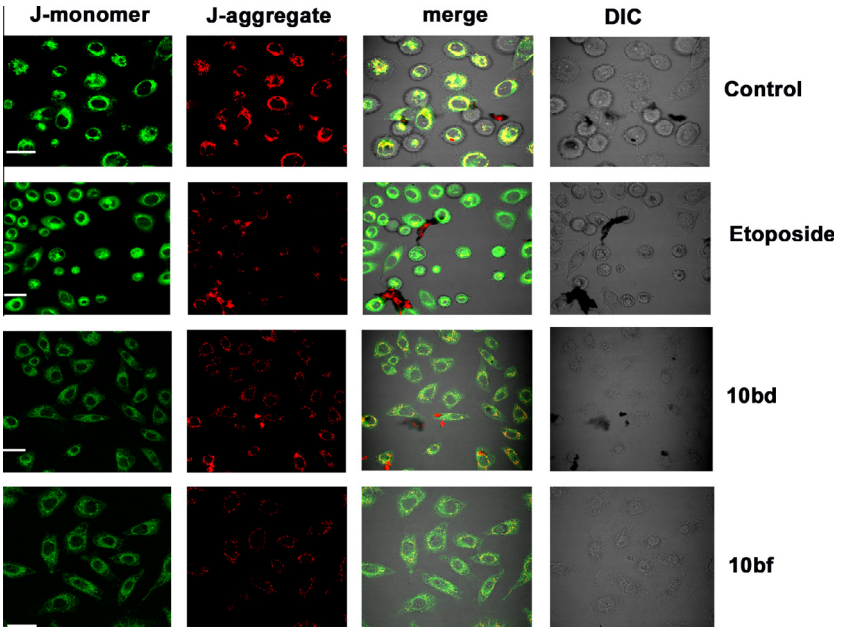


Figure 6. Effect on disruption of mitochondrial function and induction of apoptosis in cancer cells. MCF-7 cells were exposed to **10bd** and **10bf** (most effective conjugates), etoposide for 24 h at 4 μM concentration and stained with 2.5 μM JC-1 dye for 20 min at room temparature. Red fluorescence indicates mitochondria with intact MMP, whereas green fluorescence indicates loss of MMP. In healthy cells with high mitochondrial $\Delta\psi m$, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low $\Delta\psi m$, JC-1 remains in the monomeric form, which shows only green fluorescence. Scale bar is 25 μm.

observed. These results strongly suggest the effect of compounds on mitochondria in MCF-7 cells as shown in Figure 6.

3.6. Effect of conjugates on integrin protein expression

Integrins play a key role in the generation and transduction of the force to actin filament networks. Depletion of αVβIII-integrin significantly reduces the invasiveness of breast carcinoma cells and affects the epithelial–mesenchymal transition (EMT) and tumorogenic potential of the carcinoma cells.[51] To examine the effect of these conjugates on integrins, MCF-7 cells have been treated by these conjugates at 4 μM concentration for 24 h employing etoposide as standard. Interestingly, remarkable decrease in integrin protein level was observed in comparison to control cells, thus indicating significant inhibitory role during tumor cell proliferation as depicted in Figure 7.

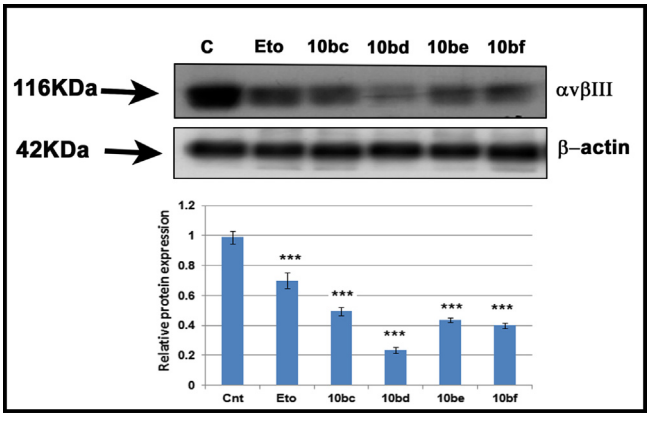


Figure 7. Effect of conjugates on integrin protein expression. MCF-7 cells were treated with most effective compounds (**10bc**, **10bd**, **10be** and **10bf**) and Etoposide (Eto) at 4 μM concentration for 24 h. The lysates were subjected to Western blot for αVβIII-integrin protein. Here β-actin was used as loading control. All the experiments are carried out in triplicates. Statistical significance was assessed using student *t*-test. ***indicates $p<0.001$.

4. Conclusion

In the present study, a series of novel quinozolino linked 4β-amidopodophyllotoxin conjugates (**10aa–af**) and (**10ba–bf**) were synthesized and evaluated for their anticancer activity. Compounds (**10bc**, **10bd**, **10be** and **10bf**) were found to be more cytotoxic than the other compounds in this series. The flow cytometry analysis also showed that **10bc**, **10bd**, **10be** and **10bf** caused significant G2/M cell-cycle arrest in MCF-7 cells. Treatment of MCF-7 cells with effective compounds (**10bc**, **10bd**, **10be** and **10bf**) for 24 h at 4 μM concentration lead to increase in expression of p53 and cyclin B1 protein with concomitant decrease in Cdk1. Therefore, these results reveal the inhibitory action of these conjugates on mitosis. An increase in caspase-9 levels is observed by these conjugates thereby leading to apoptosis. We have also examined the role of these conjugates towards the release of cytochrome-c from mitochondria, an increase of cytochrome-c expression in the cytosolic extract was observed that reveals the induction of apoptosis by these conjugates. Further, we have performed a functional assay to estimate MMP using JC-1 dye. Further, the results obtained from the functional assay relating to the mitochondrial membrane potential clearly revealed the mitochondrial mediated apoptotic event. These conjugates also caused the decrease in protein expression related to angiogenesis and invasion such as VEGF-A, STAT-3, AKT-1 ser 473 phosphorylation, ERK1/2, ERK phosphorylation (activated form of ERK) and integrin level. Thus these quinozolino linked podophyllotoxin conjugates can be considered as leads that are likely to be useful for the effective treatment against breast cancer.

