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Synthesis and Biological Evaluation of Arylcinnamide linked Combretastatin-A4 hybrids as Tubulin Polymerization Inhibitors and Apoptosis Inducing Agents

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Abstract: A series of new molecules have been designed based on a hybridization approach by combining the arylcinnamide and combretastatin pharmacophores. These were synthesized and evaluated for their cytotoxic activity, effect on inhibition of tubulin polymerization and apoptosis inducing ability. Most of the conjugates exhibited significant cytotoxic activity against some representative human cancer cell lines and two of the conjugates **6i** and 6**p** displayed potent cytotoxicity with GI_{50} values of 56 nM and 31 nM respectively against the human breast cancer cell line (MCF-7). SAR studies revealed that 3,4-substitution on the phenyl ring of the cinnamide moiety is beneficial for enhanced cytotoxicity. Moreover, G2/M cell cycle arrest was induced by these conjugates (**6i** and **6p**) apart from tubulin polymerization inhibition (IC_{50} of 1.97 μM and 1.05 μM respectively). Further, mitochondrial membrane potential, Annexin V-FITC and caspase-9 activation assays suggested that these conjugates induce cell death by apoptosis. Docking studies revealed that these conjugates interact and bind at the colchicine binding site of the tubulin.

***Keywords*:** CA-4, Cinnamides, Conjugate, Cytotoxicity, Cell cycle, Tubulin polymerization.

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Microtubules are protein biopolymers formed through polymerization of heterodimers of α and β tubulins. Tubulin, a globular protein, has emerged as one of the valuable molecular targets in anticancer drug discovery. Tubulin plays a pivotal role in many cellular processes such as maintenance of skeletal integrity of cell, cell signaling and segregation of chromosomes during mitosis.[1,2] Several chemical compounds 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, combretastatins are isolated from the bark of the South African tree *Combretum caffrum*, 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,** Fig**. 1**) is the lead compound of this class with high cytotoxic potency against murine lymphocytic leukemia, human ovarian and colon cancers. However it suffers from limitation of poor aqueous solubility and to overcome this, prodrug CA-4P (**2**, Fig**. 1**) 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 ring A and the crucial role played by *cis*-configuration of the olefinic bond to impart activity for this well-established pharmacophoric structure. The ring B has been reported to be tolerant for structural modification for enhancing the pharmacological profile of the molecule. Particularly, the aminocombretastatins such as AVE-8063 (**3**, Fig**. 1**), AVE-8062 (**4**, Fig**. 1**) with an amino and amino-serine groups replacing the hydroxyl group at the C-3 position of ring B showed potent inhibition of tubulin polymerization activity and cytotoxicity. In recent years antitumor activity of cinnamic acid derivatives was explored by many research groups.[10-13] Phenylcinnamide derivative 8H (**5**, Fig**. 1**), initially identified by Hergenrother as a potential anticancer agent,[14] induces G2/M-phase cell cycle arrest and cell death in cancer cell lines. We surmised that by integrating the phenylcinnamide pharmacophore onto the combretastatin framework may furnish new anticancer agents with enhanced activity. Our efforts along this direction validated this hypothesis and the results are presented in the following sections.

