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Design, Synthesis of Phenstatin/isocombretastatin-Oxindole Conjugates as Antimitotic agents

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Abstract :

A series of phenstatin/isocombretasatin-oxindole conjugates was synthesized and tested for their cytotoxic activity against five human cancer cells such as prostate (DU-145), lung (A549), colon (HT-29), breast (MCF-7), liver (HepG2) cancer cells with IC_{50} values ranging from 0.049-38.90 μM. Amongst them, two conjugates (**5c** and **5d**) showed broad spectrum of antiproliferative efficacy on lung cancer cells with an IC_{50} value of 79 nM and 93 nM respectively, whereas on colon cancer cells with an IC_{50} values 45 nM and 49 nM respectively. In addition, cell cycle assay revealed that these conjugates (**5c** and **5d**) arrest at the G_2/M phase and leads to apoptotic cell death which was confirmed by Annexin V-FITC and mitochondrial membrane depolarization. Further, the tubulin polymerization assay analysis results suggest that these conjugates particularly **5c** and **5d** exhibit significant inhibitory effect on the tubulin assembly with an IC_{50} value of 1.23 μM and 1.01 μM respectively. Molecular docking studies indicated that these compounds (**5c** and **5d)** occupy the colchicine binding site of the tubulin

Keywords: phenstatin/isocombretastatin-oxindole, tubulin depolymerization, Annexin V-FITC and mitochondrial membrane depolarization

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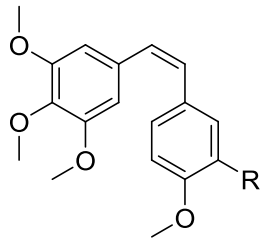
Introduction

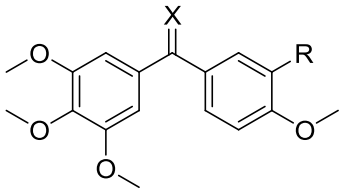
Microtubules have become an important target in chemotherapy that are present in all eukaryotic cells and play an essential role in the formation of mitotic spindles, cell division etc.[1] The mitotic spindles are generated by noncovalent polymerization of α-tubulin and β-tubulin heterodimers that have become an attractive target to treat many types of malignancies.[2] The majority of drugs that binds at β-tubulin of the microtubule, are known to inhibit tubulin polymerization or depolymerization.[3] In particular, three major binding domains on β-tubulin subunit of microtubule, namely the vinca binding site, the taxane binding site and the colchicine binding site for mostly lipophilic ligands and some high affinity agents have been developed as antiproliferative drugs.[3,4] More particularly, compounds such as the paclitaxel, docetaxal and epothilones, that bind to the taxane binding site prevent microtubule disassembly by stabilizing microtubules and are used in the treatment of carcinomas, such as lung, breast, ovarian, and bladder.[5] In contrast, compounds like colchicinium, vinca alkaloids and combretastatin A-4 (**1**, Figure 1) and nocodazole, that bind to the vinca or colchicine binding sites inhibit cancer cell proliferation and tubulin assembly by destabilizing microtubules and are used in the treatment of leukemia, lymphoma and a variety of other diseases.[6] However, many of such agents manifest different limitations in their clinical utility, therefore development of new microtubule targeting agents is of significance.

Phenstatin (**2a**, Figure 1) is a benzophenone motif reported by Pettit and coworkers and it is also well known microtubule-destabilizing agent. Phenstatin (**2a**, Figure 1) and its derivatives exhibits the most potent activity at the colchicine binding site and significantly inhibits tubulin polymerization and exerts profound antiproliferative activity against various human cancer cell lines, including multidrug-resistant cancer cells.[7] Recently, a new class of benzophenone series with insertion of small heterocyclic groups in the B-ring like indole, quinoline, carbazole, thiophenes displayed excellent cytotoxic activity as well as significant inhibition of tubulin polymerization.[8] Recently, isocombretastatin A-4 (iso CA-4, **2c**) (Figure 1) is a non isomerized C A-4, reported as antimitotic agent. In addition, isocombretastatin and its derivatives shows substantial cytotoxic activity towards selected human cancer cell lines and cells are accumulated in the G2/M phase of the cell cycle.[9]

