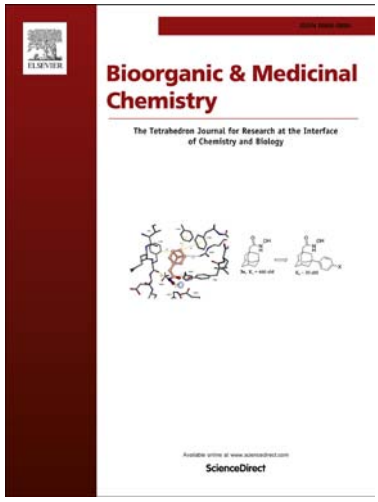


Synthesis and biological evaluation of 1,2,3-triazole linked aminocombretastatin conjugates as mitochondrial mediated apoptosis inducers

Ahmed Kamal, Bajee Shaik, V. Lakshma Nayak, Burri Nagaraju, Jeevak Sopanrao Kapure, M. Shaheer Malik, Thokhir Basha Shaik, B. Prasad

PII: S0968-0896(14)00585-9
DOI: http://dx.doi.org/10.1016/j.bmc.2014.08.008
Reference: BMC 11756

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 25 April 2014
Revised Date: 23 July 2014
Accepted Date: 11 August 2014

Please cite this article as: Kamal, A., Shaik, B., Lakshma Nayak, V., Nagaraju, B., Kapure, J.S., Shaheer Malik, M., Shaik, T.B., Prasad, B., Synthesis and biological evaluation of 1,2,3-triazole linked aminocombretastatin conjugates as mitochondrial mediated apoptosis inducers, *Bioorganic & Medicinal Chemistry* (2014), doi: http://dx.doi.org/10.1016/j.bmc.2014.08.008

Synthesis and biological evaluation of 1,2,3-triazole linked aminocombretastatin conjugates as mitochondrial mediated apoptosis inducers

Ahmed Kamal,*[a,b] Bajee Shaik,[a] V. Lakshma Nayak,[a] Burri Nagaraju,[a] Jeevak Sopanrao Kapure,[a,b] M. Shaheer Malik,[a] Thokhir Basha Shaik, B. Prasad[a]

[a]*Medicinal Chemistry and Pharmacology,* CSIR-*Indian Institute of Chemical Technology, Hyderabad 500 007, India*
[b]*Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad 500 037, India*

Abstract: A series of 1,2,3-triazole linked aminocombretastatin conjugates were synthesized and evaluated for cytotoxicity, inhibition of tubulin polymerization and apoptosis inducing ability. Most of the conjugates exhibited significant anticancer activity against some representative human cancer cell lines and two of the conjugates **6d** and **7c** displayed potent cytotoxicity with IC_{50} values of 53 nM and 44 nM against A549 human lung cancer respectively, and were comparable to combretastatin A-4 (CA-4). SAR studies revealed that 1-benzyl substituted triazole moiety with an amide linkage at 3-position of B-ring of the combretastatin subunit are more active compared to 2-position. G2/M cell cycle arrest was induced by these conjugates **6d** and **7c** and the tubulin polymerization assay (IC_{50} of 1.16 µM and 0.95 µM for **6d** and **7c**, respectively) as well as immunofluorescence analysis showed that these conjugates effectively inhibit microtubule assembly at both molecular and cellular levels in A549 cells. Colchicine competitive binding assay suggested that these conjugates bind at the colchicine binding site of tubulin as also observed from the docking studies. Further, mitochondrial membrane potential, ROS generation, caspase-3 activation assay, Hoechst staining and DNA fragmentation analysis revealed that these conjugates induce cell death by apoptosis.

***Keywords*:** CA-4, cell cycle, cytotoxicity, triazoles, tubulin polymerization.

*,[a]Corresponding authors. Tel.: +91-40-27193157; fax: +91-40-27193189 (A.K.); e-mail: ahmedkamal@iict.res.in (A. Kamal).

1. Introduction

Tubulin, a globular protein, has emerged as one of the valuable molecular targets in anticancer drug discovery. It exists as dimeric (α and β) subunits in the microtubules, which plays a pivotal role in many cellular process such as maintenance of skeletal integrity of cell, cell signaling and segregation of chromosomes during mitosis.[1,2] Several chemical agents are known to target the microtubule dynamics by binding to different domains of the tubulin protein and prevent the polymerization or depolymerization of the microtubules resulting in mitotic spindle arrest[3]. In nature, the bark of the South African tree *Combretum caffrum* contains *cis* stilbene derivatives named as combrestatins, which are well known to effectively bind at the colchicine site of the tubulin and inhibit the polymerization resulting in the arrest of cell proliferation.[4] Combretastatin A-4 (CA-4, **1**) is the lead compound of this class with high cytotoxic potency against murine lymphocytic leukemia, human ovarian and colon cancer cell lines. However it suffers from limitation of poor aqueous solubility and to overcome this, prodrug CA-4P (**2**) and other synthetic analogues have been developed, which are undergoing clinical trials.[5-9] The structure activity relationship studies of CA-4 suggested the importance of trimethoxy substitution on the ring A and the crucial role played by *cis*-configuration of the olefic bond to impart activity to this pharmacophoric structure has been well established. The ring B has been reported to be tolerant to structural modification to enhance the pharmacological profile of the molecule. Particularly, the aminocombretastatins such as AVE-8063(**3**), AVE-8062 (**4**) with an amino and amino-serine groups replacing the hydroxyl group at the C-3 position of ring B showed potent antitubulin activity and cytotoxicity.

In recent years 1,2,3-triazoles have emerged as attractive and desirable scaffolds in the development of potential drug molecules in medicinal chemistry. Molecules with triazole nucleus are known to display broad spectrum of biological properties such as antifungal, anticancer,[10-13] anti-allergic, antibacterial, anti-HIV, anticonvulsant, anti-inflammatory and antitubercular activities. Interestingly, triazole hybrids of some biologically important natural products[14-18] have been developed and recently *N*-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)arylamide (**5**) have been evaluated as antimicrotubule agents against selective cancer cell lines.[19]

<Insert Figure 1>

We have been involved in the design and synthesis of new heterocyclic based compounds as potential anticancer agents that could target the tubulin polymerization process.[20] In the present work, we have explored the structural modification of the ring B of CA-4 by incorporating the triazole moiety on different positions via an amide linkage. These conjugates are evaluated for their cytotoxic activity and studied for the mechanistic aspects which include the inhibition of tubulin polymerization and apoptosis induction.

2. Results and Discussions

2.1. Chemistry.

The synthesis of these triazole linked combretastatin conjugates (**6a-e**, **7a-e**, and **8a-e**) was accomplished as illustrated in Schemes 1 and 2. The synthesis of conjugates with amino group at C-3 of ring B (**6a-e** and **7a-e**) started with a double bond forming Wittig reaction between the nitro benzaldehydes (**10, 11**) and 3,4,5-trimethoxybenzyl triphenylphosphonium bromide (**9**) in presence of sodium hydride in anhydrous dichloromethane to provide a mixture of nitro *Z*-stilbenes (**12,13**) and *E*-stilbenes (**14,15**) as depicted in Scheme 1. The starting materials (**9**-**11**) were prepared by a standard protocol as reported in the literature.[21] The nitro *Z*-stilbenes (**12, 13**) were subjected to reduction with zinc-ammonium formate in methanol to give the corresponding amino *Z*-stilbenes (**16,17**). These amino stilbenes were coupled with different substituted benzyl-1H-1,2,3-triazole-4-carboxylic acids (**18a-e**)[22] using 3-ethyl-1(*N*,*N*-dimethyl)aminopropyl carbodiimide (EDCI) /1-hydroxybenzotriazole (HOBT) in dimethyl formamide to afford the desired conjugates (**6a-e** and **7a-e**).

<Insert scheme 1>

The desired conjugates with amino group at C-2 of ring B (**8a-e**) were synthesized following a similar synthetic route as shown in Scheme 2. In this route, a silyl protected nitrobenzaldehyde (**19**) prepared from a standard literature protocol[23] was employed as the starting material. A double bond was formed employing phosphonium bromide **9** and aldehyde **19** to afford a

mixture of nitro Z-stilbenes (**20**) and E-stilbenes (**21**), this was followed by the reduction of nitro functionality of *Z* isomer **20** to provide the amino stilbenes **22**. This was coupled with different substituted carboxylic acids (**18a-e**) and followed by deprotection of silyl group with tetra-*n*-butylammonium fluoride to afford the desired conjugates **8a-e**.

<*Insert scheme 2*>

The key benzyl-1H-1,2,3-triazole-4-carboxylic acids (**18a-e**) intermediates with different substitution were synthesized with a standard copper (I) catalyzed azide-alkyne cycloaddition protocol yielding 1,4-adduct exclusively (Scheme 3).

<*Insert scheme 3*>

3. Biology

3.1 Cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[24] was performed to evaluate the cytotoxic potential of these compounds against selected human cancer cell lines which include HeLa (cervical) HepG2 (liver) A549 (lung), ACHN (renal) and MCF-7 (breast). The results are summarized as IC_{50} values in Table 1 and are compared with CA-4. The assay revealed that these compounds showed significant cytotoxic activity with IC_{50} values ranging from 0.044-9.5 μM against different cancer cell lines. The most active compounds **6d** and **7c** with triazole moiety at the 3-position of B-ring exhibited promising antiproliferative activity with IC_{50} values ranging from 0.053 to 1.659 μM and 0.044 to 1.698 μM, respectively. Particularly the compound **6d** and **7c** exhibited remarkable cytotoxicity comparable to CA-4 in A549 cell line with IC_{50} values of 0.053 and 0.044 μM, respectively.

The electron donating alkoxy groups are present in many naturally occurring and synthetic tubulin inhibitors and apoptosis inducers which are reported as anticancer agent. In this study some selected substitutions, which includes 3 and 4-methoxy, 3-phenoxy, 3,4,5-trimethoxy and an electron withdrawing 4-fluoro, were employed on the basis of literature precedence.[19] SAR studies have revealed that the conjugates **6a-e, 7a-e** with triazole moiety attached as amide

at C-3 position of B-ring of CA-4 exhibited significantly enhanced activity than conjugates **8a-e** with triazole moiety attached at C-2 position. In addition the para substitution on the benzyl ring of the 1,2,3-triazole moiety was crucial in imparting more potency to these conjugates.

<Insert Table 1>

3.2 Cell cycle analysis

Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of cell cycle or by induction of apoptosis or a combined effect of both cycle block and apoptosis.[25,26] Furthermore regulation of the cell cycle and apoptosis are considered to be effective cancer therapeutic methods.[27] The *in vitro* screening results revealed that compounds, **6d** and **7c** showed significant antiproliferative activity against lung cancer cell line A549. Therefore, it was considered of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. In this study A549 cells were treated with compounds **6d** and **7c** at concentrations of 25 and 50 nM for 48 h. The data obtained clearly indicated that these compounds arrested cell cycle at G2/M phase as compared to the untreated control. CA-4 was used as reference compound in this study. These compounds (**6d** and **7c**) showed 17.57 and 21.37 % of cell accumulation in G2/M phase respectively at 25 nM concentration, whereas it exhibited 25.73 and 26.63 % of cell accumulation in G2/M phase at 50 nM concentration (Figure 2). Interestingly, at higher concentration both the compounds exhibited G2/M cell cycle arrest comparable to the standard CA-4 (26.48 %)

<insert Figure 2>

<insert Table 2>

3.3 Effect of compounds on tubulin polymerization

In general G2/M cell cycle arrest is strongly associated with inhibition of tubulin polymerization[28] and since compounds **6d** and **7c** cause cell cycle arrest at G2/M cell cycle arrest, their effect on microtubule inhibitory function was investigated. Tubulin subunits are known to heterodimerize and self-assemble to form microtubules in a time dependent manner. The progression of tubulin polymerization[29,30] was thus examined by monitoring the increase in

fluorescence emission at 420 nm (excitation wavelength is 360 nm) in 384 well plate for 1 h at 37 ^{o}C with and without the conjugates at 3 µM concentration in comparison with reference compound CA-4. The test compounds (**6d** and **7c**) efficiently inhibited tubulin polymerization by 66.82 and 70.86 %, respectively, comparable to 70.51 % inhibition exhibited by CA-4 (Figure 3). This was followed by evaluation of IC_{50} values for these compounds and results are shown in Table 3. The compound **7c**, which displayed higher tubulin-assembly inhibition was more potent with an IC_{50} value of 0.95 µM and was superior to standard CA-4 (IC_{50}=1.08 µM). The effect of these compounds on the inhibition of tubulin assembly correlated well with their strong antiproliferative activity.

<Insert Figure 3>

<Insert Table 3>

3.4 Competitive colchicine binding assay

Compounds **6d** and **7c** showed similar inhibitory effects on tubulin polymerization comparable to CA-4 and this prompted us to investigate whether these compounds bind to the colchicine site of tubulin as CA-4 by fluorescence based assay. Tubulin (3 µM) was incubated with the various concentrations of compounds **6d** and **7c** (0-20 µM) in the presence of colchicine (3 µM) with CA-4 as positive control at 37 ^{o}C for 60 min and the fluorescence of tubulin-colchicine complex was monitored at 435 nm when excited at 350 nm. We observed an increase in fluorescence of tubulin-colchicine complex in the presence of tested CA-4 analogues (**6d** and **7c**) and CA-4. This is in the agreement with a previous investigation in which CA-4 is reported to give fluorescence upon binding at the colchicine site.[31a, b] Therefore, the experiment was carried out both in the presence and absence of colchicine to obtain fluorescence values of the desired tubulin-colchicine complex, the fluorescence values of tubulin-test compounds complex was subtracted from the tubulin-test compounds-colchicine complex. It was observed that at low concentrations the test compounds (**6d** and **7c**) and CA-4 showed significant affinity towards colchicine site, whereas at higher concentration **7c** showed a steady increase while **6d** and CA-4 showed lower in the binding affinity. Compared to the positive control CA-4, compound **7c** exhibited more affinity towards colchicine site whereas **6d** has less affinity. Taxol was used as a negative

control, which is known to bind at different site and shows no effect on tubulin-colchicine complex. Therefore this study indicates that **6d** and **7c** bind at the colchicine site on tubulin.