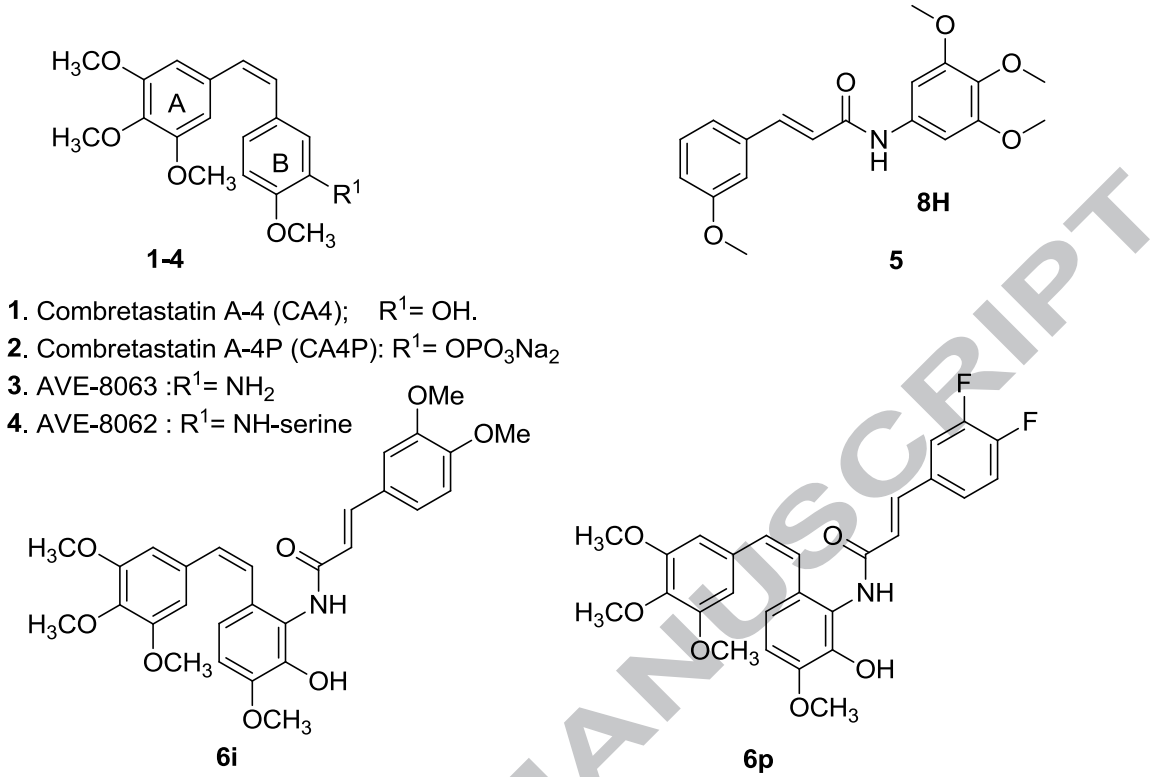
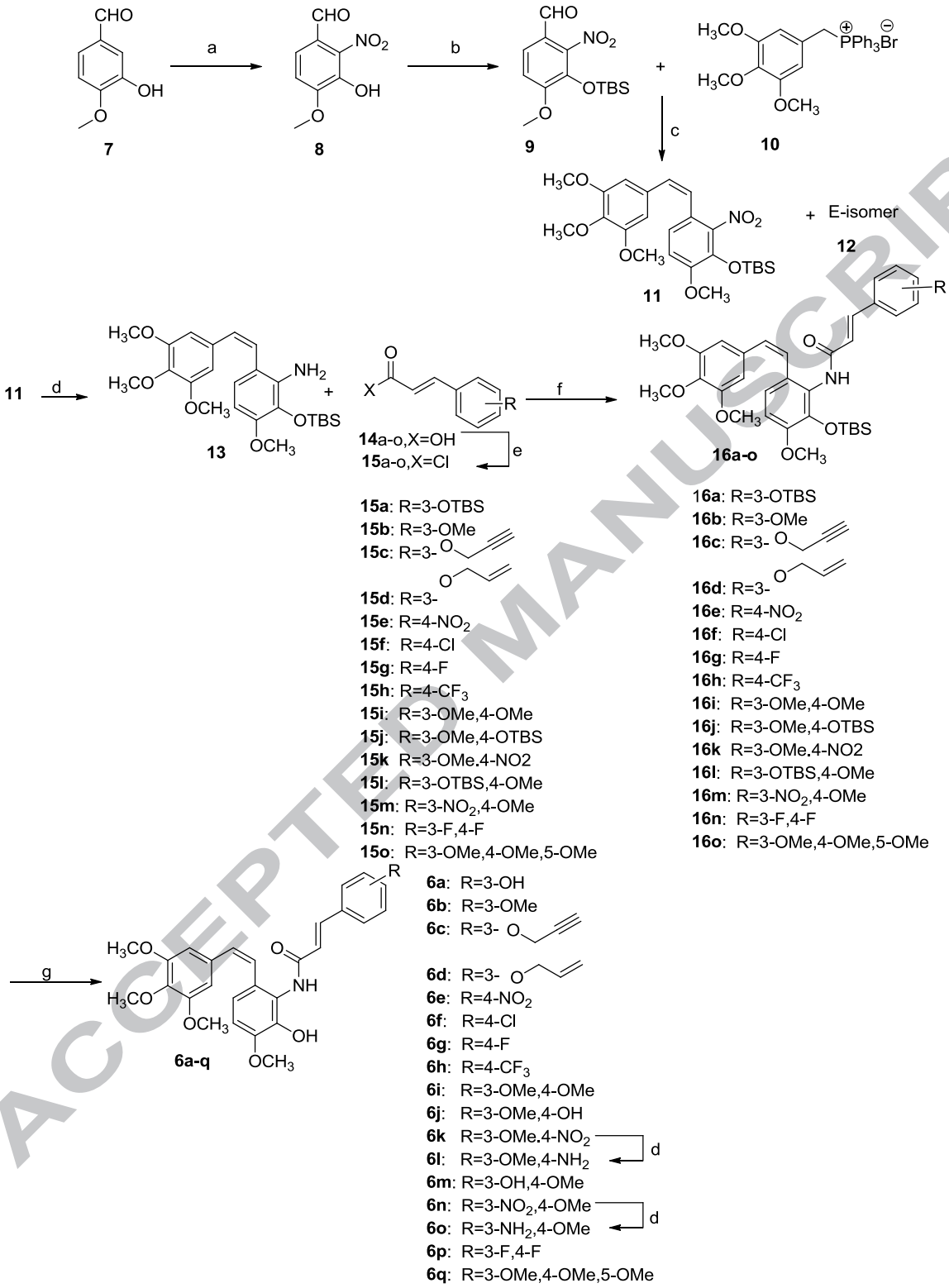


Figure 1: Tubulin polymerization inhibitors.

The synthesis of cinnamamide linked combretastatin conjugates (**6a-q**) was accomplished as illustrated in Scheme **1**. The key intermediate, silyl protected nitrobenzaldehyde (**9**) was prepared by nitration of isovanillin (**7**) with nitronium tetrafluoroborate (NO_2BF_4) in CH_2Cl_2 at -40 °C to give 2-nitro isovanillin (**8**), followed by the free OH group was protected with TBS-Cl in CH_2Cl_2 using imidazole as base. The silyl protected nitrobenzaldehyde (**9**) was subjected to Wittig reaction with 3,4,5-trimethoxybenzyl triphenylphosphonium bromide (**10**) in the presence of sodium hydride to provide a mixture of nitro [*Z*]-stilbene (**11**) and [*E*]-stilbene (**12**)[17]. The nitro [*Z*]-stilbene (**11**) was subjected to reduction with zinc-ammonium formate to give the corresponding amino [*Z*]-stilbene (**13**). The amino stilbene was coupled with different substituted cinnamic acid chlorides (**15a-o**) [generated from the corresponding cinnamic acids (**14a-o**)] in the presence of triethyl amine to afford the silyl protected cinnamamide linked combretastatin conjugates (**16a-o**). The later on deprotection with TBAF afforded the desired conjugates (**6a-q**). All the new compounds were characterized by standard spectroscopic analysis.



Scheme 1: Reagents and conditions: (a) NO_2BF_4, CH_2Cl_2, -40 °C; (b) TBSCl, imidazole, CH_2Cl_2, 0 °C to rt; (c) NaH, CH_2Cl_2, 0 °C to rt 12 h; (d) Zn, HCO_2NH_4, MeOH, rt, 6 h; (e)

$(COCl)_2$, CH_2Cl_2, DMF(cat), 0 °C to rt, 3 h; (f) Et_3N, THF,0 °C to rt, 3 h; (g) TBAF, CH_2Cl_2, 0 °C, 1 h.