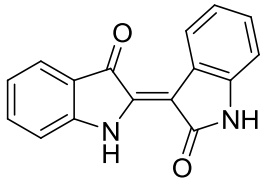
Oxindoles are versatile moieties that display diverse biological activities, including anticancer activity. Bis-indole alkaloid indirubin (**3**, Figure 1) is interesting natural pharmacophore and traditional Chinese medicine recipe used in the treatment of leukemias. This has broad spectrum of activity and it is mainly recognized as kinase inhibitor. In addition, indirubins are potent inhibitors of cyclin-dependent kinases (CDKs) like glycogen synthase kinase-3b (GSK-3b) and CDK1/cyclin B.[10] A-432411 (**4**, (*Z*)-3-((1H-pyrrol-2-yl)methylene)-6-(4-hydroxy-3-methoxyphenyl)indolin-2-one) (Figure 1) is an indolinone that is structurally different from other known synthetic microtubule inhibitors. This compound is efficacious against a variety of human cancer cell lines including drug-resistant HCT-15 that over expresses Pgp170. These compete with the colchicine-binding site on the tubulin thereby inhibiting microtubule polymerization and causing G2-M arrest and induces apoptosis.[11]



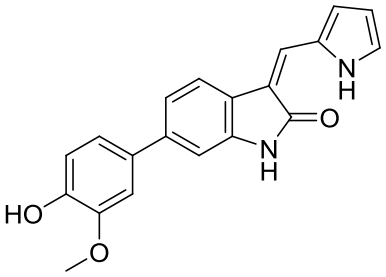
1: R = OH, CombretastatinA-4



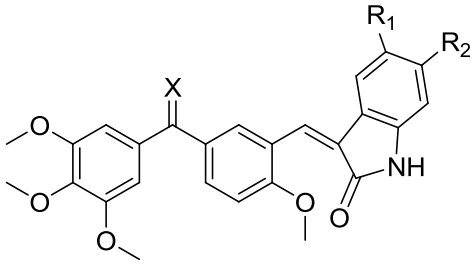
2a : X = O; R = OH; phenstatin
2b : X = O; R = NH_2; phenstatin amine
2c : X = CH_2; R = OH; *iso*CA-4
2d : X = CH_2; R = NH_2; *iso*NH_2CA-4



(**3**)



(**4**)



(**5a-h** and **6a-h**)

Figure 1. Chemical structures of microtubule targeting agents. Combretastatin A-4 (**1**), phenstatin (**2a**) phenstatin amine (**2b**) isocombretastatin A-4 (**2c**) isocombretastatin A-4 amine (**2d**), Indirubin (**3**), A-432411 (**4**) Phenstatin\isoCombretastatin-Oxindole conjugates (**5a-h** and **6a-h**)