<insert Figure 4>

3.5 Immunohistochemistry (IHC) studies on tubulin

In addition to *in vitro* tubulin polymerisation studies, we investigated alterations in the microtubule network induced by conjugates **6d** and **7c** in A549 cell culture by immunofluoresence microscopy, as most antimitotic agents affect microtubules.[32] Therefore, A549 cells were treated with these compounds at 25 nM concentration for 48 h. The test results, demonstrated a well organized microtubular network in control cells, however cells treated with these compounds (**6d**, **7c** and CA-4) showed disrupted microtubule organization as seen in Figure 4, thus demonstrating the inhibition of tubulin polymerization.

. <insert Figure 5>

3.6 Hoechst staining for the effect on apoptosis

Disruption of microtubule formation leads to cell-cycle arrest in the G2/M phase, followed by apoptotic cell death.[33] Apoptosis is one of the major pathways that lead to the process of cell death. Chromatin condensation, nuclear shrinking and fragmented nuclei are known as classic characteristics of apoptosis. In the present investigation the apoptotic inducing effect of compounds **6d** and **7c** was studied by Hoechst staining (H 33258) in A549 cancer cell line. Therefore cells were treated with these compounds at 25 nM concentration for 48 h and the results revealed typical apoptotic characteristics, including condensation of chromatin and appearance of nuclear fragmentation and apoptotic bodies. These results clearly demonstrated that compounds **6d** and **7c** were also effective in inducing cellular apoptosis (**Figure 5**).

<insert Figure 6>

3.7 Measurement of Mitochondrial Membrane Potential (ΔΨm)

The maintenance of mitochondrial membrane potential (ΔΨm) is significant for mitochondrial integrity and bio energetic function.[34] Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm), are key events that take place during drug-induced apoptosis. To determine the changes in mitochondrial membrane potential (ΔΨm), we examined the lipophilic dye JC-1, specific for mitochondria. Mitochondria that maintain normal mitochondrial membrane potential (ΔΨm) concentrate JC-1 into aggregates indicates red fluorescence. However, JC-1 forms monomers (green fluorescence) in depolarized mitochondria. A549 cells were treated with compounds **6d** and **7c** at 25 nM concentration and incubated for 48 h. After 48 h of treatment with these conjugates, it was observed that decrease in the mitochondrial membrane potential (ΔΨm) when compared to untreated control (**Figure 6**)

<insert Figure 7>

3.8 Effect of 6d and 7c on intracellular ROS generation

Generation of intracellular reactive oxygen species (ROS) is considered one of the key mediators of apoptotic signaling for the most anti tumor agents.[35] In order to demonstrate the role of test compounds on ROS generation (**6d** and **7c**) during apoptosis process, production of ROS was examined by using an oxidant-sensitive fluorescent probe, DCFDA (2',7'dichlorofluorescin diacetate). The results showed that treatment with test compounds at 25 nM concentration significantly increased the intensity of the DCF signal when compared to control cells (**Figure 7**).

<insert Figure 8>

3.9 Effect on activation of caspase 3

From literature precedence, it is well established that molecules affecting microtubule polymerization cause mitotic arrest and ultimately lead to apoptosis.[36] Caspases, are a family of cysteine-aspartic proteases that are crucial mediators of apoptosis. Among them, caspase-3 is the most studied in the mammalian caspases in terms of its specificity role in apoptosis.[37] Some reports[38,39] indicated that the cell cycle arrest at G2/M phase takes place by the induction of cellular apoptosis and in the present study an investigation to understand the correlation of

cytotoxicity and apoptosis by compounds **6d** and **7c** was undertaken. A549 cells were treated with **6d** and **7c** at 25 and 50 nM concentrations for 48 h. CA-4 (50 nM) was used as reference compound in this study and examined for the activation of caspase-3 activity. Results indicate that there was nearly 3 to 7-fold induction in caspase-3 levels compared to the control and compound **6d** exhibited superior caspase activation compared to CA-4 (**Figure 8**).

<insert Figure 9>

3.10 DNA Fragmentation analysis

Apoptosis is a programmed cell death, characterized with chromatin condensation and internucleosomal DNA fragmentation.[40] DNA fragmentation is well known and a typical biochemical hallmark of apoptotic cell death. During apoptosis DNA is cleaved into small fragments by endonucleases and these fragments can be observed by gel electrophoresis as ladders. In this context, the endonucleolytic DNA cleavage was checked by agarose gel electrophoresis to further confirm **6d** and **7c** induced cell apoptosis. A549 cells were treated with these compounds at 25 nM concentration for 48 h. After treatment it was observed that compounds **6d** and **7c** produced significant DNA fragmentation, which is indicative of apoptosis (**Figure 9**).

. <insert Figure 10>

4. Molecular modeling studies:

A molecular modeling study was performed to explain the binding mode of conjugates **6d** and **7c** within tubulin protein domains to correlate the polymerization inhibitory activities of these compounds (**Figure 10**). Coordinates of protein structure of tubulin-colchicine was obtained from the Protein Data Bank (PDB ID 3E22). Docking was accomplished into the colchicine binding sites of tubulin using AutoDock 4.2 software and the important interactions are discussed. Results showed that the triazole ring of compound **6d** exhibited important hydrophobic interaction with αSER178, αTYR224. Carbonyl oxygen of amide group present between ring **B** and triazole ring establishes hydrophobic interaction with αThr179. The ring **A**

(trimethoxyphenyl group) buried in a hydrophobic pocket interacted with the βAla250, βLys254, βLeu255, βLys352 residues similar to that of trimethoxyphenyl group of colchicine and ring B binded with αVal181, βALA250, αAsn258, αAsn101 residues. The fluorobenzyl group on triazole ring shows hydrophobic interaction with αSer178, αVal177, and αTyr224. Similar to **6d** carbonyl oxygen of amide of conjugate **7c** establishes hydrophobic interaction through a different residue βThr353. Methoxybenzyl group on triazole ring is involved in important hydrophobic interaction with αAsn101 and βLys254, whereas the ring A of conjugate **7c** occupies the same position as the corresponding moiety of the colchicine analogue. Ring B shows hydrophobic interaction with βLys352 and βLeu255, where as the triazole ring of **7c** establishes hydrophobic interaction with αSer178 and βLeu255. The molecular docking simulations suggest that the conjugates **6d** and **7c** interact with αβ tubulin interface in the colchicine binding as pocket seen from the colchicine competitive binding assay. *<Insert Figure 11>*

5. Conclusion

In conclusion, a new series of 1,2,3-triazole linked aminocombretastatin conjugates (**6a-e** to **8a-e**) have been synthesized and evaluated for their anticancer potential against selected human cancer cell lines. All the synthesized compounds showed potent anticancer activity against the tested cancer cell lines and two of them (**6d** and **7c**) showed IC_{50} values 0.053 and 0.044 µM, respectively, against A549 cancer cell line. Flow cytometric analysis of these compounds showed the arrest of the cell cycle in the G2/M phase leading to caspase-3 dependent apoptotic cell death. These active compounds (**6d** and **7c**) showed potent inhibition of tubulin polymerization with IC_{50} values 1.16 and 0.95 µM, respectively. The immunohistochemistry study of tubulin showed that the level of tubulin polymerization inhibition was comparable to that of CA-4 for the compounds **6d** and **7c**. The colchicine competitive binding assay and docking studies clearly indicate that these conjugates bind at colchicine binding site of the tubulin. Further, Hoechst staining, loss of mitochondrial membrane potential, significant activation and cleavage of caspase-3 activity, DNA fragmentation analysis and the production of reactive oxygen species (ROS) suggested that cytotoxic efficacy of these compounds is mainly due to inhibition of tubulin polymerization and apoptosis. The results demonstrate that compounds **6d** and **7c** are potential leads and could be taken up for further evaluation as in the development of tubulin polymerization inhibitors.

6. Experimental protocol

All chemicals and reagents were obtained from Aldrich (Sigma– Aldrich, S t. Louis, MO, USA), Lancaster (AlfaAesar, 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 G F -254, and visualization 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 AVANCE-300 (300 MHz) or JCAMP (300/500 MHz) instruments. Chemical shifts (δ) were reported in ppm with TMS as internal standard. EI-MS 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. The purity of tested compounds was ≥95% as determined by HPLC performed on a Shimadzu LCMS-2020 apparatus equipped with a SPD-M20A diode array detector and a Shimadzu SIL-20AC auto injector using C18 column (Phenomenex luna 5µm C18, 4.6 mm × 250 mm column). Elution conditions: mobile phase A(75%)-acetonitrile; mobile phase B(25%)-water containing 0.1% formic acid + 10 mmol NH_4OAc. The flow rate was 1.0 ml/min and the injection volume was 5 µl at 25 °C and detection at 254 nm.

6.1 Procedure for the synthesis of nitro *Z*-stilbenes (12, 13, 20)

To a stirred solution of nitrobenzaldehydes (**10**, **11**, **19**, 1 mmol) and 3,4,5-trimethoxybenzyl triphenylphosphonium bromide (**9,** 1.1 mmol) in anhydrous dichloromethane, sodium hydride (4 mmol) was added at 0 ^{o}C. The reaction mixture was stirred at room temperature for 18 h and monitored by TLC. After completion of the reaction appropriate amount of water, until foaming stopped, was added at 0 ^{o}C. The organic layer was separated and the aqueous layer was extracted with chloroform. The combined organic layers were washed with brine solution, dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure to get crude compounds, nitro *Z*-and *E*-stilbenes isomeric mixture. The isomers were separated by using column chromatography to give pure *Z*- stilbenes.

6.1.1 (*Z*)-1,2,3-Trimethoxy-5-(4-methoxy-3-nitrostyryl)benzene (12): bright yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm); 7.78 (d, *J* = 2.0 Hz, 1H), 7.44 (dd, *J* = 2.1, 8.4 Hz, 1H), 6.95 (d, *J* = 8.4 Hz, 1H), 6.58 (d, *J* = 12.8 Hz, 1H), 6.48 (s, 2H), 6.45 (d, *J* = 12.8 Hz, 1H), 3.95 (s, 3H), 3.84 (s, 3H), 3.72 (s, 6H); MS (ESI, *m/z*): 346 $[M+1]^+$.

6.1.2 (*Z*)-5-(3,4-Dimethoxy-5-nitrostyryl)-1,2,3-trimethoxybenzene(13): bright yellow solid, ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.21 (s, 1H), 6.93 (s, 1H), 6.56 (d, *J* = 11.8 Hz, 1H), 6.43 (s, 2H), 6.40 (d, *J* = 11.8 Hz, 1H), 3.91 (s, 3H), 3.79 (s, 3H), 3.71 (s, 6H), 3.66 (s, 3H); MS (ESI, *m/z*): 376 $[M+1]^+$.

6.1.3(*Z*)-tert-Butyl(6-methoxy-2-nitro-3-(3,4,5-trimethoxystyryl)phenoxy)dimethylsilane (20): yellow liquid; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm); 6.77 (s, 2H), 6.61 (d, *J* = 12.0 Hz, 1H), 6.41 (s, 2H), 6.38 (d, *J* = 12.0 Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H), 3.67 (s, 6H), 0.94 (s, 9H), 0.21 (s, 6H). MS (ESI, *m/z*): 476 $[M+1]^+$.

6.2 Procedure for the synthesis of amino *Z*-stilbenes (16, 17, 22)

Zinc powder (3 mmol) and ammoniumformate (3mmol) were added portion wise to a solution of nitro *Z*-stilbenes (**12, 13, 20,** 1 mmol) in methanol at 0 °C and the reaction mixture was stirred at room temperature for 12 hrs. After completion of reaction, the reaction mixture was filtered to remove the residual zinc and the solvent was evaporated under reduced pressure. The residue was redissolved in ethyl acetate and washed with water, brine solution and dried over anhydrous Na_2SO_4. The resulting solution was filtered and the solvent was removed under reduced pressure followed by the purification of residue by column chromatography using ethyl acetate and hexane as eluents to give pure products.

6.2.1 (*Z*)-2-Methoxy-5-(3,4,5-trimethoxystyryl)aniline (16): ^{1}H NMR (300 MHz, $CDCl_3$.) δ (ppm); 6.70 (s, 1H), 6.67 (s, 2H), 6.55 (s, 2H), 6.47 (d, *J* = 12.0 Hz, 1H,), 6.36 (d, *J* = 12.0 Hz, 1H,), 3.84 (s, 3H), 3.82 (s, 3H), 3.70 (s, 6H,); MS (ESI, *m/z*): 316 $[M+1]^+$.

6.2.2 (*Z*)-2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (17): ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm); 6.47 (s, 2H), 6.39 (d, *J* = 12.0 Hz, 1H), 6.36 (d, *J* = 12.0 Hz, 1H), 6.27 (d, *J* = 1.5 Hz,

1H), 6.19 (d, *J* = 1.5 Hz, 1H), 3.75 (s, 3H), 3.71 (s, 3H), 3.63 (s, 6H), 3.59 (s,3H); MS (ESI, *m/z*): 346 $[M+1]^+$.