The arylcinnamide linked combretastatin-A4 hybrids (**6a-q**) were then evaluated for their cytotoxic activity against selected human cancer cell lines like; MCF-7 (breast-ER positive), DU-145 (prostate), Hop-62, HeLa (cervical), K562 (LIKK), SK-OV-3, Colo-205 (colon), MAIPa-Ca-2 by using Sulforhodamine B (SRB) method.[18,19] The results of this cytotoxicity data expressed as GI_{50} values in comparison with CA-4 is summarized in Table **1**. Interestingly, these hybrids showed considerable cytotoxic activity against most of the cell lines with subnanomolar to micromolar range. Moreover, all the compounds (**6a-q**) showed more pronounced activity against MCF-7 cancer cells compared to other cell lines tested with only a few exceptions. Among them, **6i** and **6p** exhibited enhanced cytotoxic activity than CA-4 against MCF-7 cancer cell lines with GI_{50} values of 0.056 μM and 0.031 μM respectively.

Table 1: Cytotoxic activity ([a]GI_{50} in μM) data of compounds, **6a-q** by SRB method.

S.No	[b]MCF7	[c]DU145	[d]HOP62	[e]HeLa	[f]K562	[g]SK-OV-3	[h]Colo205	[i]MIA-PaCa-2
6a	0.063	0.070	0.082	0.0949	0.094	0.097	13.4	0.098
6b	59.5	>100	65.4	18.1	0.098	17.6	>100	93.9
6c	11.6	96.4	>100	>100	>100	>100	>100	>100
6d	0.083	0.085	0.060	0.0787	0.091	0.082	18.0	0.098
6e	0.072	0.085	0.061	15.7	0.097	17.0	10.3	40.8
6f	55.5	54.1	79.4	38.8	24.9	62.6	>100	>100
6g	0.066	0.083	43.0	24.0	23.5	47.5	67.6	78.1
6h	83.7	>100	>100	>100	58.0	>100	>100	>100
6i	0.056	0.060	0.090	7.5	0.094	0.099	0.099	29.9
6j	0.060	0.072	0.079	0.090	0.093	0.096	0.098	0.098
6k	0.095	0.075	0.084	0.099	0.096	7.9	0.096	23.5
6l	0.074	0.085	0.031	0.090	0.082	0.094	0.086	0.097
6m	0.095	0.117	17.1	15.9	23.7	1.8	>100	46.3
6n	19.4	64.7	71.3	49.2	46.8	52.6	>100	>100
6o	0.050	0.079	0.044	0.091	0.089	0.097	0.089	14.3
6p	0.031	0.045	43.6	29.2	0.099	29.8	74.9	74.0
6q	0.079	0.095	24.8	14.9	28.0	20.9	76.0	64.5
CA-4	0.033	0.046	0.15[j]	0.008	0.031[j]	31.6[j]	0.025	nd

Note: [a] Concentration of drug causing 50% inhibition of cell growth, [b] Human breast cancer, [c] Human prostate cancer, [d]Human lung cancer, [e] Human cervical cancer, [f] Human leukemia, [g]

Human ovarian cancer, [h] Human colon cancer [i] Human pancreatic, [j] NSC 613729, nd= not determind.

In general, the antiproliferative activities of the tested compounds were more pronounced against MCF-7 and DU-145 cells as compared with the other cancer cell lines. Moreover, many derivatives (**6a-b**, **6d-e**, **6i-m** and **6o-q**) were more active than CA-4 in SK-OV-3 cell line. Many of the tested compounds such as **6a, 6d, 6j, 6l, 6o** and **6p** possessed the excellent overall potency with GI_{50} values of 0.063-13.4, 0.06-18, 0.06-0.09, 0.03-0.09 and 0.04-14.3 and 0.03-74 μM against the tested cell lines.

As the congeners in the tested series differ only on the nature and position of substituents on the cinnamide-aryl ring, a qualitative analysis of structure-acivity relationship may be advanced as follows. Most notably, compounds possessing free OH or NH_2 groups on the cinnamide and exhibited higher potency in general (**6a**, **6j**, **6l**, **6m** and **6o**). This may be the result of either a better binding at the cellular target or facile transport across the cell membrane. When the OH group was masked with methyl (**6b**) and propargyl (**6c**) groups, significant loss of activity was observed. Enhanced in antiproliferative activity was observed when fluorine (**6g**) was replaced with nitro (**6e**) group on para position cinnamide aryl ring. Loss of activity was observed by increasing halogen size from fluorine (**6g**) to chlorine (**6f).** The presence 4-trifluoromethyl (**6h**) groups on the cinnamide aryl ring resulted in complete loss of activity. Two of the most active compounds **6i** and **6p** possess electronically dissimilar substituents (OMe and F) at the 3 and 4-positions of the cinnamide aryl ring. Thus it is appearent that the electronic nature of the cinnamide ring is not affecting the activity. Compound **6p** is notable for its excellent antiproliferative activity, which is superior to the positive control CA-4, against MCF-7, DU-145, HOP-62 and K562 cell lines. The most cytotoxic hybrids **6i** and **6p** were taken up for detailed biological studies, such as effect on inhibition of tubulin polymerization and apoptosis induction, and the results are described below.

Cytotoxic agents generally alter the regulation of the cell cycle resulting in the arrest of cell division in various phases, thereby decreasing the growth and proliferation of cancerous cells.[20] Cytotoxicity assay showed that the test compounds **6i** and **6p** induced significant inhibition of breast cancer cells with GI_{50} values 0.056 and 0.031 μM respectively. It was of interest to examine cell-cycle alterations caused by these conjugates in MCF-7 cancer cells to understand

the phase distribution. In this study, MCF-7 cells were treated with compounds, **6i** and **6p** at 50 and 100 nM concentrations for 48 h. The data obtained clearly indicated that these compounds (**6i** and **6p**) showed G2/M cell cycle arrest when compared to untreated control (Fig. **2**).