5. Experimental

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. 1H 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.

5.1. 6-Hydroxy-2-methyl-4*H*-benzo[*d*][1,3]oxazin-4-one (12)

Compound **12** was prepared by heating **11** (3.06 g, 20 mmol) in acetic anhydride (7 ml) at 150 °C for 30 min. The reaction mixture was poured in ice cold water, filtered and the precipitate was washed with water, dried and used directly without further purification to get **12** as white solid.

5.2. 6-Hydroxy-2-methyl-3-(3,4,5-trimethoxyphenyl)quinazolin-4(3*H*)-one (14a)

Equimolar amounts **12** (318.6 mg, 1.8 mmol) and 3,4,5-trimethoxy aniline (**13a)** (329.4 mg, 1.8 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 ethyl acetate. The solvent was evaporated under reduced pressure to afford the crude product. This was further purified by column chromatography (20% ethyl acetate–hexane) to get the compound **14a** as a white solid (530 mg, 86%). Mp 195–196 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.31 (s, 3H), 3.84 (s, 6H), 3.90 (s, 3H), 6.51 (s, 2H), 7.30 (d, 1H, J = 2.6 Hz), 7.57 (d, 1H, J = 8.8 Hz), 7.73 (d, 1H, J = 2.6 Hz); MS (ESI): 343 $[M+H]^+$

5.3. 3-(3,5-Dimethoxyphenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (14b)

The compound **14b** was prepared following the method described for the preparation of the compound **14a**, employing **12** (318.6 mg, 1.8 mmol) and 3,5-dimethoxyaniline (**13b**) (275.4 mg, 1.8 mmol), and the crude product was purified by column chromatography (18% ethyl acetate–hexane) to afford the compound **14b** as a white solid (500 mg, 89%); mp 170–171 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.14 (s, 3H), 3.76 (s, 3H), 3.79 (s, 3H), 6.77 (d, 1H, J = 2.9 Hz), 6.97 (dd, 1H, J = 2.9, 8.8 Hz), 7.02 (d, 1H, J = 8.8 Hz), 7.21 (dd, 1H, J = 1.9, 8.8 Hz), 7.44 (d, 2H, J = 7.9 Hz), 9.49 (s, 1H); MS (ESI): 313 $[M+H]^+$

5.4. 6-Hydroxy-3-(4-methoxyphenyl)-2-methylquinazolin-4(3*H*)-one (14c)

The compound **14c** was prepared following the method described for the preparation of the compound **14a**, employing **12** (318.6 mg, 1.8 mmol) and 4-methoxyaniline (**13c**) (221.4 mg, 1.8 mmol), and the crude product was purified by column chromatography (15% ethyl acetate–hexane) to afford the compound **14c** as a brown solid (420 mg, 82%); mp 260–261 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.15 (s, 3H), 3.80 (s, 3H), 6.86 (dd, 2H, J = 2.2, 6.7 Hz), 7.05 (dd, 2H, J = 2.2, 6.7 Hz), 7.17 (dd, 1H, J = 2.2, 6.7 Hz), 7.38 (dd, 2H, J = 2.2, 6.7 Hz); MS (ESI): 283 $[M+H]^+$

5.5. 3-(3,4-Dichlorophenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (14d)

The compound **14d** was prepared following the method described for the preparation of the compound **14a**, employing **12** (318.6 mg, 1.8 mmol) and 3,4-dichloroaniline (**13d)** (291.6 mg, 1.8 mmol), and the crude product was purified by column chromatography (17% ethyl acetate–hexane) to afford the compound **14d** as a white solid (490 mg, 84%); mp 133–134 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.08 (s, 3H), 6.96 (d, 1H, J = 8.8 Hz), 7.28 (d, 1H, J = 8.8 Hz), 7.43–7.52 (m, 2H), 7.80 (s, 1H), 8.42 (d, 1H, J = 7.9 Hz), 9.58 (s, 1H); MS (ESI): 321 [M+].