6.2.3 (*Z*)-2-(*tert*-Butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (22): ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm); 6.70 (d, *J* = 8.3 Hz, 1H), 6.51 (s, 2H), 6.47 (d, *J* = 12.0 Hz, 1H), 6.43 (d, *J* = 12.0 Hz ,1H), 6.27 (d, *J* = 8.3 Hz, 1H), 3.81 (brs, 2H), 3.81 (s, 3H), 3.76 (s, 3H), 3.63 (s, 6H), 1.00 (s, 9H), 0.17 (s, 6H), MS (ESI, *m/z*): 446 [M+1]+.

6.3 Procedure for the synthesis of benzyl-1H-1,2,3-triazole-4-carboxylic acids (18a-e)

Appropriate azide (1 mmol), sodium ascorbate (0.1mmol) and copper sulfate pentahydrate (0.01 mmol) were added sequentially to a solution of propiolic acid (1 mmol), in *tert*-butanol and water (1:1). The resulting reaction mixture was stirred at room temperature for 12h. The precipitate formed was filtered and washed with water to yield pure products.

6.3.1 1-(3-Methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (18a): ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm); 8.26 (s, 1H), 7.29 (t, *J* = 7.8 Hz, 1H), 6.90-6.85 (m, 3H), 5.57 (s, 2H), 3.78 (s, 3H); MS (ESI, *m/z*): 234 [M+1]+.

6.3.2 1-(3-Phenoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (18b): ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm); 8.27 (s, 1H), 7.37-7.30 (m, 3H), 7.12 (t, *J* = 7.3 Hz, 1H), 7.03-6.93 (m, 5H), 5.58 (s, 2H); MS (ESI, *m/z*): 296 [M+1]+.

6.3.3 1-(4-Methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (18c): ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm); 8.29 (s, H), 7.32 (d, *J* = 7.8 Hz, 2H), 6.93 (d, *J* = 7.8 Hz, 2H), 5.56 (s, 2H), 3.75 (s, 3H); MS (ESI, *m/z*): 234 [M+1]+.

6.3.4 1-(4-Fluorobenzyl)-1H-1,2,3-triazole-4-carboxylic acid (18d): ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm); 8.27 (s, 1H), 7.38-7.33 (m, 2H), 7.07 (t, *J* = 8.5 Hz, 2H), 5.60 (s, 2H); MS (ESI, *m/z*): 222 [M+1]+.

6.3.5 1-(3,4,5-Trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (18e): ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm); 8.25 (s, 1H), 6.59 (s, 2H), 5.52 (s, 2H), 3.83 (s, 6H), 3.79 (s, 3H); MS (ESI, *m/z*): 294 [M+1]+.

6.4 General procedure for the synthesis of triazole linked aminocombretastatins (6a-e, 7a-e)

To a solution of substituted benzyl-1H-1,2,3-triazole-4-carboxylic acids (**18a-e,** 1 mmol) in dry dimethylformamide, EDCI (1.2 mmol) and HOBT (1.2mmol) were added and the reaction mixture was stirred for 20 min. To the reaction mixture, amino *Z*-stilbenes (**16**, **17,** 1 mmol) was added and stirred at room temperature for 12 h. The solvent was removed and the residue was dissolved in dichloromethane (50 mL), washed with 5% HCl (2 x 20 mL), 5% $NaHCO_3$ (2 x 20 mL), and brine solution (20 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed to give a crude product which was purified by chromatography (ethyl acetate/hexanes) to give the desired product (**6a-e, 7a-e).**

6.4.1 (*Z*)-*N*-(2-Methoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (6a)

The compound **6a** was prepared according to the general procedure by employing 1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (74 mg, 0.32 mmol) and (*Z*)-2-methoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.32 mmol) as white solid. Yield 117 mg (69%); m.p: 83-85 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.49 (s, 1H), 8.43 (s, 1H), 8.02 (s, 1H), 7.31 (t, *J* = 7.9 Hz, 1H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.93-6.86 (m, 2H), 6.82-6.75 (m, 2H), 6.56-6.52 (m, 3H), 6.42 (d, *J* = 12.0 Hz, 1H), 5.54 (s, 2H), 3.91 (s, 3H), 3.82 (s, 3H), 3.79 (s, 3H), 3.68 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 160.1, 157.4, 152.7, 147.4, 144.0, 135.0, 132.7, 130.3, 129.9, 129.9, 129.0, 126.9, 125.5, 125.5, 124.5, 120.6, 120.2, 120.2, 114.4, 113.8, 109.5, 105.9, 60.8, 55.7, 55.2, 54.4; IR (KBr): $\bar{\upsilon}_{max}$/cm^{-1} = 3350, 3007, 2950, 1652, 1566, 1490, 1448, 1325, 1250, 1125, 1078, 1042, 1001, 855; MS (ESI, *m/z*): 531 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{29}H_{31}O_6N_4$: 531.22337; found: 531.22373 $[M+1]^+$. HPLC: t_R 6.21 min, purity 98.6%.

6.4.2 (*Z*)-*N*-(2-Methoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (6b)

The compound **6b** was prepared according to the general procedure by employing 1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (94 mg, 0.32 mmol) and (*Z*)-2-methoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.32 mmol) as reddish solid. Yield: 123 mg (65%); m.p; 159-161 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.48 (s, 1H), 8.43 (s, 1H), 8.03 (s, 1H), 7.38-7.29 (m, 3H), 7.14 (d, *J* = 7.5 Hz, 1H), 7.04-6.93 (m, 6H), 6.74 (s, 1H), 6.56 (d, *J* = 12.2 Hz, 1H), 6.52 (s, 2H), 6.46 (d, *J* = 12.2 Hz, 1H), 5.54 (s, 2H), 3.91 (s, 6H), 3.68 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 158.2, 156.2, 153.3, 152.8, 147.5, 144.1, 135.5, 132.7, 130.6, 129.9, 129.6, 129.1, 125.5, 124.6, 123.9, 122.5, 120.7, 119.3, 118.8, 118.1, 117.2, 110.1, 109.6, 105.9, 103.2, 60.8, 56.0, 55.8, 54.2, IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3373, 3136, 2931, 1683, 1585, 1560, 1528, 1485, 1455, 1326, 1252, 1212, 1125, 1041, 1007, 964, 899, 874, 821; MS (ESI , *m/z*): 593 $[M+1]^+$. HPLC: t_R 9.52 min, purity 95.2%.

6.4.3 (*Z*)-*N*-(2-Methoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (6c)

The compound **6c** was prepared according to the general procedure by employing 1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (94 mg, 0.32 mmol) and (*Z*)-2-methoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.32 mmol) as dark brown solid. Yield: 115 mg (68%); m.p: 85-87 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 9.48 (s, 1H), 8.43 (s, 1H), 7.97 (s, 1H), 7.26 (d, *J* = 7.7 Hz, 2H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.92-6.80 (m, 2H), 6.77 (d, *J* = 8.2 Hz, 1H), 6.55-6.52 (m, 3H), 6.43 (d, *J* = 12.0 Hz, 1H), 5.50 (s, 2H), 3.91 (s, 3H), 3.82 (s, 6H), 3.67 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 160.1, 157.5, 152.7, 147.4, 143.8, 136.9, 132.7, 129.9, 129.7, 129.6, 129.0, 126.9, 125.3, 124.5, 120.6, 114.5, 109.5, 105.9, 103.2, 60.8, 56.0, 55.7, 55.2, 54.0; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3369, 3137, 2995, 2940, 2836, 1666, 1608, 1565, 1527, 1484, 1430, 1415, 1327, 1252, 1177, 1119, 1045, 1033, 894, 877, 845; MS (ESI, *m/z*): 531 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{29}H_{31}O_6N_4$: 531.22364; found: 531.22381 $[M+1]^+$. HPLC: t_R 7.64 min, purity 96.8%.

6.4.4 (*Z*)-1-(4-Fluorobenzyl)-*N*-(2-methoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1H-1,2,3-triazole-4-carboxamide (6d)

The compound **6d** was prepared according to the general procedure by employing 1-(4-fluorobenzyl)-1H-1,2,3-triazole-4-carboxylic acid (70 mg, 0.32 mmol) and (*Z*)-2-methoxy-5-

(3,4,5-trimethoxystyryl)aniline (100 mg, 0.32 mmol) as brown red solid. Yield: 117 mg (71%); m.p: 85-87 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 9.48 (s, 1H), 8.42 (s, 1H), 8.03 (s, 1H), 7.31 (t, *J* = 8.5 Hz, 2H), 7.02 (t, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.5 Hz, 1H), 6.76 (d, *J* = 8.5 Hz, 1H), 6.52 (d, *J* = 12.0 Hz, 1H), 6.52 (s, 2H), 6.43 (d, *J* = 12.0 Hz, 1H), 5.55 (s, 2H), 3.91 (s, 3H), 3.82 (s, 3H), 3.68 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 157.4, 152.8, 147.5, 144.1, 137.0, 132.7, 130.2, 130.0, 129.6, 129.1, 127.7, 126.9, 125.4, 124.7, 120.7, 116.4, 116.1, 109.6, 106.0, 103.3, 60.8, 56.0, 55.8, 53.8; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3378, 3133, 2937, 2835, 1681, 1583, 1560, 1508, 1459, 1431,1326, 1231, 1183, 1157, 1125, 1041, 1007, 900, 874, 850; MS (ESI, *m/z*): 541 $[M+Na]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{28}H_{27}O_5N_4FNa$: 541.18598; found: 541.18585 $[M+Na]^+$. HPLC: t_R 7.90 min, purity 95.3%.

6.4.5 (*Z*)-*N*-(2-Methoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (6e)

The compound **6e** was prepared according to the general procedure by employing 1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (93 mg, 0.32 mmol) and (*Z*)-2-methoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.32 mmol) as red solid. Yield: 126 mg (67%); m.p: 90-92 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.49 (s, 1H), 8.49 (s,1H), 8.06 (s, 1H),7.02 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.62-6.52 (m, 5H), 6.44 (d, *J* = 12.0 Hz, 1H), 5.49 (s, 2H), 3.91 (s, 3H), 3.84 (s, 9H), 3.82 (s, 3H), 3.67 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-*d6*) δ (ppm): 156.3, 152.3, 151.5, 146.3, 142.9, 131.3, 128.8, 128.6, 128.3, 127.9, 126.9, 125.7, 125.1, 123.4, 118.9, 117.7, 108.7, 104.9, 104.5, 59.3, 54.9, 54.8, 54.6, 53.2; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3379, 2937, 1682, 1586, 1559, 1528, 1505, 1459, 1425, 1385, 1327, 1239, 1186, 1156, 1126, 1004, 901; MS (ESI, *m/z*): 591 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for; $C_{31}H_{35}O_8N_4$: 591.24565; found : 591.24560 [$M+1]^+$. HPLC: t_R 5.35 min, purity 95.6%.

6.4.6 (*Z*)-*N*-(2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (7a)

The compound **7a** was prepared according to the general procedure by employing 1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (67 mg, 0.29 mmol) and (*Z*)-2,3-dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.29 mmol) as light orange solid. Yield: 112 mg (69%); m.p :120-122 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.56 (s, 1H), 8.06-8.04 (m, 2H),

7.41-7.33 (m, 3H), 6.90 (t, *J* = 7.5 Hz, 1H), 6.63 (s, 1H), 6.55 (d, *J* = 12.2 Hz, 1H), 6.52 (s, 2H), 6.50 (d, *J* = 12.2 Hz, 1H), 5.54 (s, 2H), 3.92 (s, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 3.69 (s, 6H), 3.61 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 161.6, 160.1, 152.8, 151.3, 143.8, 137.0, 135.0, 133.1, 132.6, 131.4, 130.4, 129.8, 126.4, 125.6, 120.4, 114.5, 113.9, 113.5, 111.4, 108.4, 106.0, 60.9, 60.8, 55.9, 55.6, 55.3, 54.5; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3290, 3098, 1670, 1607, 1553, 1452, 1384, 1271, 1231, 1187, 1157, 1129, 1104, 985, 863; MS (ESI, m/z) : 561 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for $C_{30}H_{33}O_7N_4$: 561.23507; found: 561.23530 $[M+1]^+$. HPLC: t_R 7.76 min, purity 95.8%.

6.4.7 (*Z*)-*N*-(2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (7b)

The compound **7b** was prepared according to the general procedure by employing 1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (85 mg, 0.29 mmol) and (*Z*)-2,3-dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.29 mmol) to obtained cream white solid. Yield :117 mg (65%); m.p: 135-137 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 9.56 (s, 1H), 8.07 (s, 1H), 8.06 (s, 1H), 7.37-7.32 (m, 3H), 7.14 (t, *J* = 7.10 Hz, 1H), 7.10-6.95 (m, 5H), 6.64 (s, 1H), 6.54 (d, *J* = 12.1 Hz, 1H), 6.53 (s, 2H), 6.52 (d, *J* = 12.1 Hz, 1H), 5.45 (s, 2H), 3.92 (s, 3H), 3.81 (s, 3H), 3.70 (s, 6H), 3.62 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 158.2, 157.7, 156.2, 152.8, 151.3, 143.9, 137.2, 137.1, 135.5, 133.1, 132.6, 131.4, 130.6, 129.8, 125.6, 123.9, 122.5, 119.3, 118.8, 118.1, 113.5, 108.4, 106.1, 60.9, 60.8, 55.9, 55.6, 54.2; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3305, 3095, 2938, 1651, 1555, 1449, 1381, 1270, 1233, 1186, 1160, 1132, 1108, 988; MS (ESI, *m/z*): 623 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{35}H_{35}O_7N_4$: 623.25042; found : 623.25036 $[M+1]^+$. HPLC: t_R 7.36 min, purity 97.0%.