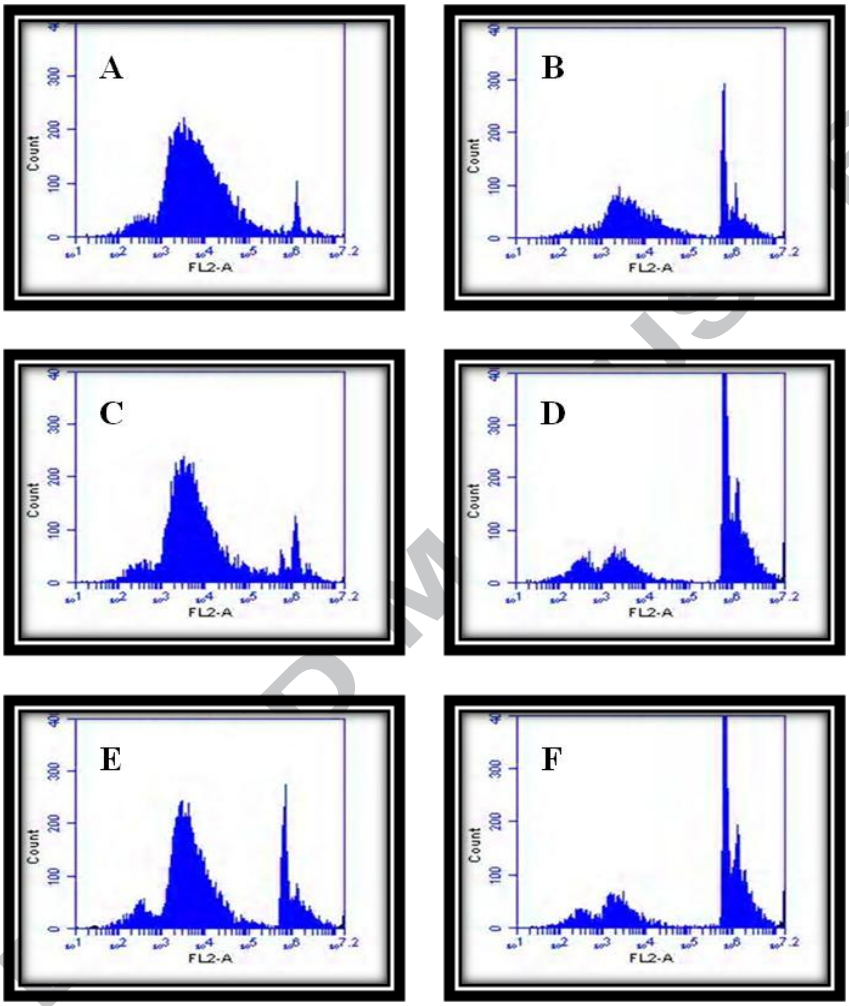


Figure 2: Cell cycle analysis of **6i** and **6p** on MCF-7 cells. A: Control cells (MCF-7), B: CA-4 (50 nM), C: **6i** (50 nM), D: **6i** (100 nM), E: **6p** (50 nM) and F: **6p** (100 nM).

Table 2: Effect of compounds **6i**, **6p**, and CA-4 on cell-cycle phase distribution in MCF-7 cells

Sample	**Sub G1 %**	**G0/G1 %**	**S %**	**G2/M %**
A: Control (MCF-7)	2.40	85.30	3.10	8.44
B: CA-4 (50 nM)	2.71	56.19	1.43	37.41

B: 6i (50 nM)	2.56	78.55	2.86	15.25
C: 6i (100 nM)	3.65	28.16	0.35	64.29
D: 6p (50 nM)	2.99	70.02	1.46	23.58
E: 6p (100 nM)	3.07	27.47	0.52	66.13

In general G2/M cell cycle arrest is strongly associated with inhibition of the tubulin polymerization [21] and since compounds **6i** and **6p** cause cell cycle arrest at G2/M phase, it was considered of interest to investigate their microtubule inhibitory function.