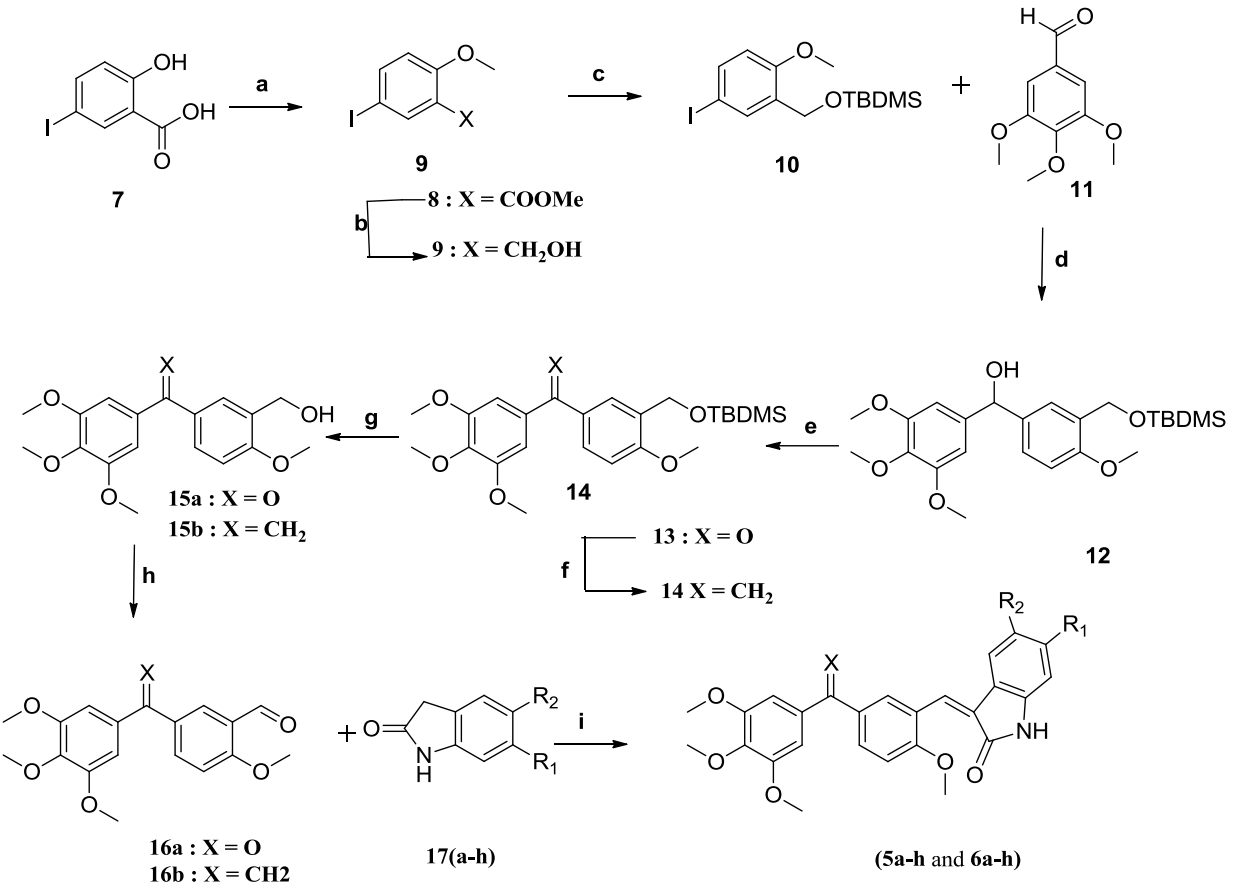
Thus Based on these observations,, it was considered of interest to explore newer antitubulin agents by linking different pharmacophores. In this study, we linked the substituted oxindoles with phenstatin and isocombretastatin A-4 to generate phenstatin/isocombretastatin A-4 oxindoles. The promising activity observed prompted us to investigate the role of these new compounds in the proliferation and apoptosis of human colon cancer cell line (HT-29). We also investigated the effect of these compounds on proteins that regulate cell-cycle progression.

Results and Discussion

Chemistry

These phenstatin/iso-combretastatin-oxindole conjugates (**5a-h** and **6a-h**) were prepared employing Knoevenagel reaction between equimolar mixtures of substituted oxindoles (**17a-h**) and phenstatin 3-aldehyde/isocombretastatin 3-aldehyde (**16a-b**) in the presence of piperidine as shown in **Scheme 1.** The compound structures were confirmed by means of ^{1}H NMR, ^{13}C NMR, HRMS and IR spectra.

The phenstatin 3-aldehyde/*iso*-combretastatin 3-aldehyde (**16a-b**) were obtained via Swern's oxidation of the corresponding (3-(hydroxymethyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (**15a**) and (2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)phenyl)methanol (**15b**). The ketone **13** is generated from the benzhydrol derivative **12** via oxidation and subsequent Witting methylation afforded the olefin **14**. The benzhydrol derivative **12** was in turn, obtained via the addition of the aryl lithium reagent generated from the iodide **10** to aldehyde **11**. The aryl iodide **10** was obtained via a three step synthetic manipulation of 5-iodo salicylic acid **7** (**Scheme 1**).