6.4.8 (*Z*)-*N*-(2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (7c)

The compound **7c** was prepared according to the general procedure by employing 1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (67 mg, 0.29 mmol) and (*Z*)-2,3-dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.29 mmol) as white solid. Yield: 103 mg (64%); m.p: 115-117 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.54 (s, 1H), 8.04 (s, 1H), 7.99 (s, 1H), 7.27 (d, *J* = 8.6 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 2H), 6.63 (s, 1H), 6.58-6.45 (m, 4H), 5.50 (s, 2H),

3.91 (s, 3H), 3.81 (s, 3H), 3.81 (s, 3H), 3.69 (s, 6H), 3.61 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 160.1, 157.3, 152.7,151.3, 143.7, 136.9, 133.0, 132.6, 131.4, 129.8, 129.8, 129.7, 125.5, 125.3, 120.8, 114.5, 113.4, 108.2, 105.8, 60.9, 60.8, 55.8, 55.5, 55.3, 54.1; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3284, 3095, 1673, 1615, 1556, 1450, 1392, 1359, 1270, 1233, 1185, 1160, 1132, 1108, 989, 845, 859, 845; MS (ESI, *m/z*): 561 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{30}H_{33}O_7N_4$: 561.23503; found: 561.23516 $[M+1]^+$. HPLC: t_R 7.33 min, purity 98.9%.

6.4.9 (*Z*)-N-(2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(4-fluorobenzyl)-1H-1,2,3-triazole-4-carboxamide (7d)

The compound **7d** was prepared according to the general procedure by employing 1-(4-fluorobenzyl)-1H-1,2,3-triazole-4-carboxylic acid (64 mg, 0.29 mmol) and (*Z*)-2,3-dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.29 mmol) as white solid. Yield: 111 mg (70%); m.p: 85-87 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.54 (s, 1H), 8.05 (s, 1H),8.03 (s, 1H), 7.35-7.28 (m, 2H), 7.13-7.05 (m, 2H), 6.64 (s, 1H), 6.56 (d, *J* = 12.2 Hz, 1H), 6.52 (s, 2H), 6.48 (d, *J* = 12.2 Hz, 1H), 5.55 (s, 2H), 3.91 (s, 3H), 3.81 (s, 3H), 3.69 (s, 6H), 3.62 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 164.6, 161.3, 157.6, 152.8, 151.3, 144.0, 137.1, 133.1, 132.6, 131.3, 130.1, 129.8, 129.6, 125.5, 116.4, 116.1, 113.5, 108.5, 106.1, 60.9, 60.8, 55.9, 55.6, 53.8; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3359, 3122, 2935, 2836, 1670, 1586, 1565, 1511, 1457, 1424, 1330, 1235, 1183, 1129, 1080, 1044, 998, 883, 862; MS (ESI, *m/z*): 549 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for $C_{29}H_{30}O_6N_4F$: 549.21491; found 549.21490 $[M+1]^+$. HPLC: t_R 7.52 min, purity 96.7%.

6.4.10 (*Z*)-N-(2,3-Dimethoxy-5-(3,4,5-trimethoxystyryl)phenyl)-1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (7e)

The compound **7e** was prepared according to the general procedure by employing 1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (84 mg, 0.29 mmol) and (*Z*)-2,3-dimethoxy-5-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.29 mmol) as yellow solid. Yield: 129 mg (72%); m.p: 96-98 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.56 (s, 1H), 8.06 - 8.05 (m, 2H), 6.63 (s, 1H), 6.59-6.46 (m, 6H), 5.49 (s, 2H), 3.92 (s, 3H), 3.84 (s, 9H), 3.81 (s, 3H), 3.69 (s, 6H) 3.62 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 157.7, 153.7, 152.8, 151.3, 143.8, 138.6, 137.1, 137.0, 133.1, 132.6, 131.3, 129.8, 129.0, 125.5, 113.4, 108.4, 106.0, 105.5, 60.9,

60.7, 56.1, 55.8, 55.5, 54.8; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3373, 2937, 2835, 1651, 1590, 1561, 1530, 1505, 1462, 1423, 1327, 1236, 1183, 1126, 1079, 1044, 1000, 878; MS (ESI, *m/z*): 621 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for $C_{32}H_{37}O_9N_4$: 621.25659; found: 621.25657 $[M+1]^+$. HPLC: t_R 6.31 min, purity 96.9%.

6.5 Procedure for the synthesis of triazole linked aminocombretastatins (8a-e)

a) To a solution of substituted benzyl-1H-1,2,3-triazole-4-carboxylic acids (**18a-e,**1 mmol) in dry dimethylformamide, EDCI (1.2 mmol) and HOBT (1.2mmol) were added and the reaction mixture was stirred for 20 min. To the reaction mixture amino *Z*-stilbene (**22**, 1 mmol) was added and stirred at room temperature for 12 h. The solvent was removed and the residue was dissolved in dichloromethane (50 mL), washed with 5% HCl (2 x 20 mL), 5% $NaHCO_3$ (2 x 20 mL), and brine solution (20 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed to give crude products (**23a-e**), which was used without purification.

b) To a solution of compounds (**23a-e,** 1 mmol) in dry tetrahydrofuran, TBAF (1.0 M in THF, 2.5 mmol) was added at 0 ^oC and stirred at room temperature for 1h. After completion of reaction, the reaction mixture was diluted with ethyl acetate, washed with water and brine solution, dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure followed by the purification of reside by column chromatography using ethyl acetate and hexane as eluents to afford respective desired products (**8a-e**).

6.5.1 (*Z*)-*N*-(2-Hydroxy-3-methoxy-6-(3,4,5-trimethoxystyryl)phenyl)-1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (8a)

The compound **8a** was prepared according to the general procedure by employing 1-(3-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (50 mg, 0.22 mmol) and (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.22 mmol) as brown solid. Yield: 84 mg (70%); m.p: 153-155 °C; ^1H NMR (500 MHz, $CDCl_3$) δ (ppm): 9.05 (s, 1H), 9.02 (s, 1H), 7.85 (s, 1H), 7.35-7.30 (m, 1H), 6.95-6.82 (m, 5H), 6.67 (d, *J* = 12.0 Hz, 1H), 6.56 (d, *J* = 12.0 Hz, 1H), 6.29 (s, 2H), 5.48 (s, 2H), 3.91 (s, 3H), 3.80 (s, 3H), 3.70 (s, 3H), 3.53 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 160.2, 159.3, 152.3, 141.7, 139.9, 137.1, 133.0, 131.0, 130.4. 128.9, 127.8, 126.0, 125.8, 124.2, 121.0, 120.9, 120.5, 114.5, 114.2, 109.6,

105.5, 60.7, 56.2, 55.3, 54.4; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3355, 3138, 2933, 2835, 1650, 1577, 1494, 1459, 1327, 1240, 1125, 1076, 1043, 1002, 856; MS (ESI, m/z): 547 $[M+1]^+$; HRMS (ESI, m/z) Calculated for, $C_{29}H_{31}O_7N_4$: 547.21928; found: 547.21919 $[M+1]^+$. HPLC: t_R 4.86 min, purity 95.7%.

6.5.2 (*Z*)-N-(2-Hydroxy-3-methoxy-6-(3,4,5-trimethoxystyryl)phenyl)-1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (8b)

The compound **8b** was prepared according to the general procedure by employing 1-(3-phenoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (64 mg, 0.22 mmol) and (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.22 mmol) as yellow solid. Yield: 90 mg (68%); m.p: 151-152 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.03 (s, 1H), 9.01 (s, 1H), 7.87 (s, 1H), 7.36 (d, *J* = 7.8 Hz, 1H), 7.34 (d, *J* = 7.8 Hz, 1H), 7.17-7.13 (m, 1H), 7.03-6.95 (m, 5H), 6.89-6.83 (m, 3H), 6.66 (d, *J* = 12.6 Hz, 1H), 6.55 (d, *J* = 12.6 Hz, 1H), 6.29 (s, 2H), 5.48 (s, 2H), 3.92 (s, 3H), 3.70 (s, 3H), 3.53 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 159.4, 153.8, 152.3, 150.1, 141.7, 139.9, 138.6, 137.5, 133.0, 132.9, 131.6, 130.7, 129.9, 128.4, 125.8, 125.7, 124.3, 124.0, 123.6, 123.1, 122.6, 121.0, 119.4, 119.4, 118.8, 109.6, 105.7, 60.8, 56.1, 55.5, 54.8; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3350, 2936, 2835, 1649, 1580, 1504, 1460, 1422, 1328, 1241, 1186, 1126, 1042, 1003, 856; MS (ESI, *m/z*): 609 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{34}H_{33}O_7N_4$: 609.23496; found: 609.23569 $[M+1]^+$. HPLC: t_R 4.34 min, purity 95.2%.

6.5.3 (*Z*)-*N*-(2-Hydroxy-3-methoxy-6-(3,4,5-trimethoxystyryl)phenyl)-1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (8c)

The compound **8c** was prepared according to the general procedure by employing 1-(4-methoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (51 mg, 0.22 mmol) and (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.22 mmol) as brick red solid. Yield: 85 mg (71%); m.p: 142-144 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.07 (s, 1H), 9.02 (s, 1H), 7.79 (s, 1H), 7.27 (d, *J* = 9.4 Hz, 2H), 6.93-6.82 (m, 4H), 6.66 (d, *J* = 12.6 Hz, 1H), 6.56 (d, *J* = 12.6Hz, 1H), 6.29 (s, 2H), 5.45 (s, 2H), 3.91 (s, 3H), 3.81 (s, 3H), 3.70 (s, 3H), 3.53 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 160.2, 159.4, 152.3, 150.1, 141.6, 139.9, 137.5, 133.0, 131.6, 130.1, 125.6, 125.0, 124.2, 123.5, 123.2, 120.9, 114.7, 109.6, 105.5,

60.7, 56.2, 55.5, 55.3, 54.1; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3246, 3136, 3007, 2835, 1646, 1606, 1575, 1513, 1505, 1460, 1428, 1331, 1243, 1178, 1121, 1121, 1080, 1043, 1023, 997, 854. MS (ESI, *m/z*): 547$[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{29}H_{31}O_7N4$: 547.21874; found: 547.21919 $[M+1]^+$. HPLC: t_R 4.77 min, purity 95.2%.

6.5.4 (*Z*)-1-(4-Fluorobenzyl)-*N*-(2-hydroxy-3-methoxy-6-(3,4,5-trimethoxystyryl)phenyl)-1H-1,2,3-triazole-4-carboxamide (8d)

The compound **8d** was prepared according to the general procedure by employing 1-(4-fluorobenzyl)-1H-1,2,3-triazole-4-carboxylic acid (48 mg, 0.22 mmol) and (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.22 mmol) as white solid. Yield: 88 mg (75%); m.p: 141-143 °C; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ (ppm): 9.37 (s, 1H), 8.45 (s, 1H), 8.41 (s, 1H), 7.40 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 8.3Hz, 1H), 7.21-7.06 (m, 4H), 6.94-6.87 (m, 2H), 6.68 (s, 2H), 5.62 (s, 2H), 3.91 (s, 3H), 3.81 (s, 6H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6) δ (ppm):158.9, 152.7, 151.3, 150.5, 143.6, 141.3, 140.8, 136.7, 128.7, 126.0, 125.1, 122.8, 121.3, 121.1, 117.9, 114.1, 113.8, 108.9, 101.8, 58.5, 54.3, 54.0, 52.7; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3462, 3355, 3152, 2934, 2835, 1650, 1574, 1509, 1461, 1325, 1234, 1130, 1073, 854; MS (ESI, *m/z*): 535 $[M+1]^+$; HRMS (ESI, *m/z*) Calculated for, $C_{28}H_{28}O_6N_4F$: 535.19897; found: 535.19902 $[M+1]^+$. HPLC: t_R 4.87 min, purity 95.6%.

6.5.5 (*Z*)-N-(2-Hydroxy-3-methoxy-6-(3,4,5-trimethoxystyryl)phenyl)-1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxamide (8e)

The compound **8e** was prepared according to the general procedure by employing 1-(3,4,5-trimethoxybenzyl)-1H-1,2,3-triazole-4-carboxylic acid (64 mg, 0.22 mmol) and (*Z*)-2-(*tert*-butyldimethylsilyloxy)-3-methoxy-6-(3,4,5-trimethoxystyryl)aniline (100 mg, 0.22 mmol) as brick red solid. Yield: 93mg (70%); m.p:95-97 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 9.03 (s, 1H), 9.01 (s, 1H), 7.87 (s, 1H) ,7.36 (d, *J* = 8.0 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H) 7.27 (s, 2H), 6.69 (d, *J* = 12.0 Hz, 1H), 6.55 (d, *J* = 12.0 Hz, 1H), 6.29 (s, 2H), 5.84 (s, 2H), 3.92 (s, 6H), 3.70 (s, 6H), 3.53 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 158.3, 152.3, 151.9, 135.0, 133.0, 131.6, 130.7, 129.9, 129.5, 125.8, 124.2, 124.0, 123.1, 122.6, 121.0, 119.4, 118.8, 118.3, 109.7, 105.6, 60.7, 56.2, 55.5, 54.2; IR (KBr): $\bar{\upsilon}_{max}/cm^{-1}$ = 3343, 2932, 2834, 1648, 1579, 1489, 1455, 1327, 1244, 1125, 1077, 1043, 1003, 858; MS (ESI, *m/z*): 607 $[M+1]^+$; HRMS (ESI, *m/z*)

Calculated for, $C_{31}H_{35}O_9N_4$: 607.24140; found: 607.24149 $[M+1]^+$. HPLC: t_R 7.49 min, purity 96.0%.