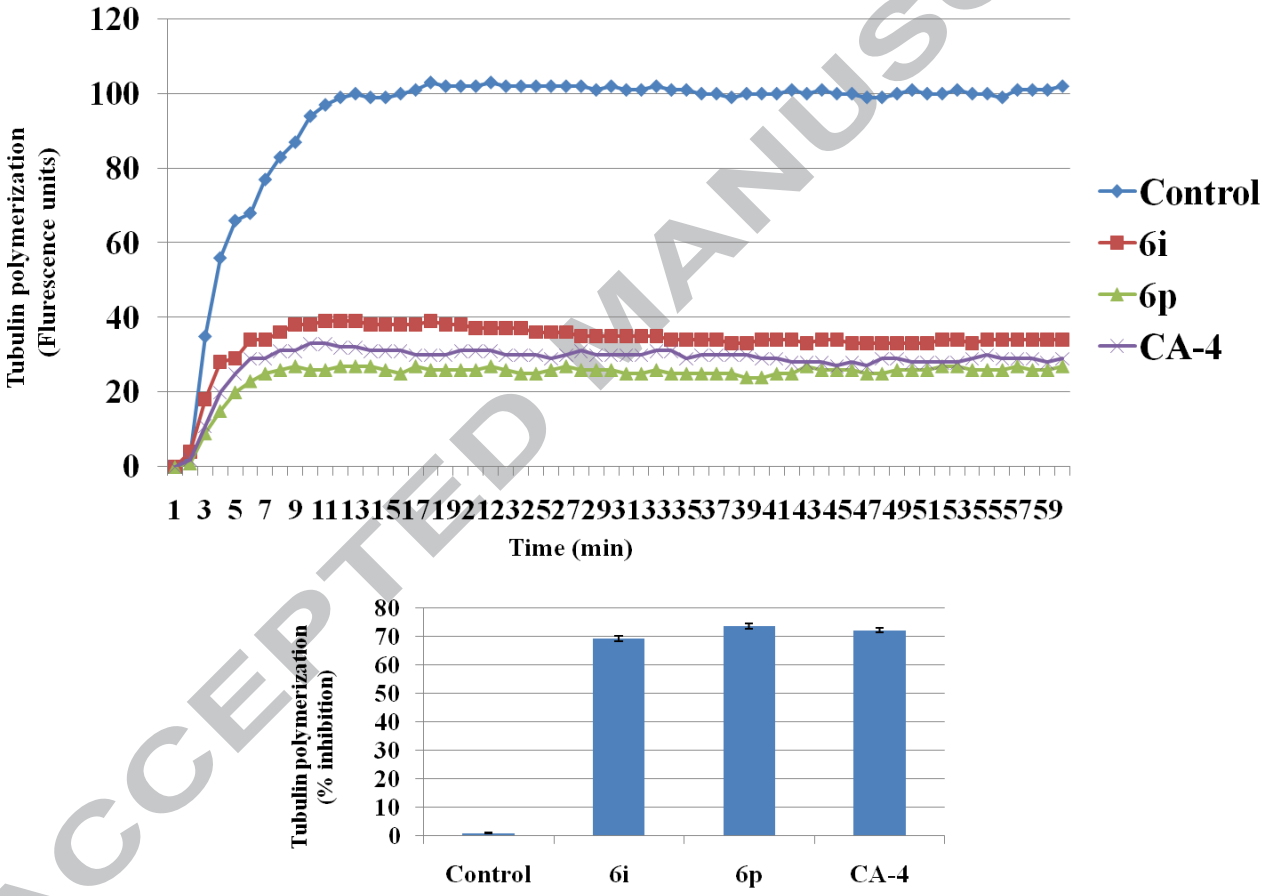


Figure 3: Effect of conjugates **6i** and **6p** on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C. All the compounds were included at a final concentration of 3 µM. CA-4 was used as a positive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

Tubulin subunits are known to heterodimerize and self-assemble to form microtubules in a time dependent manner. The progression of tubulin polymerization[22,23] 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 °C with and without the conjugate at 3 µM concentration in comparison with CA-4 as a reference. The test compounds (**6i** and **6p**) significantly inhibited tubulin polymerization by 69.25 and 73.71 % respectively, whereas CA-4 exhibited 72.18 % inhibition (Fig. **3**). This was followed by evaluation of IC_{50} values for these compounds and the results are shown in Table **2**. The test compounds **6i** and **6p** showed significant tubulin-assembly inhibition with IC_{50} values of 1.97 and 1.05 µM respectively and this data indicates that the IC_{50} value of **6p** is better than that of CA-4 (IC_{50} =1.48 µM).

Table 3: Inhibition of tubulin polymerization (IC_{50}) of compounds **6i, 6p** and **CA-4**.

Compound	**IC_{50} [a] ± SD (in µM)**
6i	1.97±0.02
6p	1.05±0.07
CA-4	1.48±0.05

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

The maintenance of mitochondrial membrane potential (ΔΨm) is significant for mitochondrial integrity and bioenergetic function.[24] Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm) are key events that take place during drug-induced apoptosis. Mitochondrial injury by compounds **6i** and **6p** was evaluated by detecting drops in mitochondrial membrane potential (ΔΨm). In this study we have investigated the involvement of mitochondria in the induction of apoptosis by these compounds. After 48 h of drug treatment with these compounds, it was observed that they reduced mitochondrial membrane potential (ΔΨm) of MCF-7 cells, assessed by JC-1 staining (Fig. **4**). The apoptotic effect of **6i** and **6p** was also evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[25] to examine the occurrence of phosphatidylserine externalization and also to understand whether it is due to physiological apoptosis or nonspecific necrosis.