	R_1	R_2	X		R_1	R_2	X
5a	H	H	O	**6a**	H	H	CH_2
5b	H	NO_2	O	**6b**	H	NO_2	CH_2
5c	H	OMe	O	**6c**	H	OMe	CH_2
5d	H	F	O	**6d**	H	F	CH_2
5e	H	Cl	O	**6e**	H	Cl	CH_2
5f	Cl	H	O	**6f**	Cl	H	CH_2
5g	H	Br	O	**6g**	H	Br	CH_2
5h	Br	H	O	**6h**	Br	H	CH_2

Scheme 1. ***Reagents and Conditions*****:** (a) DMS, K_2CO_3, Acetone, 60 ºC, 8 h; (b) DIBAL-H (1N), THF 05 ºC, 3 h; (c) TBDMSCl, imidazole, CH_2Cl_2 3 h; (d) n-But-Li (1.6N), THF -78 ºC; (e) IBX, DMSO, 10 ºC, 2 h; (g) TBAF (1N), THF 05 ºC, 6 h; (g) CH_2PPh_3I, t-ButOK, dry THF 05 ºC, 8 h; (h) Oxalyl chloride, DMSO, -78 ºC to rt, Et_3N; (i) piperidine, EtOH, reflux 3–8 h.

Biological studies

Antiproliferative activity

In an attempt to examine the structure activity relationship of phenstatin/iso-combretastatin-oxindole conjugates (**5a-h** and **6a-h**) consisting A, B, C, D-rings and X (Fig 2) as

shown in scheme 1. Sixteen compounds were prepared with respect to different modifications made on the D-ring and X which were evaluated for cytotoxic activity against a panel of five human cancer cell lines such as A549 (lung), DU-145 (prostate cancer), HT-29 (colon cancer), MCF-7 (breast cancer) and HepG2 (liver cancer) by employing MTT assay.[12] Combretastatin A-4 was used as reference drug and the results are summarized in Table 1 and expressed as IC_{50} values.

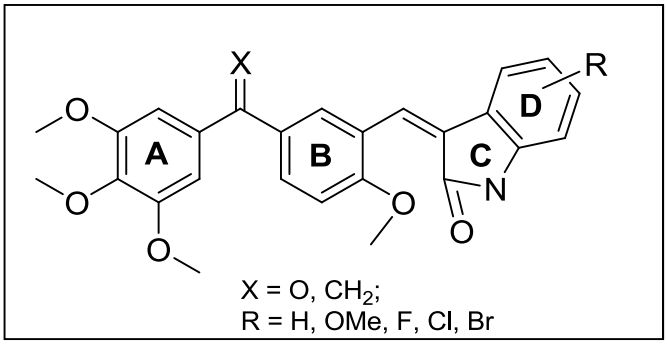


Table 1: [a]IC_{50} values (in μM) for compounds in selected human cancer cell lines

Compound	DU-145[b]	A549[c]	HT-29[d]	MCF-7[e]	HepG2[f]
5a	26.78	6.698	2.728	10.70	3.801
5b	35.55	8.912	5.128	12.27	6.918
5c	7.943	0.079	0.045	0.269	0.095
5d	9.705	0.093	0.049	0.386	0.186
5e	29.21	9.660	3.235	14.25	2.511
5f	32.64	11.93	13.42	15.88	15.84
5g	19.14	9.772	8.609	30.82	8.128
5h	12.02	4.570	3.715	14.69	4.017
6a	25.60	11.38	6.456	20.32	7.673
6b	19.49	5.754	1.339	13.18	3.715
6c	5.035	1.479	0.107	3.162	0.897
6d	3.133	1.174	0.081	3.981	0.084
6e	38.90	10.93	4.265	15.16	5.623
6f	11.40	7.413	2.269	11.74	3.235
6g	19.14	7.430	5.623	18.20	8.317
6h	20.70	12.58	8.317	21.00	10.47
CA-4	0.007	0.051	0.049	0.046	0.023

[a] 50% Inhibitory concentration after 48 h of drug treatment; [b] human prostate cancer; [c] human lung cancer; [d] human colon cancer; [e] human breast cancer; [f] human liver cancer.