5.6. 6-Hydroxy-2-methyl-3-(4-nitrophenyl)quinazolin-4(3*H*)-one (14e)

The compound **14e** was prepared following the method described for the preparation of the compound **14a**, employing **12** (318.6 mg, 1.8 mmol) and 4-nitroaniline **(13e)** (248.4 mg, 1.8 mmol), and the crude product was purified by column chromatography (18% ethyl acetate–hexane) to afford the compound **14e** as a white solid (415 mg, 77%); mp 265-266 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.18 (s, 3H), 7.23–7.37 (m, 5H), 7.57 (dd, 1H, J = 5.1, 8.8 Hz), 7.83 (s, 1H), 9.68 (s, 1H); MS (ESI): 298 $[M+H]^+$

5.7. 3-(4-Fluorophenyl)-6-hydroxy-2-methylquinazolin-4(3*H*)-one (14f)

The compound **14f** was prepared following the method described for the preparation of the compound **14a**, employing **12** (318.6 mg, 1.8 mmol) and 4-fluoroaniline **(13f**) (199.1 mg, 1.8 mmol), and the crude product was purified by column chromatography (14% ethyl acetate–hexane) to afford the compound **14f** as a white solid (400 mg, 82%); mp 179–180 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.23 (s, 3H), 7.24 (s, 3H), 7.31 (dd, 2H, J = 2.8, 8.8 Hz), 7.59 (d, 1H, J = 8.8 Hz), 7.73 (d, 1H, J = 2.6 Hz); MS (ESI): 270 $[M]^+$

5.8. 4β-[5-Bromopentamido]-4-desoxy-podophyllotoxin (16a)

To a solution of 5-bromovaleric acid (724 mg, 4 mmol) in dry dichloromethane (20 ml) was added oxalyl chloride (0.52 ml, 1.5 mmol), and 2–3 drops of DMF at 0 °C. Reaction mixture was stirred for 7–9 h at room temperature. After completion of the reaction checked by TLC, the solvent was evaporated under vacuum to get 5-bromopentanoyl chloride as yellow solid. Then dissolved the acid chloride in dry dichloromethane (20 ml) and was added compound **15** (1.65 g, 4 mmol) and triethylamine (1.12 ml, 8 mmol) at 0 °C. Reaction mixture was stirred for 10 h at room temperature. After completion of reaction, water (20 ml) was added and compound was extracted with dichloromethane (2×20 ml). The organic phase were washed with water followed by brine solution, dried over Na_2SO_4 and evaporated under vacuum to obtain crude compound. This was further purified by column chromatography (23% ethyl acetate–hexane) to get the compound **16a** as brown solid (1.84 g, 80%); mp 115–116 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62–1.75 (m, 2H), 1.78–1.95 (m, 2H), 2.24–2.40 (m, 2H), 2.65–2.87 (m, 2H), 2.89–3.03 (m, 1H), 3.43 (t, 1H, J = 6.2 Hz), 3.74 (s, 3H), 3.76 (s, 3H), 3.80 (s, 3H), 3.93–4.12 (m, 1H),), 4.31–4.46 (m, 1H), 4.55–4.63 (m, 1H), 5.21–5.27 (m, 1H), 5.94 (m, 2H), 6.2 (s, 1H), 6.28 (s, 1H), 6.45-6.57 (m, 1H), 6.75 (s, 1H), MS (ESI): 576 $[M+H]^+$.

5.9. 4β-[6-Bromohexamido]-4-desoxy-podophyllotoxin (16b)

The compound **16b** was prepared following the method described for the preparation of the compound **16a**, employing 6-bromohaxanoic acid (780 mg, 4 mmol) and oxalyl chloride (0.52 ml, 1.5 mmol) and the crude product was purified by column chromatography (22% ethyl acetate–hexane) to afford the compound **16b** as a white solid (1.92 g, 81%); mp 119–120 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.39–1.54 (m, 2H), 1.58-1.73 (m, 2H), 1.76–1.94 (m, 2H), 2.20 (t, 2H, J = 7.3 Hz), 2.78 (dd, 1H, J = 4.7, 14.3 Hz), 2.83–2.96 (m, 1H), 3.38 (t, 1H, J = 6.6 Hz), 3.51 (t, 1H, J = 6.4 Hz), 3.71 (s, 6H), 3.74 (s, 3H), 4.08 (dd, 1H, J = 7.1, 14.3 Hz), 4.31–4.48 (m, 2H), 5.16–5.25 (m, 1H), 5.96 (d, 2H, J = 7.9 Hz), 6.20 (s, 2H), 6.47 (s, 1H), 6.74 (s, 1H); MS (ESI): 591 $[M+H]^+$.

5.10. 4β-[5-(3,4-Dihydro-3-(3,4,5-trimethoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy) pentana-mide]-4-desoxy-podophyllotoxin (10aa)

To a solution of compound **14a** (180 mg, 0.52 mmol) in acetone (25 ml), anhydrous K_2CO_3 (287 mg, 2.08 mmol) and **16a** (362 mg, 0.63 mmol) were added and the mixture was refluxed for 12 h. The reaction was monitored by TLC. After completion of the reaction, K_2CO_3 was removed by filtration and the organic layer was dried with Na_2SO_4 and evaporated under reduced pressure to get the crude product which was further purified by column chromatography (50% ethyl acetate–hexane) to obtain the pure product (**10aa**) as white solid (330 mg, 75%); mp 134–135 °C; $[\alpha]_D^{25}$ −68.0 (c 1.0 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.77–2.09 (m, 4H), 2.55 (s, 3H), 2.29–2.41 (m, 2H), 3.07–3.26 (m, 1H), 3.34–3.42 (t, 1H, J = 6.7 Hz), 3.75 (s, 6H), 3.77 (s, 6H), 3.82 (s, 3H), 3.90 (s, 3H), 3.95–4.03 (m, 2H), 4.10–4.35 (m, 3H), 5.36–5.44 (m, 1H), 5.89 (d, 2H, J = 5.0 Hz), 6.21 (s, 2H), 6.38 (s, 1H), 6.53 (s, 2H), 6.61–6.75 (m, 2H), 7.13 (d, 1H, J = 7.9 Hz), 7.30 (s, 1H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 21.9, 22.2, 23.6, 28.3, 35.7, 37.9, 44.8, 45.2, 47.6, 56.1, 56.2, 60.7, 60.8, 67.7, 68.6, 101.2, 104.7, 105.1, 106.3, 106.8, 109.8, 121.1, 124.9, 128.1, 128.4, 130.3, 133.3, 137.6, 137.8, 141.7, 147.2, 147.5, 152, 153.4, 154, 157.4, 162.1, 173.1, 178.6; MS (ESI): 838 $[M+H]^+$; HRMS (ESI) calcd for $C_{45}H_{48}O_{13}N_3$ $[M+H]^+$ 838.3172.; found: 838.3181.