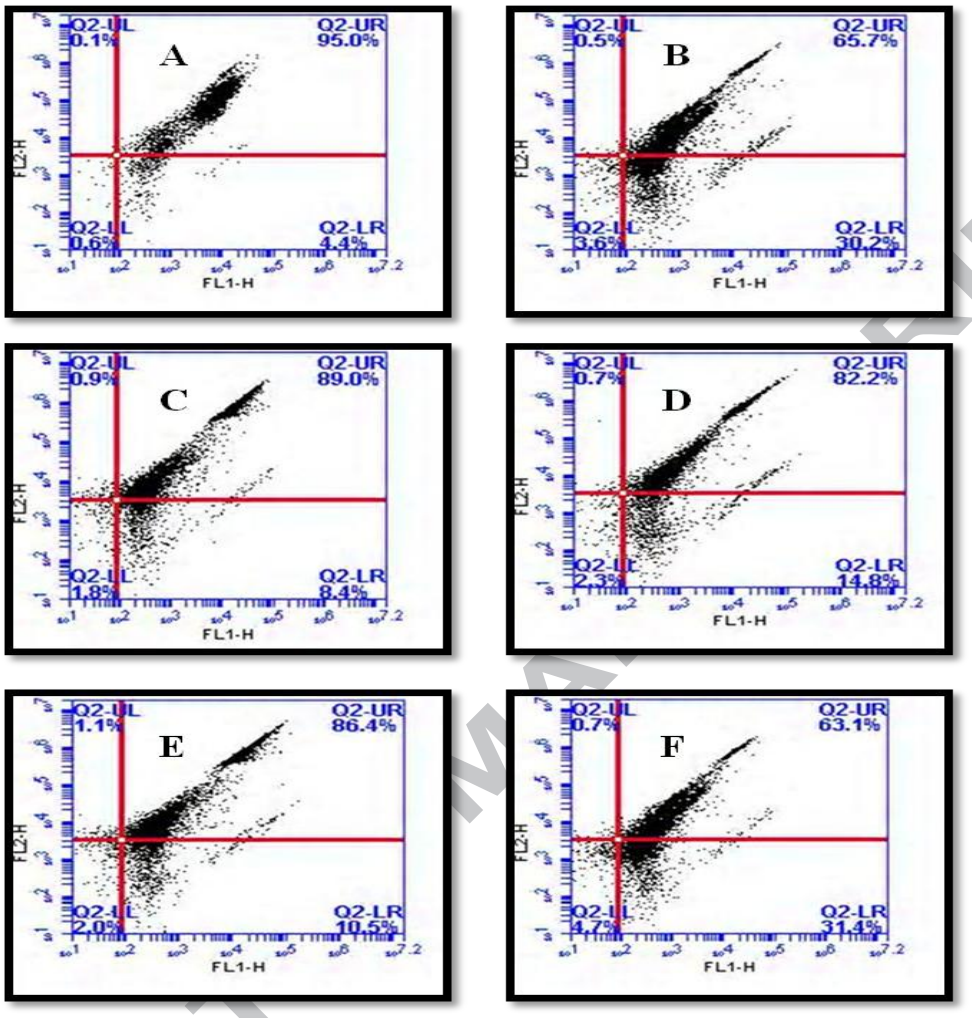


Figure 4: Compounds **6i** and **6p** triggers mitochondrial injury. Drops in membrane potential (ΔΨm) was assessed by JC-1 staining of MCF-7 cells treated with test compound and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential. A: Control cells (MCF-7), B: CA-4 (50 nM), C: **6i** (50 nM), D: **6i** (100 nM), E: **6p** (50 nM) and F: **6p** (100 nM).

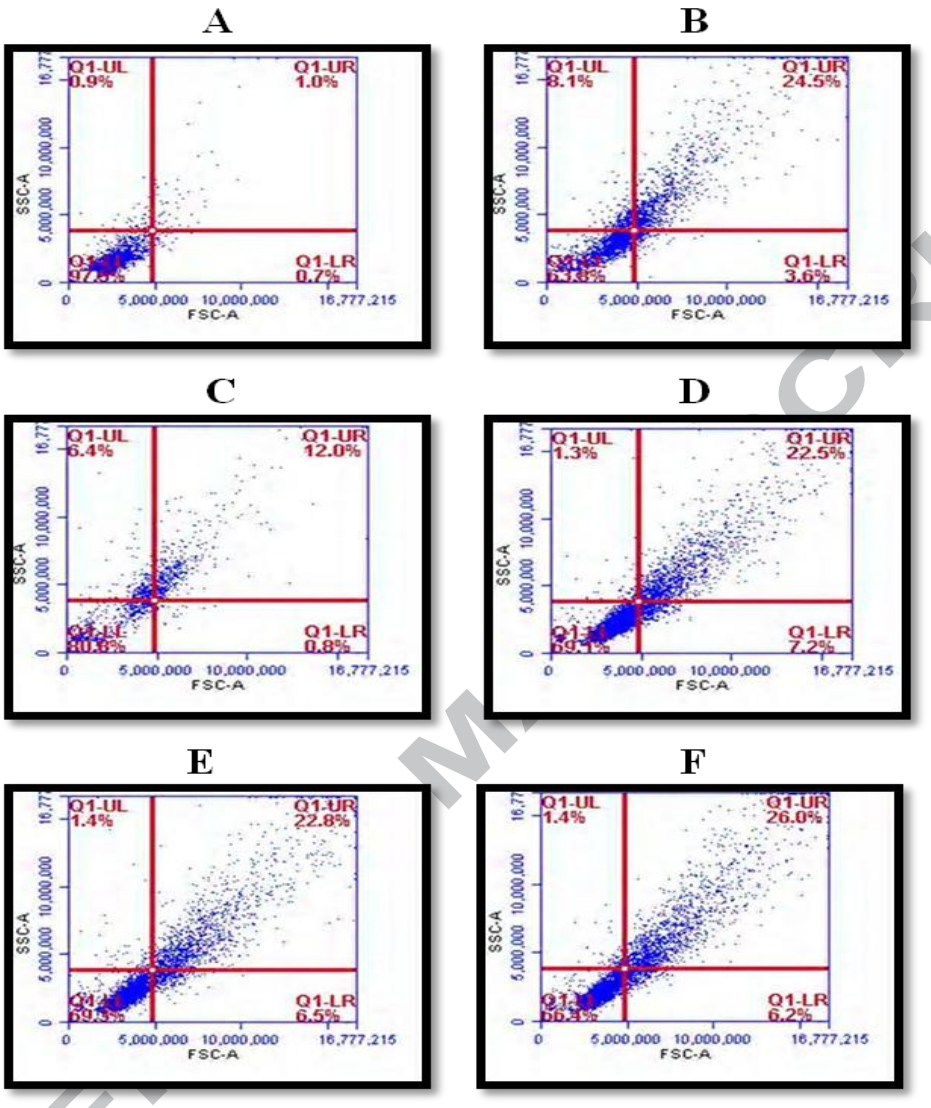


Figure 5: Annexin V-FITC staining. A: Control cells (MCF-7), B: CA-4 (50 nM), C: **6i** (50 nM), D: **6i** (100 nM), E: **6p** (50 nM) and F: **6p** (100 nM).

Table 4: Distribution of apoptotic cells in Annexin-V FITC experiment.