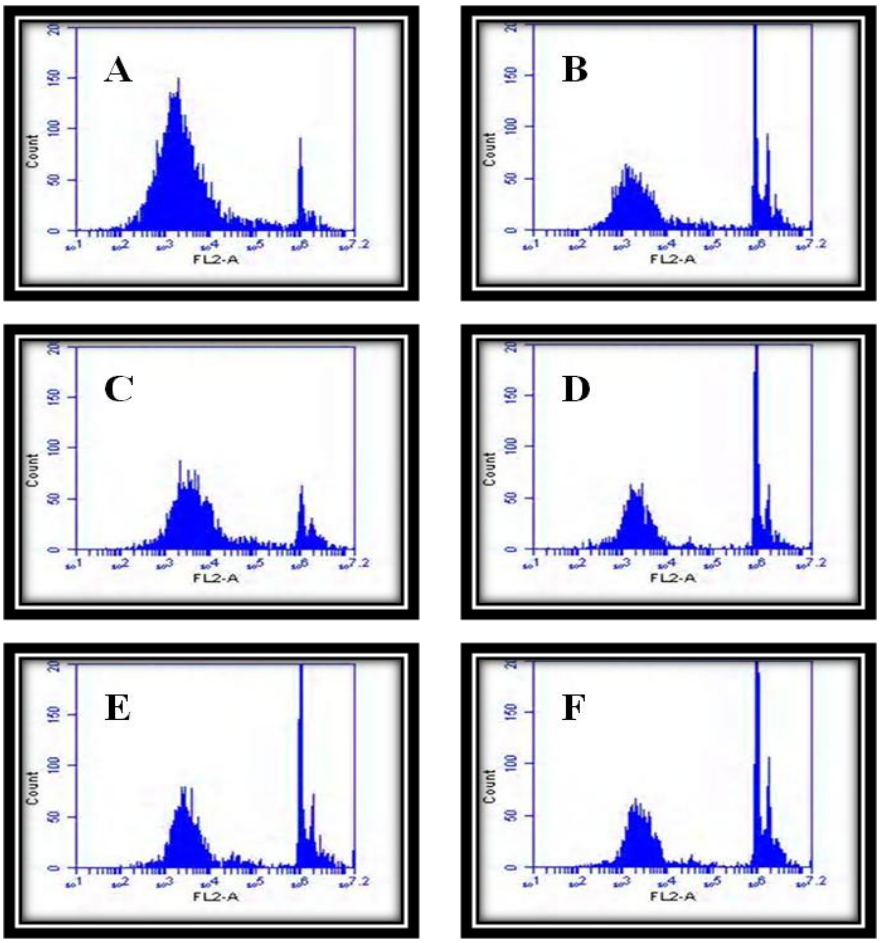
The *in vitro* screening results revealed that these conjugates possess excellent to moderate cytotoxic activity with IC_{50} values ranging from 0.049-38.90 μM. Particularly, the

phenstatin-oxindole conjugates harboring carbonyl group in between A and B rings showed profound cytotoxic activity with an IC_{50} value in the range 0.045-35.55 μM. More particularly, the conjugate 5**c** having electron donating substituent like para-methoxy group on D-ring possess excellent cytotoxicity against lung and colon cancer cells with IC_{50} value 79 nM and 45 nM respectively. In contrast, substitution with nitro at para position of D-ring as in **5b** proved deleterious for its antiproliferative activity against human prostate cancer like Du145 cells (IC_{50} value is 35.55 μM). However, isocombretastatin-oxindole conjugates (**6a-h**) harboring ethylene group in between A and B ring displayed potent antiproliferative activity with an IC_{50} value in the range 0.081-38.9 μM. In addition, the conjugate **6c** having para-methoxy group on D-ring as electron donating substituent exhibited significant cytotoxic activity towards colon cancer (HT29) with an IC_{50} value 81 nM.

These results suggest that phenstatin-oxindole conjugates exhibit better cytotoxicity than iso-combretastatin-oxindoles (**6a-h**) against tested cell lines.

Cell cycle analysis

Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of the cell cycle or by induction of apoptosis or a combined effect of both cycle block and apoptosis.[13-14] Furthermore, regulation of the cell cycle and apoptosis are considered to be effective cancer therapeutic methods.[15] Therefore, it was considered of interest to understand whether this inhibition of cell growth was on account of cell cycle arrest. In this study DU-145 cells were treated with compounds **5c** and **5d** at 10 and 50 nM concentrations for 48 h, and CA-4 was used a reference compound in this study. The data obtained clearly indicated that this compound arrested cell cycle at G2/M phase as compared to the untreated control (Figure 1). These compounds (**5c** and **5d**) showed 21.69 and 47.12 % of cell accumulation in G2/M phase, respectively, at 10 nM concentration, whereas it exhibited 49.80 and 56.82 % of the cell accumulation in G2/M phase at 50 nM concentration (Figure 2).