7. Biology

7.1 Evaluation of in vitro anti-cancer activity

The cytotoxic activity of the compounds was determined using MTT assay. 1×10^4 cells/well were seeded in 200 µl DMEM, supplemented with 10% FBS in each well of 96-well microculture plates and incubated for 24 h at 37^0 C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium, were added to the wells with respective vehicle control. After 48 h of incubation, 10 µl MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Then the supernatant from each well was carefully removed, formazon crystals were dissolved in 100 µl of DMSO and absorbance at 570 nm wavelength was recorded.

7.2 Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell-cycle phases. A549 cells, Lung cancer cells were incubated for 48 h with compounds **6d** and **7c** at concentrations of 25 and 50 nM, and also with CA-4 at 50 nM concentration. Untreated and treated cells were harvested, washed with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol, and stained with propidium iodide (Sigma–Aldrich). Cell-cycle analysis was performed by flow cytometry (Becton Dickinson FACS Caliber instrument).[41]

7.3 Tubulin polymerization assay

A fluorescence based in vitro tubulin polymerization assay was performed according to the manufacturer's protocol (BK011, Cytoskeleton, Inc.). Briefly, the reaction mixture in a total volume of 10 µL contained PEM buffer, GTP (1 µM) in the presence or absence of test compounds (final concentration of 3 µM). Tubulin polymerization was followed by a time

dependent increase in fluorescence due to the incorporation of a fluorescence reporter into microtubules as polymerization proceeds. Fluorescence emission at 420 nm (excitation wavelength is 360 nm) was measured by using a Varioscan multimode plate reader (Thermo scientific Inc.). CA-4 was used as positive control in each assay. The IC_{50} value was defined as the drug concentration required inhibiting 50% of tubulin assembly compared to control. The reaction mixture for these experiments include: tubulin (3 mg/ml) in PEM buffer, GTP (1 µM), in the presence or absence of test compounds at 2.5, 5, 10, and 15 µM concentrations. Polymerization was monitored by increase in the Fluorescence as mentioned above at 37 ^{0}C.

7.4 Colchicine competition assay

The test compounds (**6d**, **7f**) of various concentrations 5 µM, 10 µM, 15 µM and 20 µM were incubated with 3 µM tubulin in the presence and absence of 3 µM colchicine in 30 mM Tris buffer for 60 min at 37 ^{o}C. CA-4 was used as a positive control whereas taxol was used as negative control which binds at the taxane site. After incubation the fluorescence of tubulin-colchicine complex was determined by using Tecan multimode reader with excitation wavelength of 350 nm and emission wavelength of 435 nm. 30 mM Tris buffer was used as blank which was subtracted from all the samples and the fluorescence values are normalized to DMSO (control)[42].

7.5 Immunohistochemistry

A549 cells were seeded on glass cover slips, incubated for 48 h in the presence or absence of test compounds **6d** and **7c** 25 nM concentrations. Following the termination of incubation, cells were fixed with 3% paraformaldehyde, 0.02% glutaraldehyde in PBS and permeabilized by dipping the cells in 100% methanol followed by overnight incubation in _4^{0}C. Later, cover slips were blocked with 1% BSA in phosphate buffered saline for 1 h followed by incubation with a primary anti tubulin (mouse monoclonal) antibody and FITC conjugated secondary mouse anti IgG antibody. Photographs were taken using the fluorescence microscope, equipped with FITC settings and the pictures were analyzed for the integrity of microtubule network. In parallel experiments, CA-4 (25 nM) was used as positive control for analyzing microtubule integrity

7.6 Morphological analysis for apoptosis with Hoechst staining

A549 cells were seeded at a density of 10,000 cells over 18-mm cover slips and incubated for 24 h. After incubation, cells were treated with the compounds **6d** and **7c** at 25 nM concentration for 48 h. Hoechst 33258 (Sigma Aldrich) was added to the cells at a concentration of 0.5 mg/mL and incubated for 30 min at 37^0C. Later, cells were washed with phosphate buffered saline (PBS). Cells from each cover slip were captured from randomly selected fields under fluorescent microscope (Leica, Germany) to qualitatively determine the proportion of viable and apoptotic cells based on their relative fluorescence and nuclear fragmentation.[43]

7.7 Mesurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential (ΔΨm) was determined by using JC-1 dye. Cell-permeable cationic carbocyanine dye JC-1 (Sigma aldrich India, Catlog- CS0390), also known as 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodine, emits green fluorescence (525 nm) in its monomeric form. However, upon transfer to the membrane environment of a functionally active mitochondrion, it exhibits an aggregation dependent orange-red fluorescence (emission at 590 nm). Briefly, cultures were treated with the test drugs for 48 h. After drug treatment the cells were incubated with JC-1 dye for 20 min at 37 °C. After incubation cultures were used for the measuring mitochondrial membrane potiential (ΔΨm), according to the manufacturer's instructions.

7.8 ROS generation

To evaluate intracellular reactive oxygen species (ROS) levels, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) fluorescent dye was used to clarify this issue. The nonpolar DCFH-DA is converted to the polar derivative DCFH by esterases when it is taken up by the cell. DCFH is nonfluorescent but is rapidly oxidized to the highly fluorescent DCF by intracellular H_2O_2 or nitric oxide. A549 cells were treated with compound **6d, 7c** and **CA-4** at 25 nM concentration for 48 h. To determine the production of ROS, cells were treated with 5 µM of DCFH2-DA at 37^0C for 30 min, and the fluorescence of DCF was measured at 530 nm after excitation at 485 nm (DCFH2-DA, after deacetylation to DCFH2, is oxidized intracellularly to its fluorescent derivative, DCF).[44]

7.9 Caspase 3 activity

Caspase-3 assay was conducted for detection of apoptosis in Lung cancer cell line (A549). The commercially available apoptosis detection kit (Sigma-Caspase 3 Assay kit, Colorimetric) was used. A549 cells were treated with compounds **6d** and **7c** at 25 and 50 nM concentrations for 48 h. After 48 h of treatment, cells were collected by centrifugation, washed once with PBS, and cell pellets were collected. Suspended the cell pellet in lysis buffer and incubated for 15 min. After incubation, cells were centrifuge at 20,000 rpm for 15 min and collected the supernatant. Supernatants were used for measuring caspase 3 activities using an ELISA-based assay, according to the manufacturer's instructions.

7.10 Determination of the Apoptotic DNA Ladder

Cells were seeded (1×10^6) in six well plates and incubated for 24 h. After incubation, cells were treated with compounds **6d** and **7c** along with CA-4 at 25 nM concentration for 48 h. After 48 h of drug treatment cells were collected and centrifuged at 2500 rpm for 5 min at 4^0C. Pellet was collected and washed with Phosphate buffered saline (PBS). And added 100 µl of Lysis buffer, centrifuged at 3000 rpm for 5 min at 4 ^{o}C and collected supernant. And add 10 µl of 10% SDS and 10 µl of (50 mg/mL) RNase-A and incubated for 2 h at 56^0c. After that 10 µl of Proteinase K (25 mg/mL) was added and incubated at 37^0c for 2 h. After incubation, 65 µl of 10 M Ammonium acetate and 500 µl of ice cold ethanol was added and mixed well. And this sample was incubated in _80 ^{o}C for 1 h. After that samples were centrifuged at 12000 rpm for 20 min at 4^0c and washed with 80% ethanol followed by air dried for 10 min at room temperature. Dissolved pellet in 50µl of TE buffer. After that, DNA laddering was determined by 2% agarose gel electrophoresis.[45]

8.0 Molecular modeling studies

All the compounds under study and reference compounds 3D structures were built and optimised using Gaussian 03W. These optimised 3D structures were utilised for Docking. All the compounds were Docked using AutoDockTools[46] software package. The co crystallised structure

of colchicine site tubulin downloaded from the PDB data bank (http:// www.rcsb.org/pdb/index.html; PDB code: 3E22).[47]

Acknowledgement

B.S acknowledge CSIR-UGC, New Delhi and for the award of Senior Research Fellowship. We also acknowledge CSIR for financial support under the 12th Five Year plan project "Affordable Cancer Therapeutics (ACT)" (CSC0301).

References

1. Jordan, MA.; Wilson, L. *Nat. Rev. Cancer*. **2004**, *4*, 253.
2. Prasad, V. J.; Christoph, L. *Nature Reviews Cancer.* **2001**, *1*, 109.
3. Jordan, A.; Hadfield, J. A.; Lawrence, N. J.; McGown, A. T. *Med. Res. Rev.* **1998**, *18*, 259.
4. McGown, A. T.; Fox, B. W. *Anti-Cancer Drug Design*. **1989,** *3*, 249.
5. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.;Genazzani, A. A. *J. Med. Chem.* **2006**, *49*, 3033.
6. Pettit, G. R.; Lippert, J. W., III Anti-cancer Drug Des. **2000**, *15*, 203.
7. Pettit, G. R.; Temple, C. Jr.; Narayanan, V. L.; Varma, R.; Simpson, M. J.; Boyd, M. R.; Rener, G. A.; Bansal, N. *Anti-cancer Drug Des.* **1995**, *10*, 299.
8. Nihei, Y.; Suzuki, M.; Okano, A.; Tsuji, T.; Akiyama, Y.; Tsuruo, T.; Saito, S.; Hori,K.; Sato, Y.*Jpn J. Cancer Res.* **1999**, *90*, 1387.
9. Thorpe, P. E.; Chaplin, D. J.; Blakey, D. C. *Cancer Res.* **2003**, *63*, 1144.
10. Kamal, A.; Prabhakar, S.; Ramaiah, M. J.; Reddy, P. V.; Reddy, C. R.; Mallareddy, A.; Shankaraiah, N.; Reddy, T. L. N.; Pushpavalli, S. N. C. V. L.; Bhadra, M. P. *Eur. J. Med. Chem.* **2011**, *46,* 3820.
11. Thomas, K. D.; Adhikari, A. V.; Chowdhury, I. H.; Sumesh, E.; Pal, N. K. *Eur. J. Med. Chem.* **2011**, *46,* 2503.
12. He, R.; Chen, Y.; Chen, Y.; Ougolkov, A. V.; Zhang, J. S.; Savoy, D. N.;. Billadeau, D. D; Kozikowski, A. P. *J. Med. Chem.* **2010**, *53,* 1347.

13. Kamal, A.; Shankaraiah, N.; Devaiah, V.; Laxma Reddy, K.; Juvekar, A.; Sen, S.; Kurianb, N.; Zingdeb, S. *Bioorg. Med. Chem. Lett.* **2008***, 18,* 1468.
14. Amol, H. K.; Pravin, V. S.; Atul, H. K.; Sharad, K. P.; Bapurao, B. S.; Murlidhar, S. S.; *Eur. J. Med. Chem*. **2010**, *45*, 3142.
15. Hong, C.; Song, Z.; Xiaochen, W.; Xiaowei, T.; Ming, Z.; Yanling, L.; Liting, C.; Jing, L.; Yongfeng, L.; Dailin, L.; Shi, Z.; Tan, L. *Eur. J. Med. Chem* . **2011**, *46*, 4709.
16. Chung, Y. C.; Pei, H. L.; Yong, Y L.; Wen, T. Y.; Wan, P. H.; Chia, C. H.; Ying, T. L.; Long, S. C.; Cheng, T. H.; Jeh, J. W.; Mei, I. C. *Bioorg. Med. Chem. Lett* .**2013**, *23*, 6854.
17. Xiaolian, L.; Yanjie, L.; Qianqian, W.; Yukun, Y.; Hua, Z.; Xuhong, Q. *Eur. J. Med. Chem.* **2011**, *46*, 1274.
18. Kristin, O.; Jérémie, F. D. C.; Sylvie, D.; Osman, A. B. S. M. G.;, Ingebrigt, S.; Trond, V. H. *Bioorg. Med. Chem.* **2010,** *18*, 6874.
19. Stefely, J. A.; Palchaudhuri, R.; Miller, P. A.; Peterson, R. J.; Moraski, G. C.; Hergenrother, P. J.; Miller, M. J. *J. Med. Chem.* **2010***, 53,* 3389.
20. a. Kamal, A.; Reddy, N.V.S.; Lakshma, N. V.; Saidi, R. V.; B. Prasad, B.; Vijaykumar D, N.; Srinivasulu, V.; Vishnuvardhan, M. V. P. S.; Suresh, R. C. *ChemMedChem* **2014**, *9*, 117; b. Kamal, A.; Reddy, V. S; Santosh, K.; Sumit, S. C; Shaik, A. B.; Bharath Kumar, G.; Kishor, C.; Reddy, M. K.; Rao, M. P. N.; Nagabhushana, A.; Kallaganti, V.; Ramakrishna, S.; Anthony, A.; Srigiridhar, K. *ChemMedChem* **2013**, *8*, 2015; c. Kamal, A.; Ashraf, Md.; Vishnuvardhan, M. V. P. S.; Faazil, S.; Lakshma, N. V. *Bioorg. Med. Chem. Lett.* **2014,** *24*, 147; d. Kamal, A.; Jaki , R. T.; Janaki, R. M.; Adil, S. F.; Pushpavalli, S. N. C. V. L.; Ganesh, R.; Sarma, P.; Bhadra, U.; Bhadra, M.P. *Bioorg. Med. Chem* **2013***,* 21, 6414; e. Kamal, A.; Mallareddy, A.; Janaki, R. M.; Pushpavalli, S.N.C.V.L.; Suresh, P.; Kishor, C.; Murty, J.N.S.R.C N.; Rao, S.; Sowjanya, G.; Anthony, A.; Bhadra, M.P.; *Eur. J. Med. Chem.* **2012**, 56, 166. f. , Kamal, A.; Reddy, M. K.; Shaik, T. B.; Rajender; Srikanth, Y.V.V.; Reddy, V. S.; Kumar, G. B.;. Kalivendi, S. V. *Eur. J. Med. Chem.* **2012**, *50*, 9.
21. a. Pettit, G. R.; Moser, B. R.; Boyd, M. R.; Schmidt, J. M.; Pettit, R. K.; Chapuis, J. C. *Anti- Cancer Drug Des*. **2001**, *16*, 185; b. Johnson, T. B.; Bengls, R.; Hydantoins. *J. Am.*