Sample	UL %	UR %	LL%	LR %
A: Control	0.88	0.95	97.50	0.66
B: CA-4 (50 nM)	8.13	24.49	63.83	3.55
C: 6i (50 nM)	6.38	12.03	80.75	0.84
D: 6i (100 nM)	1.26	22.45	69.11	7.19

E: 6p (50 nM)	1.43	22.79	69.31	6.48
F: 6p (100 nM)	1.38	26.03	66.43	6.16

In this study MCF-7 cells were treated with these compounds for 48 h at 50 and 100 nM concentrations to examine the apoptotic effect. The percentage of apoptotic cells (upper right and lower right quadrant of Fig. 3) were increased significantly in treated cells. Results indicated that compounds **6i** and **6p** showed 12.87 and 29.27 % (upper right and lower right of Fig. **5**) at 50 nM concentration, whereas they exhibited 29.64 and 32.19 % (upper right and lower right of Fig. **5**) apoptosis at 100 nM concentration respectively for 48 h.

The activation of caspases play an important role in the process of programmed cell death or apoptosis. Caspases, or cysteine aspartic protease, are a family of cysteine proteases that are crucial mediators of apoptosis. The MCF-7 cells lack endogenous caspase-3, whereas caspase-9 plays an important role in mediating drug-induced apoptosis.[26] MCF-7 cells were treated with these compounds (**6i** and **6p**) at 100 nM concentration for 48 h. The results demonstrate that there is 2-3 fold induction in caspase-9 activity when compared to untreated control (Fig. **6**), thereby suggesting that they have the ability to induce cell death by apoptosis in MCF-7 cells.

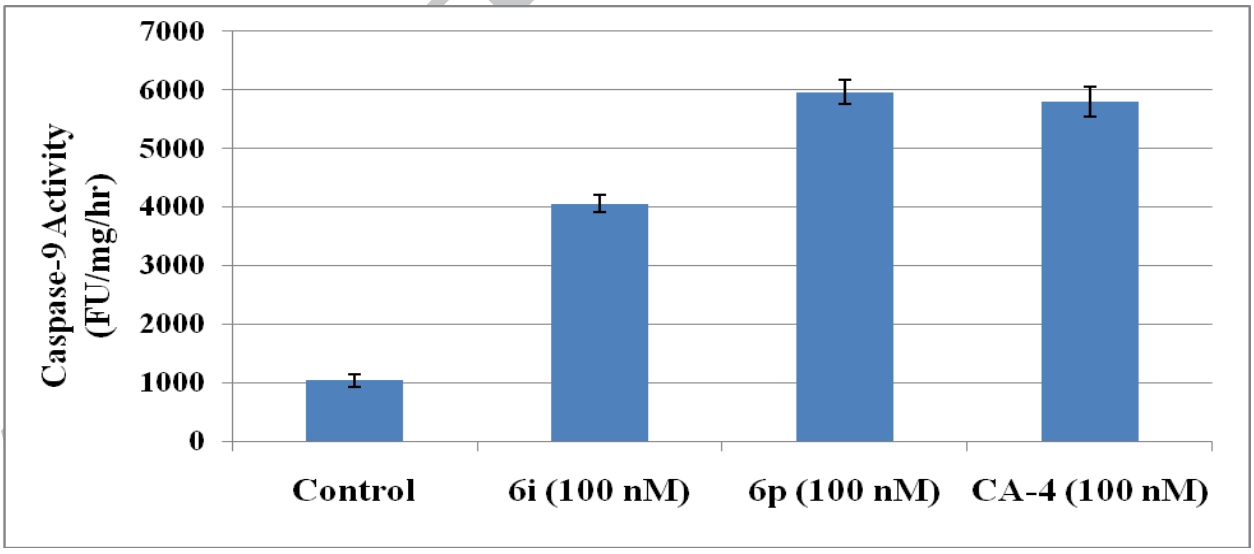
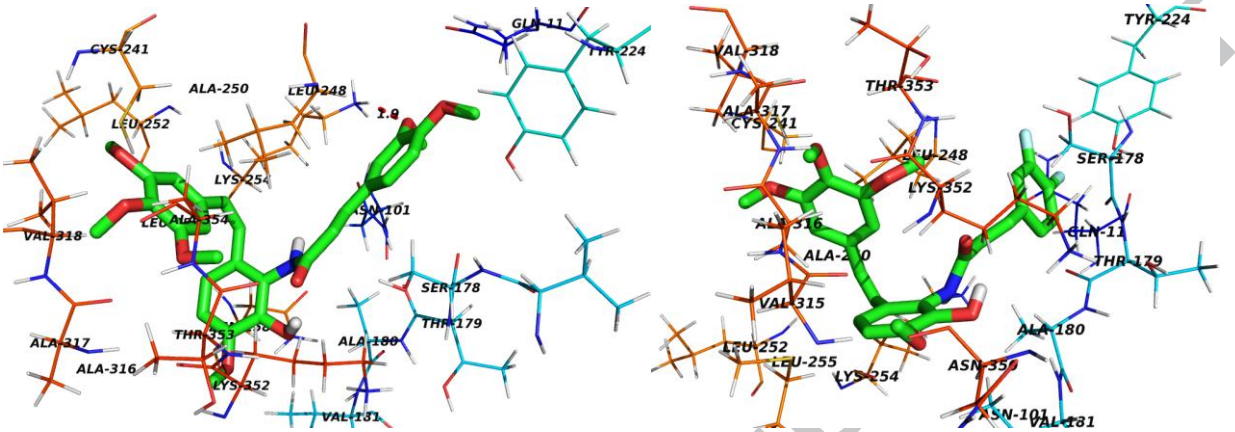


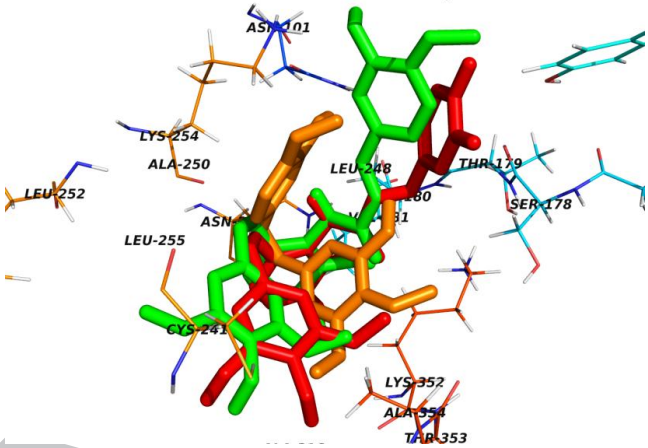
Figure 6: Effect of compounds **6i** and **6p** on caspase-9 activity; MCF-7 breast cancer cells were treated with these compounds at 100 nM concentration for 48 h. Values indicated are the mean ± SD of two different experiments performed in triplicates.