5.11. 4β-[5-(3,4-Dihydro-3-(3,5-dimethoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy)pentan amide]-4-desoxy-podophyllotoxin (10ab)

The compound **10ab** was prepared following the method described for the preparation of the compound **10aa**, employing **14b** (162 mg, 0.52 mmol) and **16a** (362 mg, 0.63 mmol), and the crude product was purified by column chromatography (48% ethyl acetate–hexane) to afford the compound **10ab** as a white solid (350 mg, 83%); mp 136–137 °C; $[\alpha]_D^{25}$ −14.4 (c 1.0 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.71–1.87 (m, 4H), 2.11–2.14 (m, 2H), 2.16 (s, 3H), 3.12-3.20 (m, 1H), 3.26 (d, 1H, J = 9.8 Hz), 3.75 (s, 6H), 3.76 (s, 9H), 3.90–4.03 (m, 2H), 4.05–4.16 (m, 2H), 4.33 (s, 1H), 5.30–5.39 (m, 1H), 5.77–5.90 (m, 2H), 6.26 (d, 2H, J = 3.9 Hz), 6.51 (s, 1H), 6.58 (dd, 1H, J = 8.9, 14.8 Hz), 6.66 (d, 1H, J = 8.9 Hz), 6.76 (d, 1H, J = 13 Hz), 6.94 (s, 3H), 7.49 (d, 1H, J = 8.9 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ 22.3, 23.1, 28.3, 35.7, 37.9, 45, 45.2, 47.6, 55.8, 56.1, 60.7, 67.7, 68.7, 101.2, 104.6, 106.3, 106.9, 109.8, 113, 115, 115.5, 115.6, 121.2, 124.8, 126.6, 128.2, 128.5, 130.2, 136.7, 137.7, 137.8, 142.1, 147.2, 147.4, 148.5, 152.4, 153.4, 153.9, 157.2, 161.8, 173.1, 178.7; MS (ESI): 808 $[M+H]^+$; HRMS (ESI) calcd for $C_{44}H_{46}O_{12}N_3$ $[M+H]^+$ 808.3076; found: 808.3058.

5.12. 4β-[5-(3,4-Dihydro-3-(4-methoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy)pentanami de]-4-desoxy-podophyllotoxin (10ac)

The compound **10ac** was prepared following the method described for the preparation of the compound **10aa**, employing **14c** (146.6 mg, 0.52 mmol) and **16a** (362 mg, 0.63 mmol), and the crude product was purified by column chromatography (48% ethyl acetate–hexane) to afford the compound **10ac** as a white solid (320 mg, 79%); mp 121–122 °C; $[\alpha]_D^{25}$ −104.0 (c 1.0 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.78–1.87 (m, 4H), 2.17 (s, 3H), 2.18-2.25 (m, 2H), 3.06–3.14 (m, 1H), 3.24 (dd, 1H, J = 2.5, 10.2 Hz), 3.77 (s, 6H), 3.78 (s, 3H), 3.87 (s, 3H), 4.01–4.12 (m, 4H), 4.33 (d, 1H, J = 2.5 Hz), 5.36 (dd, 1H, J = 5.1, 8.5 Hz), 5.88 (d, 2H, J = 12 Hz), 6.26 (s, 2H), 6.53 (d, 2H, J = 9.4 Hz), 6.64 (s, 1H), 6.96–7.06 (m, 3H), 7.17 (dd, 1H, J = 2.5, 8.5 Hz), 7.39 (d, 1H, J = 2.5 Hz), 7.48 (d, 1H, J = 9.4 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ 22.1, 24, 28.2, 35.6, 37.8, 45.1, 45.2, 47.4, 55.4, 56.1, 60.7, 67.7, 68.6, 101.2, 104.7, 106.2, 107, 109.7, 115.1, 121.1, 124.8, 128.2, 128.5, 128.8, 128.9, 130.1, 130.2, 137.5, 141.9, 147.2, 147.4, 152.3, 153.4, 157.4, 159.9, 162.4, 173.2, 178.7; MS (ESI): 778 $[M+H]^+$; HRMS (ESI) calcd for $C_{43}H_{44}O_{11}N_3$ $[M+H]^+$ 778.2970.; found: 778.2968.