Chem. Soc. **1912**, *34*, 1054; c. Firnau, G.; Nahmias, C.; Garnett, S. *J. Med. Chem* ***1973,*** *16(4);416.*

22. Dengfeng, D.; Guijia, H,; Sivakoteswara, R. M.; Sridhar, A.; Yunjeong, K.; Kyeong, O. C.; William, C.; Groutas. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 377.
23. Keith, A.; Monk.; Rogelio, S.; Mallinath, B. H.; Benon, E. M.; Freeland, A. J.; Scott, W. S.; Klaus, E.; Mary, L. T.; Charles, M. G.; Monte, R. R.; George, R. P.; Kevin, G. P.; *Bioorg. Med. Chem.* **2006**, *14*, 3231.
24. Kamal, A.; Mallareddy, A.; Suresh, P.; Nayak, V. L.; Shetti, R. V. C. R. N. C.; Rao, N. S.; Tamboli, J. R.; Shaik, T. B.; Vishnuvardhan, M. V. P. S.; Ramakrishna, S. *Eur. J.* M*ed. Chem.* **2012**, *47*, 530.
25. Chan, K. T.; Meng, F. Y.; Li, Q.; Ho, C. Y.; Lam, T. S.; To, Y.; Lee, W. H.; Li, M.; Chu, K. H.; Toh, M. *Cancer Lett*, , **2010**, *294,* 118.
26. Shen, J. K.; Du, H. P.; Yang, M.; Wang, Y. G.; and Jin, J. *Ann. Hematol*. **2009**, *88,* 52.
27. Blank, M.; Shiloh, Y. *Cell Cycle.* **2007**, *6*, 686.
28. Kanthou, C.; Greco, O.; Stanford, A.; Cook, I.; Knight, R.; Benzakour, O.; Tozer, G.; *Am. J. Pathol.* **2004**, *165*, 1401.
29. Huber, K.; Patel, P.; Zhang, L.; Evans, H.; Westwell, A. D.;Fischer, P. M.; Chan, S.;. Martin, S. *Mol. Cancer Ther.* **2008**, *7*, 143.
30. Kamal, A.; Srikanth, Y. V. V.; Shaik, T. B.; Khan, M. N. A.; Ashraf, M.; Reddy, M. K.; Kumar, K. A.;.Kalivendi, S. V. *Med. Chem. Commun.* **2011**, *2*, 819.
31. a. Catherine, R.; Pascale, B.; Ve´ronique, B. R.; Catherine, G.;| Robert, G.; Manon, C.; Se´bastien, C.; Jean-Pierre, F.; and Vincent, P. *Biochemistry.* **2006**, *45,* 9210. b. Surela, B.; N. Maruthi, K.; Arnab, G.; Mukund, P. T.; Dalip, K.; Gopal, C. *PLOS ONE.* **2013**, 8, e76286.
32. Leoni, L. M.; Hamel, E.; Genini, D.; Shih, H.; Carrera, C. J.; Cottam, H. B.; Carson, D. A. *J. Natl. Cancer Inst*. **2000**, *92*, 217.
33. Pasquier, E.; Kavallaris, M. *IUBMB Life.* **2008**, *60*, 165.
34. Gonda, K.; Tsuchiya, H.; Sakabe, T.; Akechi, Y.; Ikeda, R.; Nishio, R.; Terabayashi, K.; Ishii, K.; Matsumi, Y.; Ashla, A. A.; Okamoto, H.; Takubo, K.; Matsuoka, S.; Watanabe, Y.; Hoshikawa, Y.; Kurimasa, A.; Shiota, G. *Biochem. Biophys. Res. Commun.* **2008**, *370*, 629.

35. Fernando, T; Jose, ´ Q; Javier, C; Juan, F. L; Francisco, L; Jaime, B; Francisco, E. *Cancer Letters* **2008**, *269*, 139.

36. Zhang, L. H.; Wu, L.; Raymon, H. K.; Chen, R. S.; Corral, L.; Shirley, M. A.; Narla, R. K.; Gamez, J.; Muller, G. W.; Stirling, D. I.; Bartlett, B. J.; Schafer, P. H.; Payvandi, F .*Cancer Res.* **2006**, *66*, 951.

37. Weir, N. M.; Selvendiran, K.; Kutala, V. K.; Tong, L.; Vishwanath, S.; Rajaram, M.; Tridandapani, S.; Anant, S.; Kuppusamy, P. *Cancer Biol. Ther.* **2007**, *6*, 178.

38. Iyer, S.; Chaplin, D. J.; Rosenthal, D. S.; Boulares, A. M.; Li, L.; Smulson, M. E. *Cancer Res.***1998**, *58*, 4510.

39. Zhu, H.; Zhang, J.; Xue, N.; Hu, Y.; Yang, B.; He, Q. *Invest. New Drugs.* **2010**, *28*, 493.

40. Hou, Q.; Zhao, T. B.; Zhang, H. L.; Lu, H. X.; Zhang, Q. X.; Sun, L.; Fan, Z. *Mol. Immunol*. **2008**, *45*, 1044.

41. Szumilak, M.; Szulawska, M. A.; Koprowska, K.; Stasiak, M.; Lewgowd, W.; Stanczak, A.; Czyz, M. *Eur. J. Med. Chem*. **2010**, *45*, 5744.

42. Brett, M. I.; Luca, L.; Esther, A.; Cornelius, J. O. C.; Yaw S. T.; Huw, M.L. D.; Grahame. Mc.; Ashok. R. V.; and David. R. S. *Nature Communications.* **2013**, 5, 3155.

43. Shankar, R.; Chakravarti, B.; Singh, U. S.; Ansari, M. I.; Deshpande, S.; Dwivedi, S. K. D.; Bid, H. K.; Konwar, R.; Kharkwal, G.; Chandra, V.; Dwivedi, A.; Hajela, K. *Bioorg. Med. Chem*. **2009**, *17*, 3847.

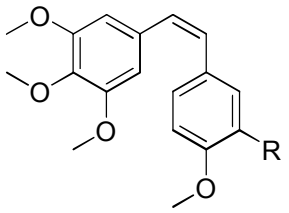
44. Aneta, R; Agnieszka, M; Arkadiusz, G; Marzena, S; Agnieszka, S; Józef, D; Zofia, J. *Toxicology in Vitro* **2013**, *27*, 239.

45. Kamal, A.; Ramakrishna, G.; Lakshma, N. V.; Raju, P.; Subba, R. A.V.;. Viswanath, A.; Vishnuvardhan, M.V.P.S.; Ramakrishna, S.; Srinivas, G.; *Bioorg. Med. Chem*. **2012**, *20* 789.

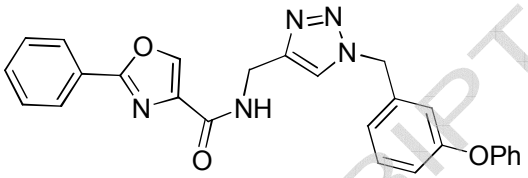
46. Warren L. D. The PyMOL Molecular Graphics System, DeLano Scientific LLC, San Carlos, CA, http://www.pymol.org.

47. Ravelli, R. B.; Gigant, B.; Curmi, P. A.; Jourdain, I.; Lachkar, S.; Sobel, A.; Knossow, M. *Nature* **2004**, 428, 198.

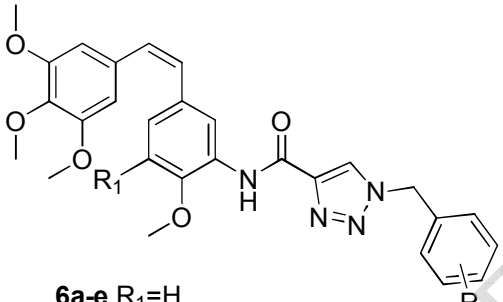
Figures



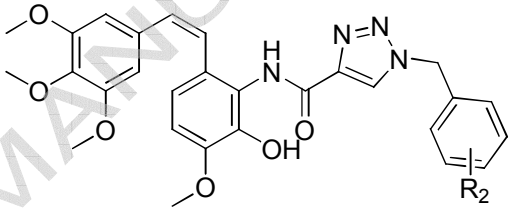
1 Combretastatin A-4 (CA4); R=OH.
2 Combretastatin A-4P (CA4P): R=OPO_3Na_2
3 AVE-8063 :R =NH2
4 AVE-8062 :R=NH-serine



5



6a-e,R_1=H.
7a-e,R_1=OMe
R_2 = 3-OMe, 3-OPh, 4-OMe, 4-F, 3,4,5-tri-OMe



8a-e

R_2=3-OMe, 3-OPh, 4-OMe, 4-F, 3,4,5-Tri-OMe

Figure 1: Chemical structures of combertastatin derivatives (**1-4**), 1,2,3-triazole- derivative (**5**), and compounds (**6-8a-e**).

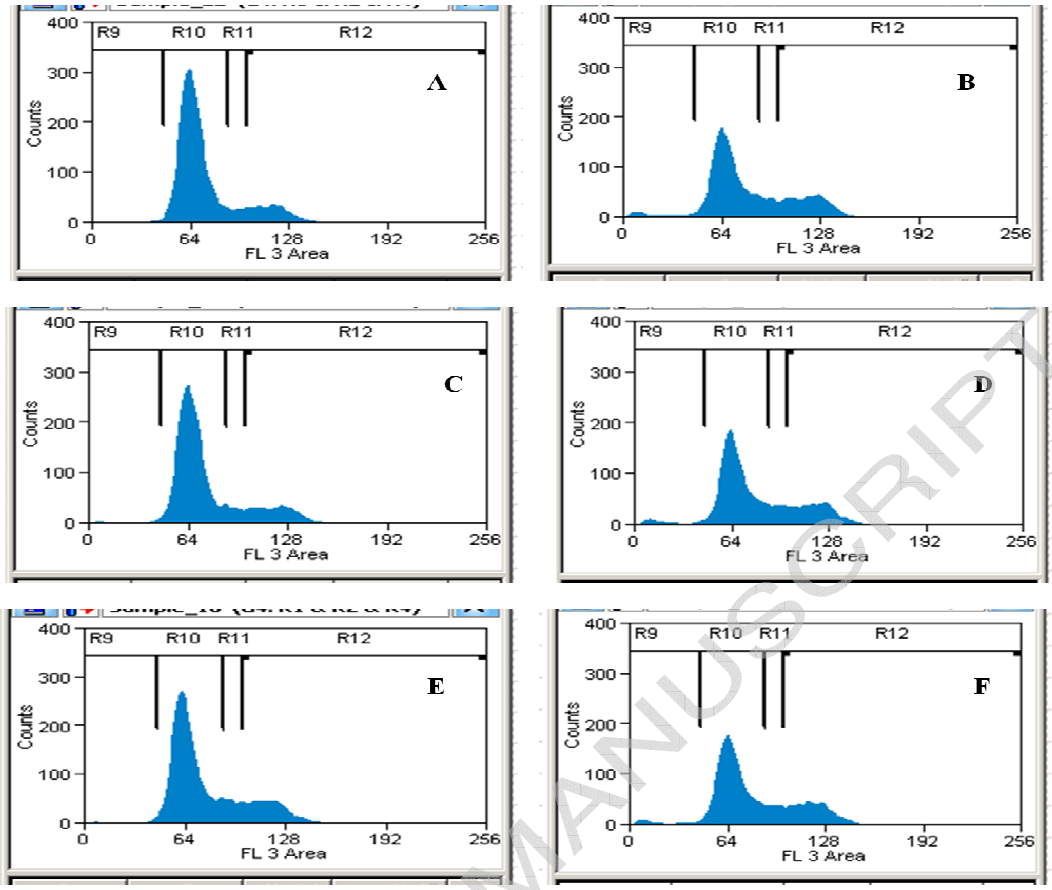


Figure 2: Flow cytometric analysis in A549 lung cancer cell lines after treatment with compound **6d** and **7c** at 25 and 50 nM concentrations for 48 h; **A:** Control cells (A549), **B:** CA-4 (50 nM), **C: 6d** (25 nM), **D: 6d** (50 nM), **E: 7c** (25 nM) and **F: 7c** (50 nM).