The hybrids synthesized in the present study are designed based on the combretastatin A-4 scaffold, which is well known inhibitor of tubulin polymerization that targets the colchicine site.[27]



6i **6p**

Figure 7(A): Interaction of the conjugate **6i** and **6p** with colchicines binding site of tubulin. The probable hydrogen bonds found were shown in red color. This figure has been generated using the software PYMOL from the tubulin-colchicine crystal structure.



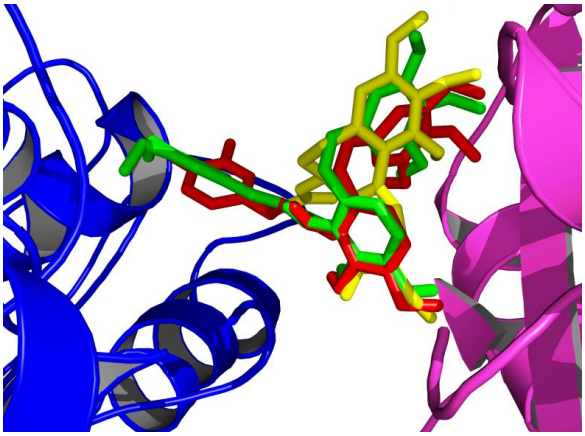


Figure 7 (B) **Figure 7 (c)**

Figure 7 (B): Overlapping of conjugate **6i** (green), **6p** (red) and combretastatin A-4 (orange) **Figure 7 (C)**: Overlay of conjugate **6i** (green), **6p** (red) and colchicines (yellow). **6i** and **6p** is expected to bind in Colchicines-binding domain with different orientation. β-tubulin shows in pink color and α-tubulin shows in blue color.

Docking studies were performed for promising conjugates (**6i** and **6p)** of the series to investigate the possible binding mode on the colchicine binding site of the tubulin. Coordinates of protein structure of tubulin-colchicine were obtained from the Protein Data Bank (PDB ID 3E22). Docking was accomplished into the colchicine binding site of tubulin using AutoDock 4.2 software. Fig. **7(A)** shows that trimethoxyphenyl ring of **6i** is buried in the hydrophobic pocket by βLys254, βLeu255, βAla250, βLeu252, βAla316, βLeu248 and βCys241 residues located in the β-tubulin in a similar manner to that of trimethoxyphenyl group of the colchicine. Dimethoxyphenyl ring of cinnamide moiety is buried at α,β interface of the tubulin surrounded by αTyr224, αAsn101, Gly11 and βLeu248. Cinnamide group and B-ring of combretastatin A-4 is also buried at α,β interface of the tubulin and surrounded by βLys352, βThr353, αSer178, αThr179, αAla180 βAsn258 and αVal181. Whereas the methoxy groups of dimethoxyphenyl ring established hydrogen bonding interaction with βLys254. In case of **6p**, trimethoxyphenyl ring is buried in hydrophobic pocket surrounded by βCys241, βLeu242, βLeu248, βLys352, βAla250, βAla316, βThr353 residues located in β-tubulin. Cinnamide group and B-ring of combretastatin A-4 are buried at α,β interface of the tubulin similar to that of conjugate **6i** and surrounded by βLys252, βLeu255, βAsn350, α Ala180, α Asn101 and α Val181. Diflurophenyl ring is buried more towards α-tubulin and surrounded by αSer178, αThr179, αGly11 and αTyr224. Fig. 7 **(B)** shows molecular docking pose of **6i** and **6p** overlaying with combretastatin A-4, whereas Fig. 7 **(C)** shows that proposed binding pose of **6i** (green) and **6p** (red) share similar binding site as of colchicine (yellow) with different orientation where α-tubulin shown in blue and β-tubulin shown in red color. These studies suggest that conjugate **6i** and **6p** can interact with both α-and β-tubulin in colchicine binding pocket.

In conclusion, a new series of CA-4 linked arylcinnamide conjugates (**6a-q)** have been synthesized and evaluated for their cytotoxic potential against selected human cancer cell lines. All the synthesized compounds showed potent cytotoxic activity against the tested cancer cell lines and two of them (**6i** and **6p**) showed GI_{50} values 0.056 and 0.031 µM, respectively against MCF-7 cancer cell line. Flow cytometric analysis of these compounds showed the arrest of the cell cycle in the G2/M phase leading to caspase dependent apoptotic cell death. These active compounds (**6i** and **6p**) showed potent inhibition of tubulin polymerization with IC_{50} values 1.97 and 1.05 µM, respectively. Further studies like, loss of mitochondrial membrane potential, significant activation and cleavage of caspase-9 activity and Annexin V-FITC assay suggested

that cytotoxic efficacy of these compounds is mainly due to inhibition of tubulin polymerization and subsequent apoptosis. Moreover, docking experiments showed that **6i** and **6p** interact and bind efficiently at the colchicine-binding site of tubulin. The results demonstrate that both **6i** and **6p** are promising leads and could be taken up for further structural modifications in the development of improved tubulin polymerization inhibitors

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Synthesis and Biological Evaluation of Arylcinnamide linked Combretastatin-A4 hybrids as Tubulin Polymerization Inhibitors and Apoptosis Inducing Agents

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