5.13. 4β-[5-(3-(3,4-Dichlorophenyl)-3,4-dihydro-2-methyl-4-oxoquinazolin-6-yloxy)pentana mide]-4-desoxy-podophyllotoxin (10ad)

The compound **10ad** was prepared following the method described for the preparation of the compound **10aa**, employing **14d** (167 mg, 0.52 mmol) and **16a** (362 mg, 0.63 mmol), and the crude product was purified by column chromatography (44% ethyl acetate–hexane) to afford the compound **10ad** as a white solid (350 mg, 82.5%); mp 130–131 °C; $[\alpha]_D^{25}$ −49.6 (c 1.0 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.73–1.90 (m, 4H), 2.14 (s, 2H), 2.19 (s, 3H), 2.88–3.01 (m, 1H), 3.11–3.28 (m, 1H), 3.77 (s, 9H), 3.87–4.05 (m, 2H), 4.08–4.21 (m, 2H), 4.26–4.32 (m, 1H), 5.29–5.40 (m, 1H), 5.81–5.96 (m, 2H), 6.25 (d, 2H, J = 3.0 Hz), 6.45–6.55 (m, 2H), 6.66 (d, 1H, J = 8.3 Hz), 7.22–7.25 (m, 1H), 7.29 (dd, 1H, J = 3.0, 9.8 Hz), 7.43 (d, 1H, J = 9.0 Hz), 7.52 (dd, 1H, J = 2.2, 12.8 Hz), 7.60–7.68 (m, 1H); MS (ESI): 817 $[M+H]^+$.

5.14. 4β-[5-(3,4-Dihydro-2-methyl-3-(4-nitrophenyl)-4-oxoquinazolin-6-yloxy)pentanamide]-4-desoxy-podophyllotoxin (10ae)

The compound **10ae** was prepared following the method described for the preparation of the compound **10aa**, employing **14e** (154.5 mg, 0.52 mmol) and **16a** (362 mg, 0.63 mmol), and the crude product was purified by column chromatography (49% ethyl acetate–hexane) to afford the compound **10ae** as a white solid (300 mg, 72%); mp 128–129 °C; $[\alpha]_D^{25}$ −87.5 (c 1.0 in $CHCl_3$), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.80–1.92 (m, 4H), 2.20 (s, 3H), 2.28–2.44 (m, 2H), 3.23-3.29 (m, 1H), 3.57–3.65 (m, 1H), 3.78 (s, 6H), 3.88 (s, 3H), 4.06–4.19 (m, 3H), 4.27 (t, 1H, J = 7.5 Hz), 4.47 (s, 1H), 5.29 (dd, 1H, J = 6.0, 8.3 Hz), 5.98 (d, 2H, J = 3.7 Hz), 6.40 (s, 2H), 6.72 (s, 1H), 6.88 (s, 1H), 7.06 (d, 2H, J = 9.0 Hz), 7.21 (d, 2H, J = 9.0 Hz), 7.35 (dd, 1H, J = 2.2, 9.0 Hz), 7.51–7.60 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 22.1, 24, 28.2, 35.7, 37.9, 45, 45.2, 47.5, 56.1, 60.7, 67.7, 68.6, 101.2, 104.7, 106.3, 107, 109.8, 115.1, 121.2, 124.9, 128.2, 128.5, 128.8, 128.9, 129.9, 130.2, 136.8, 137.7, 141.9, 147.2, 147.5, 152.4, 153.4, 157.4, 159.9, 162.4, 173.1, 178.7; MS (ESI): 793 $[M+H]^+$; HRMS (ESI) calcd for $C_{42}H_{41}O_{12}N_4$ $[M+H]^+$ 793.2715.; found: 793.2724.

5.15. 4β-[5-(3-(4-Fluorophenyl)-3,4-dihydro-2-methyl-4-oxoquinazolin-6-yloxy)pentanamide]-4-desoxy-podophyllotoxin (10af)

The compound **14f** was prepared following the method described for the preparation of the compound **14a**, employing **14f** (140.4 mg, 0.52 mmol) and **16a** (362 mg, 0.63 mmol), and the crude product was purified by column chromatography (46% ethyl acetate–hexane) to afford the compound **14f** as a white solid (320 mg, 80%); mp 119–120 °C; $[\alpha]_D^{25}$ −28.4 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.76–1.89 (m, 4H), 2.15 (s, 3H), 2.20–2.26 (m, 2H), 3.21 (dd, 1H, *J* = 1.7, 10.2 Hz), 3.36 (t, 1H, *J* = 6.8 Hz), 3.77 (s, 9H), 4.00–4.12 (m, 4H), 4.30–4.32 (m, 1H), 5.35 (dd, 1H, *J* = 5.1, 7.6 Hz), 5.88 (d, 2H, *J* = 12.2 Hz), 6.26 (s, 2H), 6.33 (d, 1H, *J* = 8.5 Hz), 6.49 (s, 1H), 6.65 (s, 1H), 7.19–7.24 (m, 3H), 7.27–7.31 (m, 1H), 7.37 (d, 1H, *J* = 1.7 Hz), 7.46 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ 22.1, 24, 28.2, 35.7, 37.9, 44.9, 45.2, 47.5, 56.1, 60.7, 67.7, 68.6, 101.2, 104.6, 106.3, 106.9, 109.8, 116.8, 117.1, 121, 125, 128.3, 129.6, 129.8, 129.9, 130.2, 133.6, 137.7, 141.8, 147.2, 147.4, 151.5, 153.4,157.7, 162.1,173, 178.7; MS (ESI): 766 $[M+H]^+$; HRMS (ESI) calcd for $C_{42}H_{41}O_{10}N_3F$ $[M+H]^+$ 766.2746.; found: 766.2757.