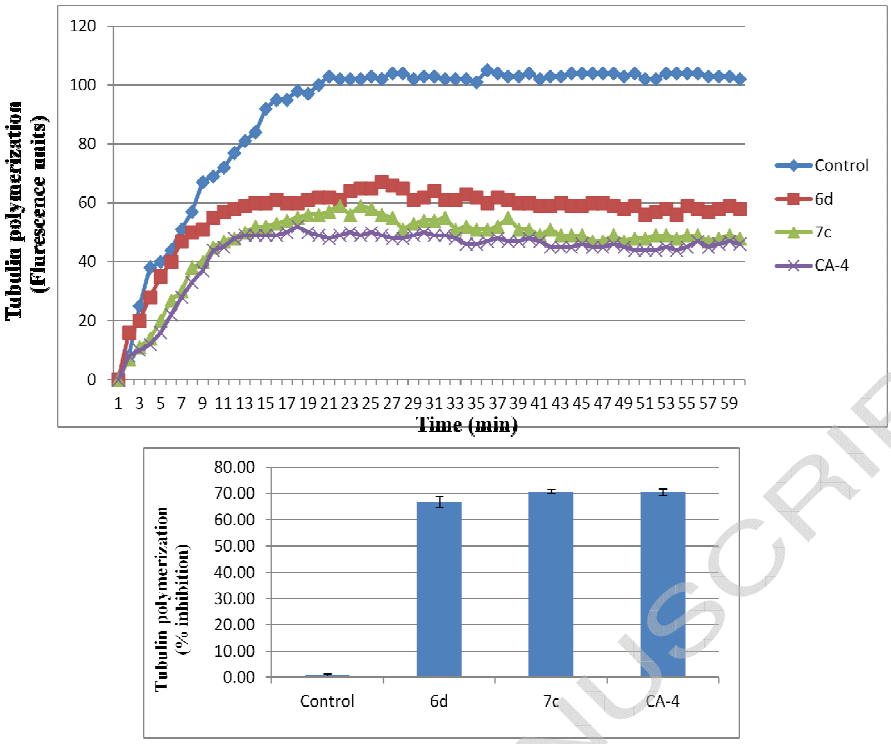


Figure 3: Effect of conjugates **6d** and **7c** on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37°C. All the compounds were included at a final concentration of 3 µM. CA-4 was used as a positive control. Values indicated are the mean ± SD of two different experiments performed in triplicates.

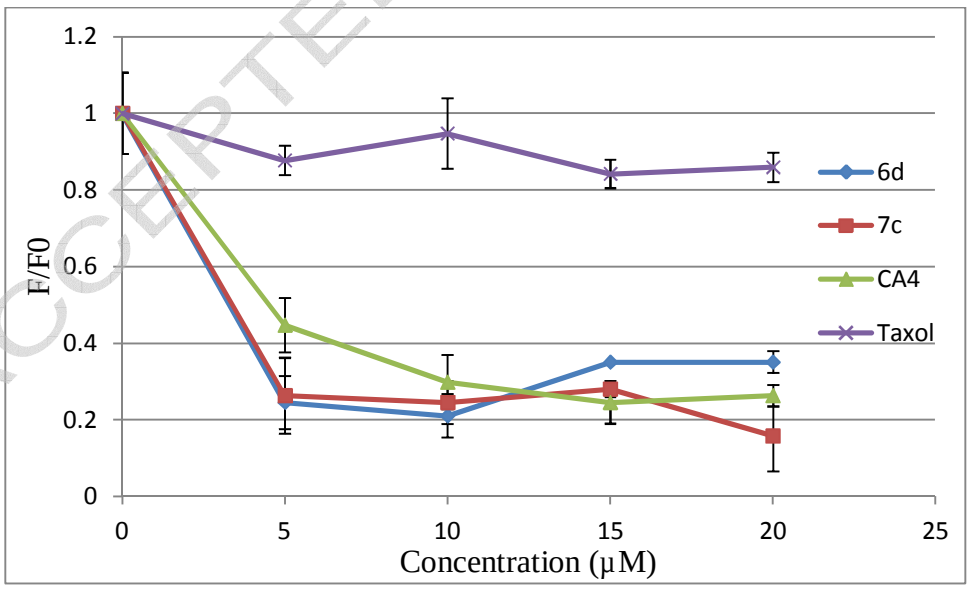


Figure 4: Fluorescence based colchicine competitive binding assay of conjugates **6d** and **7c** were carried out at various concentrations containing 3 µM of tubulin and colchicine for 60 min

at 37 ^{o}C. Combretastatin A4 was used as a positive control where as taxol was used as negative control which binds at taxane site. Fluorescence values are normalized to DMSO (control).

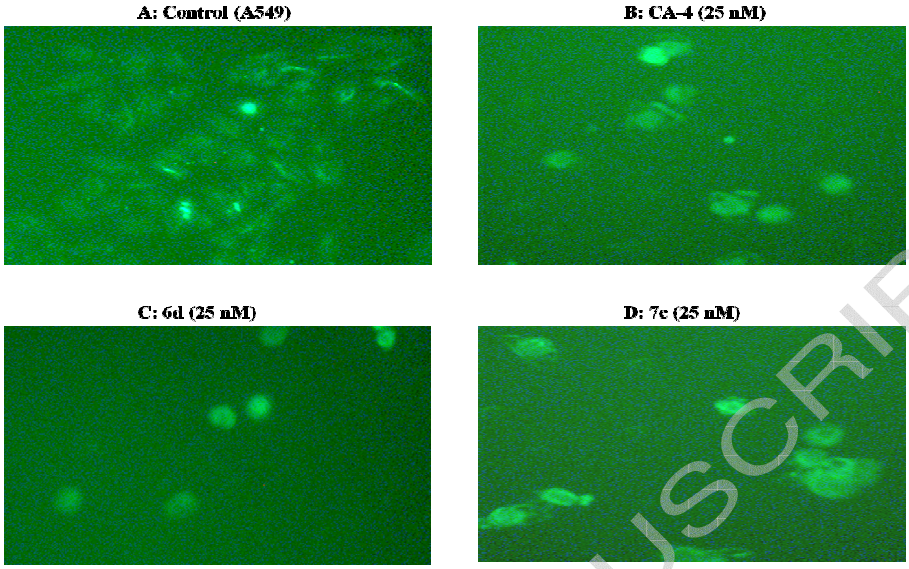


Figure 5: Immunohistochemistry (IHC) analysis of conjugates on the microtubule network: A549 cells were treated with conjugates **6d** and **7c** 25 nM concentration for 48 h followed by staining with α tubulin antibody. CA-4 was used as the reference compound

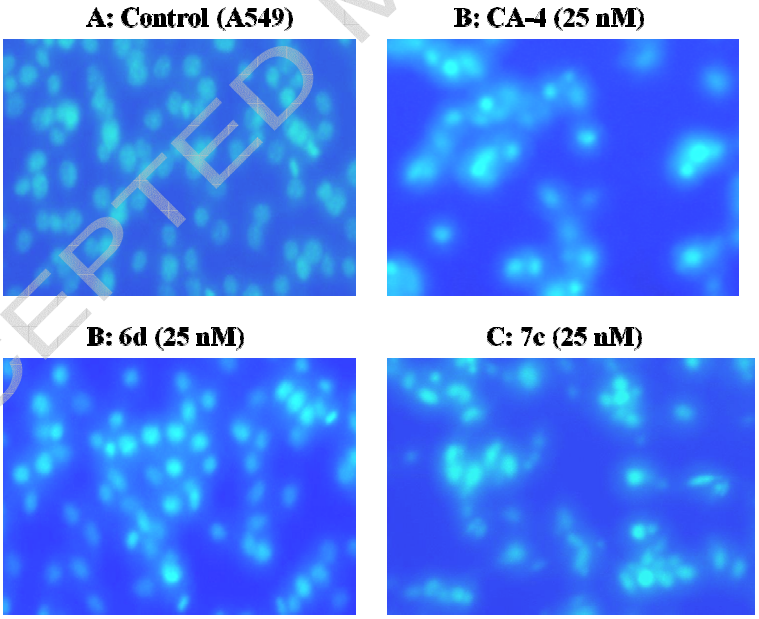


Figure 6: Hoechst staining in A549 lung cancer cell line. **A:** Control cells (A549), **B:** CA-4 (25 nM), C: **6d** (25 nM) and D: **7c** (25 nM).

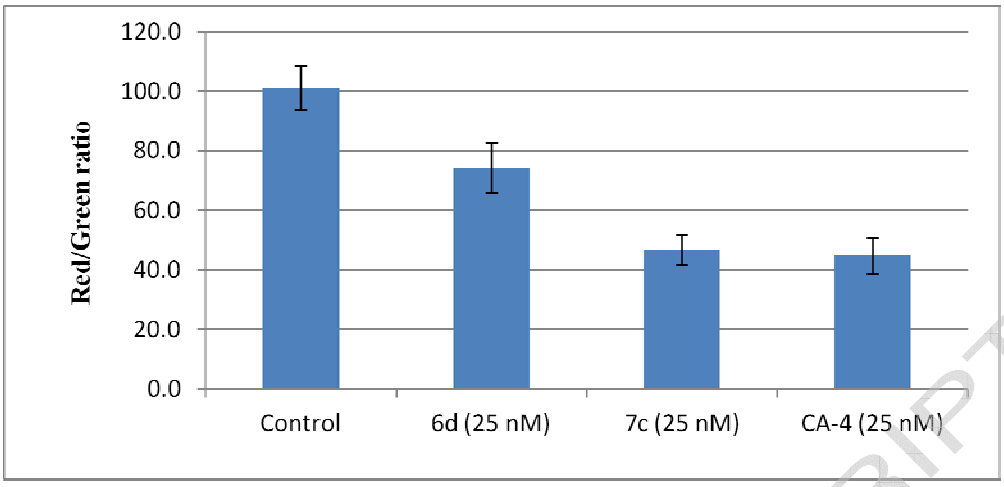


Figure 7: Figure represents the loss of mitochondrial membrane potential in human lung cancer cells, A549 cells after 48 h of drug treatment with compounds 6d, 7c and CA-4 at 25 nM concentration.

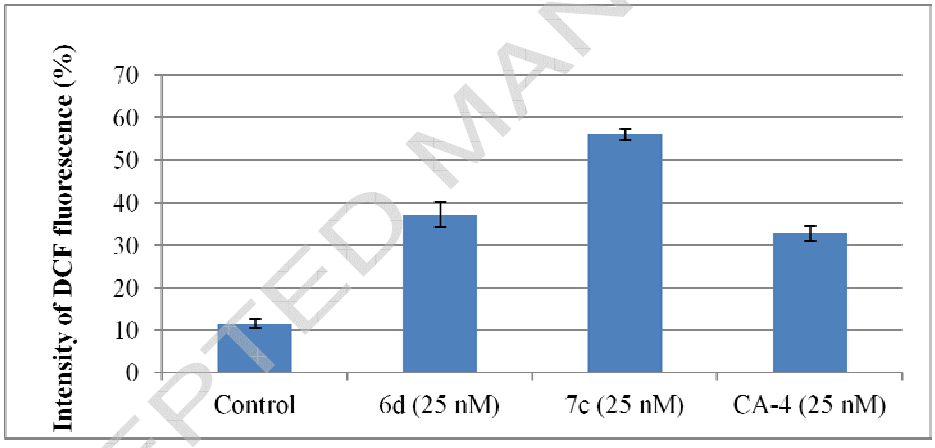


Figure 8: The effect of **6d** and **7c** on the ROS production in human lung cancer cell line A549

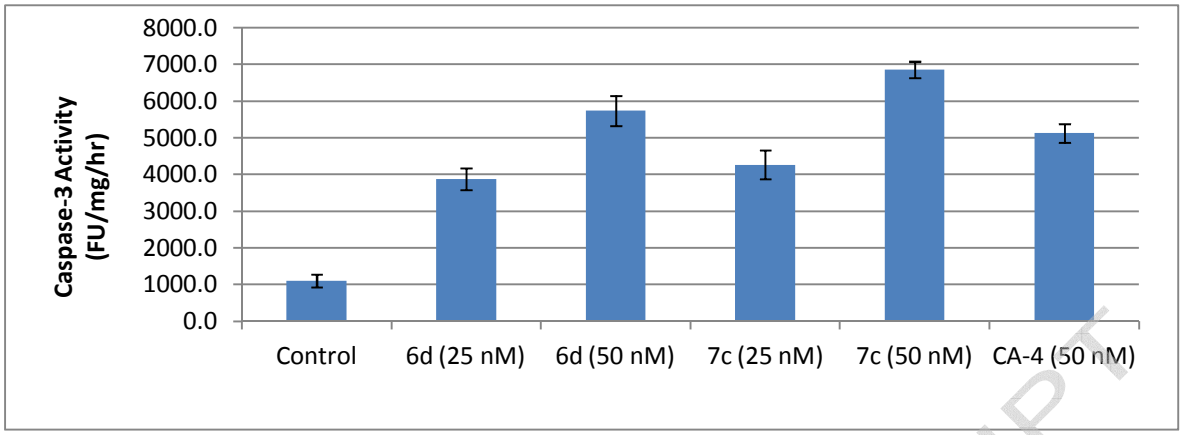


Figure 9: Effect of compounds **6d**, **7c** and CA-4 on caspase-3 activity: A549 cells were treated with compounds, **6d** and **7c** at 25 and 50 nM concentrations for 48 h. Values indicated are the mean ± SD of two different experiments performed in triplicates.