5.16. 4β-[6-(3,4-Dihydro-3-(3,4,5-trimethoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy)hexana mide]-4-desoxy-podophyllotoxin (10ba)

The compound **10ba** was prepared following the method described for the preparation of the compound **10aa**, employing **14a** (180 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (50% ethyl acetate–hexane) to afford the compound **10ba** as a white solid (330 mg, 74%); mp 139–140 °C; $[\alpha]_D^{25}$ −43.4 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.52–1.60 (m, 2H), 1.62–1.70 (m, 4H), 2.20–2.28 (m, 2H), 2.30 (s, 3H), 3.14–3.26 (m, 1H), 3.28–3.35 (m, 1H), 3.81 (s, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 3.85 (s, 9H), 4.08 (t, 2H, *J* = 6.0 Hz), 4.22–4.43 (m, 3H), 5.39–5.48 (m, 1H), 5.94–5.98 (m, 2H), 6.37 (t, 2H, *J* = 2.2 Hz), 6.48 (d, 1H, *J* = 2.2 Hz), 6.51-6.57 (m, 1H), 6.75 (d, 1H, *J* = 2.2 Hz), 6.81 (s, 1H), 7.16 (s, 1H), 7.35 (dd, 1H, *J* = 3.0, 9.0 Hz), 7.59–7.63 (m, 1H); MS (ESI): 852 $[M+H]^+$; HRMS (ESI) calcd for $C_{46}H_{50}O_{13}N_3$ $[M+H]^+$ 852.3338.; found: 852.3325.

5.17. 4β-[6-(3,4-Dihydro-3-(3,5-dimethoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy)hexana mide]-4-desoxy-podophyllotoxin (10bb)

The compound **10bb** was prepared following the method described for the preparation of the compound **10aa**, employing **14b** (162 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (47% ethyl acetate–hexane) to afford the compound **10bb** as a white solid (325 mg, 76%); mp 141–142 °C; $[\alpha]_D^{25}$ −22.4 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.45–1.57 (m, 4H), 1.69–1.88 (m, 4H), 2.18 (s, 3H), 3.19–3.31(m, 2H), 3.70 (s, 3H), 3.73 (s, 3H), 3.78 (s, 3H), 3.79 (s, 6H), 4.01–4.16 (m, 4H), 4.35 (br s, 1H), 5.31–5.40 (m, 1H), 5.91 (d, 2H, *J* = 9.0 Hz), 6.29 (s, 2H), 6.57 (d, 1H, *J* = 8.3 Hz), 6.65–6.70 (m, 2H), 6.86 (dd, 1H, *J* = 3.0, 9.0 Hz), 6.91–6.95 (m, 1H), 7.27–7.32 (m, 1H), 7.50–7.60 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 23, 25.1, 25.4, 28, 36.1, 37.7, 45.2, 45.3, 47.5, 55.6, 55.7, 56.1, 60.7, 67.9, 68.6, 101.2, 104.6, 106.2, 106.9, 107, 109.7, 109.8, 112.9, 115.5, 121.2, 124.9, 126.5, 128.1, 128.4, 128.5, 130, 136.6, 137.6, 141.9, 147.2, 147.4, 152.4, 153.3, 157.3, 161.8, 173.4, 178.8; MS (ESI): 822 $[M+H]^+$; HRMS (ESI) calcd for $C_{45}H_{48}O_{12}N_3$ $[M+H]^+$ 822.3205.; found: 822.3208.

5.18. 4β-[6-(3,4-Dihydro-3-(4-methoxyphenyl)-2-methyl-4-oxoquinazolin-6-yloxy)hexanamide]-4-desoxy-podophyllotoxin (10bc)

The compound **10bc** was prepared following the method described for the preparation of the compound **10aa**, employing **14c** (146.6 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (48% ethyl acetate–hexane) to afford the compound **10bc** as a white solid (330 mg, 80%); mp 129–130 °C; $[\alpha]_D^{25}$ −89.0 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.45–1.60 (m, 2H), 1.70–1.90 (m, 4H), 2.19 (s, 3H), 2.22–2.36 (m, 2H), 3.05–3.16 (m, 1H), 3.27 (dd, 1H, *J* = 1.8, 10.7 Hz), 3.78 (s, 9H), 3.80 (s, 3H), 3.97–4.17 (m, 4H), 4.36 (s, 1H), 5.36 (dd, 1H, *J* = 5.4, 8.4 Hz), 5.94 (d, 2H, *J* = 13 Hz), 6.26 (s, 2H), 6.59 (d, 2H, *J* = 6.7 Hz), 6.83–6.97 (m, 2H), 7.0–7.12 (m, 2H), 7.29 (dd, 1H, *J* = 2.6, 8.8 Hz), 7.49–7.60 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 24, 25, 25.3, 27.9, 36.2, 37.8, 45.2, 45.3, 47.3, 55.4, 56.1, 60.7, 67.8, 68.6, 101.2, 104.6, 106..2, 106.8, 109.7, 114.9, 115.2, 121.1, 125.1, 128.3, 128.5, 128.7, 128.8, 129.9, 130.1, 136.7, 137.6, 141.8, 147.2, 147.4, 152.3, 153.3, 157.4, 159.8, 173.4, 178.7; MS (ESI): 792 $[M+H]^+$; HRMS (ESI) calcd for $C_{44}H_{46}O_{11}N_3$ $[M+H]^+$ 792.3126; found: 792.3108.