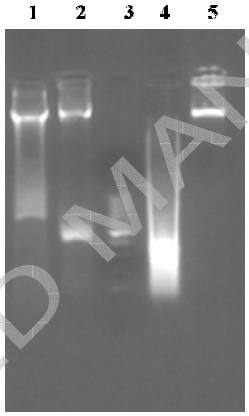
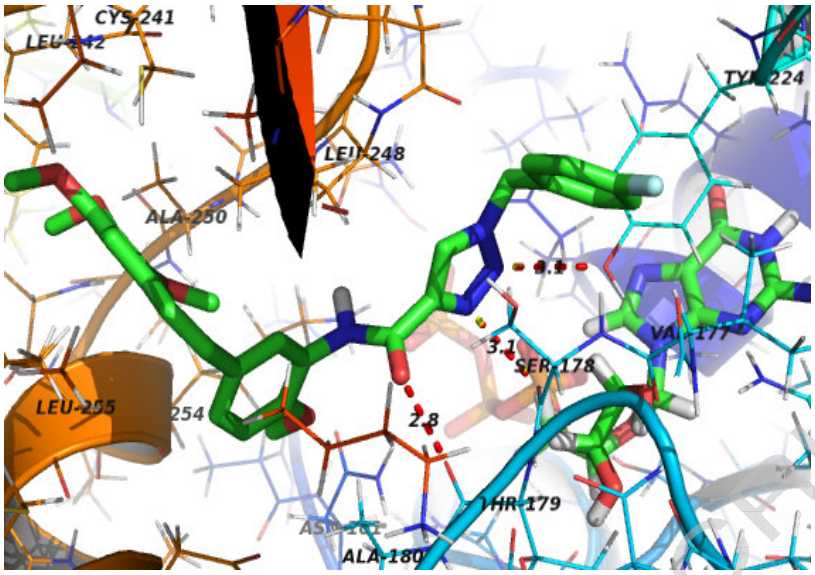
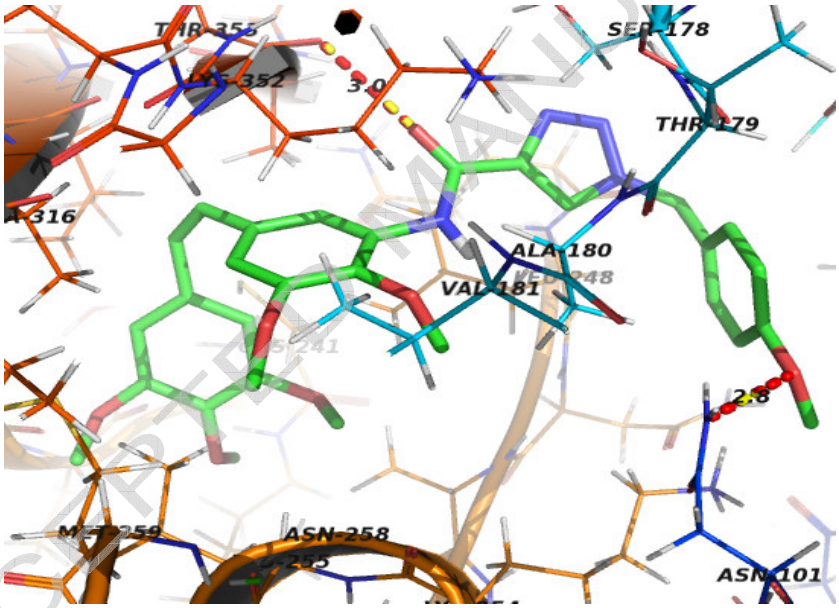


Figure 10: DNA fragmentation of compounds **6d**, **7c** and CA-4 in A-549 lung cancer cells: Lane-1: CA-4 (25 nM), Lane-2: **6d** (25 nM), Lane-3: Marker (100 bp), Lane-4: **7c** (25 nM) and Lane-5: Untreated control



6d



7c

Figure 11: Interaction of the compound **6d** and **7c** with colchicines binding site of tubulin. The Hydrogen bonding interactions are shown in red color.

Table 1: IC_{50} values[a](in µM) for compounds **6a-e, 7a-e, 8a-e** in selected human cancer cell lines

Compounds	Hela[b]	HepG2[c]	A549[d]	ACHN[e]	MCF-7[f]
6a	1.140	1.799	1.862	5.272	1.606
6b	3.230	3.715	2.570	5.744	4.187

6c	2.950	2.084	2.137	3.311	3.715
6d	0.068	0.058	0.053	1.659	0.597
6e	0.190	0.620	0.089	2.041	1.513
7a	0.570	0.513	0.406	2.890	1.273
7b	5.880	3.990	2.679	6.606	4.265
7c	0.081	0.098	0.044	1.698	0.818
7d	0.320	1.364	0.066	3.090	0.897
7e	0.720	3.388	0.181	4.764	1.380
8a	4.571	1.949	1.622	9.506	1.936
8b	5.010	4.677	3.631	7.413	4.265
8c	3.540	1.202	1.452	5.888	2.618
8d	0.330	0.354	0.568	3.388	1.862
8e	0.770	1.698	0.830	5.128	2.398
CA-4	0.018	0.008	0.052	0.005	0.072

[a] 50% Inhibitory concentration after 48 h of drug treatment and the values are average of three individual experiments. [b] Human cervical cancer, [c] Human liver cancer, [d] Human lung cancer, [e] Human renal cell carcinoma [f] Human breast cancer

Table 2: Effect of compounds **6d**, **7c**, and CA-4 on cell-cycle phase distribution in A549 cells.

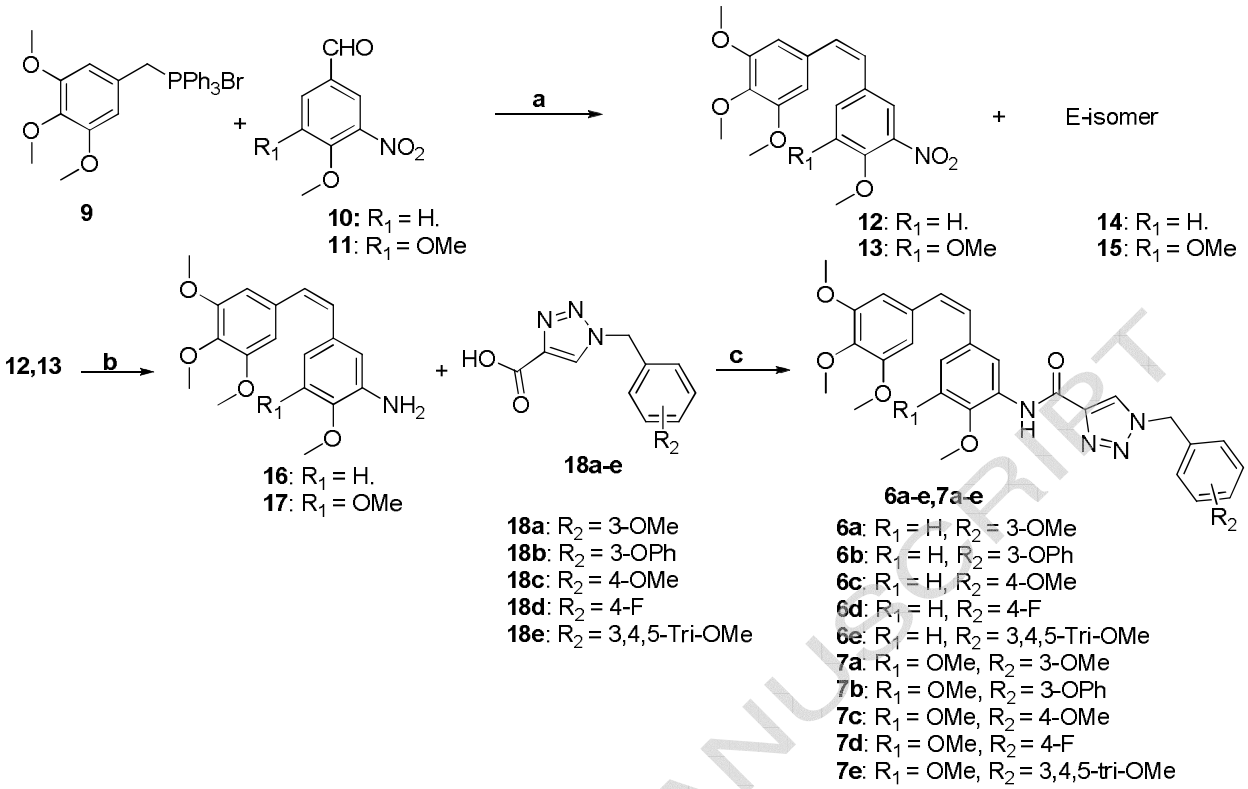
Compound	**SubG1 %**	**G0/G1 %**	**S %**	**G2/M %**
A: Control (A549)	1.41	87.75	4.69	6.15
B: CA-4 (50 nM)	4.11	61.32	8.09	26.48
C: 6d (25 nM)	1.52	75.42	5.49	17.57
D: 6d (50 nM)	4.36	61.99	7.93	25.73
E: 7c (25 nM)	1.20	69.59	7.84	21.37
F: 7c (50 nM)	3.46	62.01	7.90	26.63

Table 3: Inhibition of tubulin polymerization (IC_{50}) of compounds **6d** and **7c**.

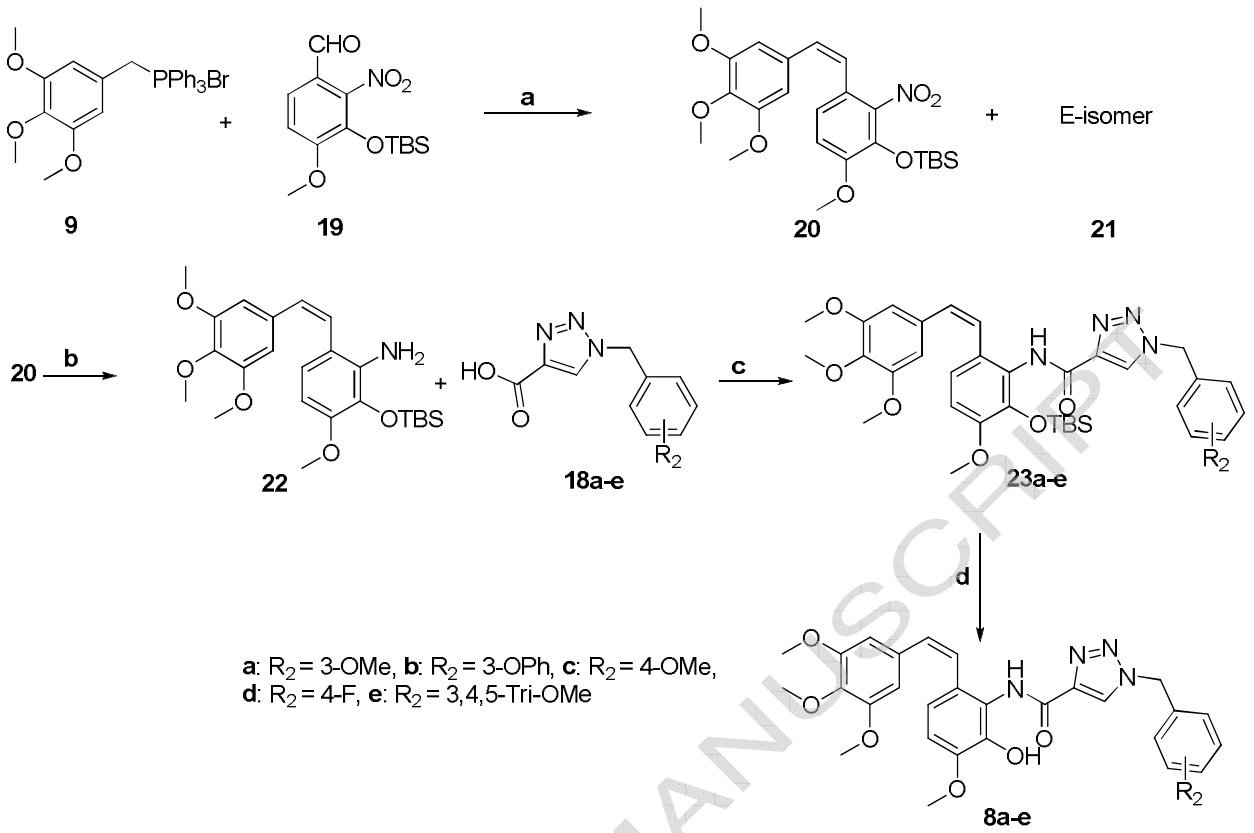
Compound	**IC_{50}[a] ± SD (in µM)**
6d	1.16±0.40
7c	0.95±0.11
CA-4	1.08±0.33

[a] Concentration of drug to inhibit 50% of tubulin assembly.

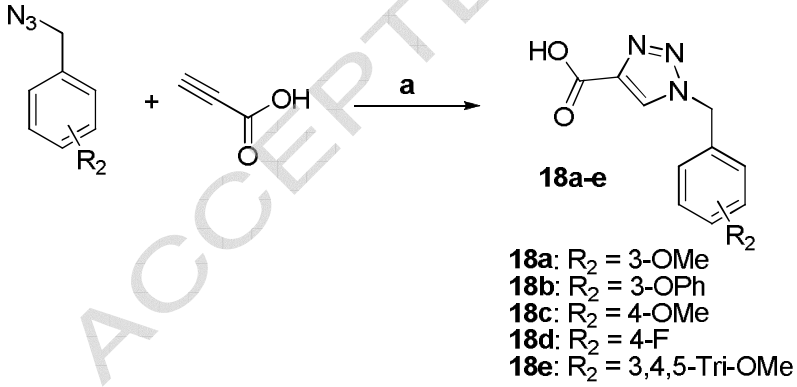
Schemes



Scheme 1 *Reagents and conditions*: (a) NaH, dry CH_2Cl_2, 0 ^oC to rt 12 h; (b) Zn, HCO_2NH_4, MeOH, rt, 6 h; (c) EDCI, HOBT, DMF, 0 ^oC to rt 12 h;



Scheme 2 *Reagents and conditions*: (a) NaH, dry CH_2Cl_2, 0 ^oC to rt 12 h; (b) Zn, HCO_2NH_4, MeOH, rt, 6 h; (c) EDCI, HOBT, DMF, 0 ^oC to rt 12 h; (d) TBAF, THF, 0 ^oC, 1 h.



Scheme 3 *Reagents and conditions*: (a) sodium ascorbate/$CuSO_4$/*t*-BuOH/H_2O, 12 h.

Graphical Abstract

Synthesis and biological evaluation of 1,2,3-triazole linked aminocombretastatin conjugates as mitochondrial mediated apoptosis inducers

Ahmed Kamal,*[a,b] Bajee Shaik,[a] V. Lakshma Nayak,[a] Burri Nagaraju,[a] Jeevak Sopanrao Kapure,[a,b] M. Shaheer Malik,[a] B. Prasad[a]