5.19. 4β-[6-(3,4-Dichlorophenyl)3,4-dihydro-2-methyl-4-oxoquinazolin-6-yloxy)hexanamide]-4-desoxy-podophyllotoxin (10bd)

The compound **10bd** was prepared following the method described for the preparation of the compound **10aa**, employing **14d** (167 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (43% ethyl acetate–hexane) to afford the compound **10bd** as a white solid (335 mg, 77%); mp 123–124 °C; $[\alpha]_D^{25}$ −17.4 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.43–1.58 (m, 2H), 1.66–1.78 (m, 2H), 1.79–1.89 (m, 2H), 2.21 (s, 3H), 2.22–2.29 (m, 2H), 2.98–3.15 (m, 1H), 3.16–3.26 (m, 1H), 3.79 (s, 9H), 4.01–4.22 (m, 4H), 4.33 (s, 1H), 5.33–5.42 (m, 1H), 5.90 (d, 2H, *J* = 6.9 Hz), 6.29 (d, 2H, *J* = 2.8 Hz), 6.52 (s, 1H), 6.67 (s, 1H), 7.06–7.19 (m, 1H), 7.30 (dd, 1H, *J* = 2.6, 8.8 Hz), 7.40 (t, 1H, *J* = 2.4 Hz), 7.47 (t, 1H, *J* = 3.3 Hz), 7.51–7.63 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 24, 25.2, 25.6, 28.4, 36.4, 38, 44.8, 45.3, 45.9, 47.7, 47.8, 56.2, 60.8, 68.1, 68.7, 95.9, 101.3, 104.8, 106.4, 107, 109.9, 114.8, 125.3, 127.6, 127.7, 128.4, 128.5, 130.2, 130.3, 131.6, 137, 138.2, 138.7, 141.7, 147.3, 150.7, 157.8, 162.6, 173.3, 178.3; MS (ESI): 830 $[M+H]^+$; HRMS (ESI) calcd for $C_{43}H_{42}O_{10}N_3Cl_2$ $[M+H]^+$ 830.2241; found: 830.2244.

5.20. 4β-[6-(3,4-Dihydro-2-methyl-3-(4-nitrophenyl)-4-oxoquinazolin-6-yloxy)hexanamide]-4-desoxy-podophyllotoxin (10be)

The compound **10be** was prepared following the method described for the preparation of the compound **10aa**, employing **14e** (154.5 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (48% ethyl acetate–hexane) to afford the compound **10be** as a white solid (345 mg, 82%); mp 132–133 °C; $[\alpha]_D^{25}$ −12.9 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.49–1.59 (m, 2H), 1.68–1.78 (m, 2H), 1.79-1.89 (m, 2H), 2.17 (s, 3H), 2.25 (t, 2H, *J* = 7.5 Hz), 3.04–3.16 (m, 1H), 3.21(dd, 1H, *J* = 3.0, 9.8 Hz), 3.78 (s, 9H), 4.0–4.17 (m, 5H), 5.37 (dd, 1H, *J* = 6.0, 9.0 Hz), 5.91 (d, 2H, *J* = 6.7 Hz), 6.28 (s, 2H), 6.52 (s, 1H), 6.64 (s, 1H), 7.33 (dd, 1H, *J* = 3.0, 9.0 Hz), 7.39–7.60 (m, 4H), 8.34-8.43 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): δ 24, 25.1, 25.4, 28.2, 36.4, 37.9, 44.8, 45.2, 47.6, 56.2, 60.7, 68.1, 68.6, 101.3, 104.8, 106.3, 107, 109.9, 125.1, 125.2, 125.4, 128.4, 128.6, 129.5, 129.6, 130.3, 136.9, 138, 141.7, 143.4, 147.2, 147.6, 148, 149.9, 153.5, 157.9, 161.8, 173.1, 178.4; MS (ESI): 807

$[M+H]^+$; HRMS (ESI) calcd for $C_{43}H_{43}O_{12}N_4$ $[M+H]^+$ 807.2872; found: 807.2868.

5.21. 4β-[6-(4-Fluorophenyl)3,4-dihydro-2-methyl-4oxoquinazolin-6-yloxy)hexanamide]-4-desoxy-podophyllotoxin (10bf)

The compound **10bf** was prepared following the method described for the preparation of the compound **10aa**, employing **14f** (140.4 mg, 0.52 mmol) and **16b** (371 mg, 0.63 mmol), and the crude product was purified by column chromatography (45% ethyl acetate–hexane) to afford the compound **10bf** as a white solid (350 mg, 86%); mp 126–127 °C; $[\alpha]_D^{25}$ −78.0 (*c* 1.0 in $CHCl_3$), ^1H NMR (300 MHz, $CDCl_3$): δ 1.40–1.58 (m, 4H), 1.59–1.72 (m, 2H), 1.73–1.87 (m, 2H), 2.19 (s, 3H), 3.02–3.26 (m, 2H), 3.78 (s, 6H), 3.80 (s, 3H), 3.93 (dd, 1H, *J* = 5.2, 8.3 Hz), 4.00–4.11 (m, 2H), 4.24–4.37 (m, 2H), 5.34–5.42 (m, 1H), 5.93 (d, 2H, *J* = 5.2 Hz), 6.30 (d, 2H, *J* = 12.8 Hz), 6.50 (s, 1H), 6.55 (s, 1H), 6.67 (d, 1H, *J* = 6.7 Hz), 7.16–7.32 (m, 4H), 7.48 (d, 1H, *J* = 9.0 Hz) 7.55 (d, 1H, *J* = 3.0 Hz); ^{13}C NMR (300 MHz, $CDCl_3$): δ 24, 25.1, 26.3, 28.2, 36.3, 37.9, 44.7, 45.3, 47.7, 56.2, 60.7, 68, 101.2, 104.8, 104.9, 106.3, 107, 109.8, 117.1, 121.1, 125.1, 128.3, 128.4, 129.6, 129.8, 129.9, 130, 130.3, 137.9, 141.8, 147.2, 151.5, 153.4, 153.5, 157.6, 162.2, 173.2, 178; MS (ESI): 780 $[M+H]^+$; HRMS (ESI) calcd for $C_{43}H_{43}O_{10}N_3F$ $[M+H]^+$ 780.2927; found: 780.2926.

6. Biology

6.1. Cell culture

Human breast cancer cell line MCF-7 cells, breast adenocarcinoma (MDA-MB-231, pancreatic carcinoma cells (Panc-1) were purchased from American type culture collection were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI (Invitrogen), supplemented with 2 mM glutamax (Invitrogen), 10% fetal calf serum and 100 U/ml Pencillin and 100 mg/ml streptomycin sulfate (Sigma). The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator. HUVEC cells purchased from Lonza company were grown on 60 mm cell culture dishes in endothelial cells medium (ECM) supplemented with 5% FBS and 100U/ml penicillin, 100 mg/ml streptomycin and 1% ECGS (endothelial cell growth supplement). The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

6.2. MTT assay

Cell viability was assessed by MTT assay, a mitochondrial function assay. It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. MCF-7, MDA-MB-231 and Panc-1 cells were seeded in a 96-well plate at a density of 10,000 cells/well. After overnight incubation, cells were treated with compounds **10aa**–**af**, **10ba**–**bf** and etoposide (Eto), the standard drug at 0.5–32 μM concentration and incubated for 24 h. Medium was then discarded and replaced with 10 μL MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubilised in 100 μL extraction buffer. The optical density (O.D) was read at 570 nm with micro plate reader (Multi-mode Varioskan instrument-Themo Scientific). The percentage of cell viability was monitored.

6.3. Cell cycle analysis

5×10^5 MCF-7 cells were seeded in 60 mm dish and were allowed to grow for 24 h. Compounds **10aa**–**af**, **10ba**–**bf**, etoposide were added at a final concentration of 4 μM 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 RNase A 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.

6.4. Angiogenesis inhibition assay

5×10^3 HUVEC cells (human umbilical vein endothelial cells) were seeded on 96 well plate containing EC matrix solidified at 37 °C. Add 150 μL endothelial growth media and allowed to grow for 12 h. The compounds [etoposide (Eto), **10bc**, **10bd**, **10be** and **10bf**] at 1 μM final concentration were incubated for about 6 h. Then cells were analysed under inverted microscope. We have examined mainly the effect of compound on tube formation. In control cells, we have noticed excellent tube formation. But disruption of tube formation was observed after compound treatment.

6.5. Protein extraction and Western blot analysis

Total cell lysates from cultured MCF-7 cells treated with compounds **10bc**, **10bd**, **10be**, **10bf** and etoposide at 4 μM for 24 h were obtained by lysing the cells in ice-cold RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) and containing 100 mg/mL PMSF, 5 mg/mL Aprotinin, 5 mg/mL leupeptin, 5 mg/mL pepstatin and 100 mg/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). Seventy five micrograms of protein per lane was applied in 12% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidine difluoride (PVDF) membrane (GE 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, primary antibody was added and incubated at 4 °C overnight (O/N). p53, β-actin was purchased from Imgenex, USA. Cyclin B, Cdk1, STAT-1, STAT-3 antibodies were purchased from Millipore Company. Caspase-9, cytochrome-c, αVβIII integrin, VEGF-A, ERK1/2P, ERK1/2, AKT1, AKT1 ser 473 phosphorylation were purchased from cell signaling company. 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 blots were visualized with chemiluminescence reagent (Thermo Fischer Scientifics Ltd). The X-ray films were developed with developer and fixed with fixer solution (Kodak Company Ltd). Cytosolic and mitochondrial fractions were obtained using Mitochondria/CYTOSOL FRACTIONATION KIT (PROMOKINE)[PK-CA-577-K256-100] and is used for probing cytochrome-c. The kit provides an easy-to-use procedure and unique reagents for separating a highly enriched mitochondria fraction from cytosol. Cytochrome-c released from mitochondria into cytosol is then determined by Western blotting using the cytochrome c antibody.

6.6. Effect on mitochondrial membrane potential (MMP)

MCF-7 cells were exposed to etoposide, 10bd and 10bf conjugate for 24 h at 4 μM concentration and stained with 2.5 μM of JC-1 dye for 20 min at room temperature. JC-1 dye was used to

estimate a spatial variation of mitochondrial potential. JC-1 dye accumulates in mitochondria as aggregate (red), whereas in apoptotic cells it cannot accumulate and will be in monomeric form (green). The loss of mitochondrial potential is indicated by red to green shift. Red fluorescence indicates mitochondria with intact membrane potential while green fluorescence indicates the mitochondria with loss of MMP.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.08.051.

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