Cell cycle distribution of HT29 cells in presence of Compound **5c**, **5d** and **CA-4** at concentrate dependant manner				
Sample	**Sub G1 %**	**G0/G1 %**	**S %**	**G2/M %**
A: Control	1.00	83.01	3.02	12.47
B: CA-4 (10 nM)	0.16	51.46	2.62	45.76
B: **5c** (10 nM)	0.43	73.61	4.87	21.69
C: **5c** (50 nM)	0.62	48.01	0.91	49.80
D: **5d** (10 nM)	0.35	50.06	2.47	47.12
E: **5d** (50 nM)	0.18	41.27	1.36	56.82

Figure 2. Cell cycle analysis of **5c** and **5d** on HT-29 cells. A: Control cells (HT-29), B: Ca-4 (10 nM), C: **5c** (10 nM), D: **5c** (50 nM), E: **5d** (10 nM) and F: **5d** (50 nM).

Effect of compounds on tubulin polymerization

In general G2/M cell cycle arrest is strongly associated with inhibition of tubulin polymerization.[16] Since compounds like **5c** and **5d** cause cell cycle arrest at G2/M phase cell

cycle arrest, it was considered of interest to investigate their microtubule inhibitory function. Tubulin subunits are known to heterodimerize and self-assemble to form microtubules in a time dependent manner. The progression of tubulin polymerization[17-18] 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 conjugate in comparison with reference compound CA-4. The test compounds (**5c** and **5d**) efficiently inhibited tubulin polymerization by 68.78 and 72.06%, respectively, comparable to 71.52 % inhibition exhibited by CA-4 (Figure. 3). This was followed by the evaluation of IC_{50} values for these compounds and results indicate that compounds **5c** and **5d** showed better tubulin-assembly inhibition with an IC50 value of 1.23 µM and 1.01 µM respectively.

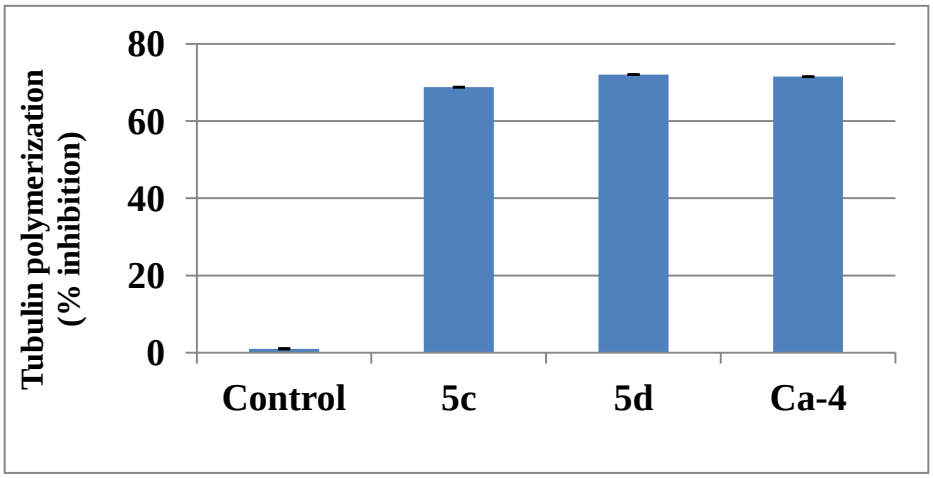


Figure 3. Effect of compounds on tubulin polymerization: tubulin polymerization was monitored by the increase in fluorescence at 360 nm (excitation) and 420 nm (emission) for 1 h at 37 °C. CA-4 was used as the reference compound in this study. Values indicated are the mean ± SD of two different experiments performed in triplicate.

Table 2. Inhibition of tubulin polymerization (IC_{50}) for compounds **5c** and **5d**.

Compound	**[a] IC_{50} ± SD (in µM)**
5c	1.23±0.12
5d	1.01±0.08
Ca-4	1.06±0.12

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

Measurement of mitochondrial membrane potential (ΔΨm)

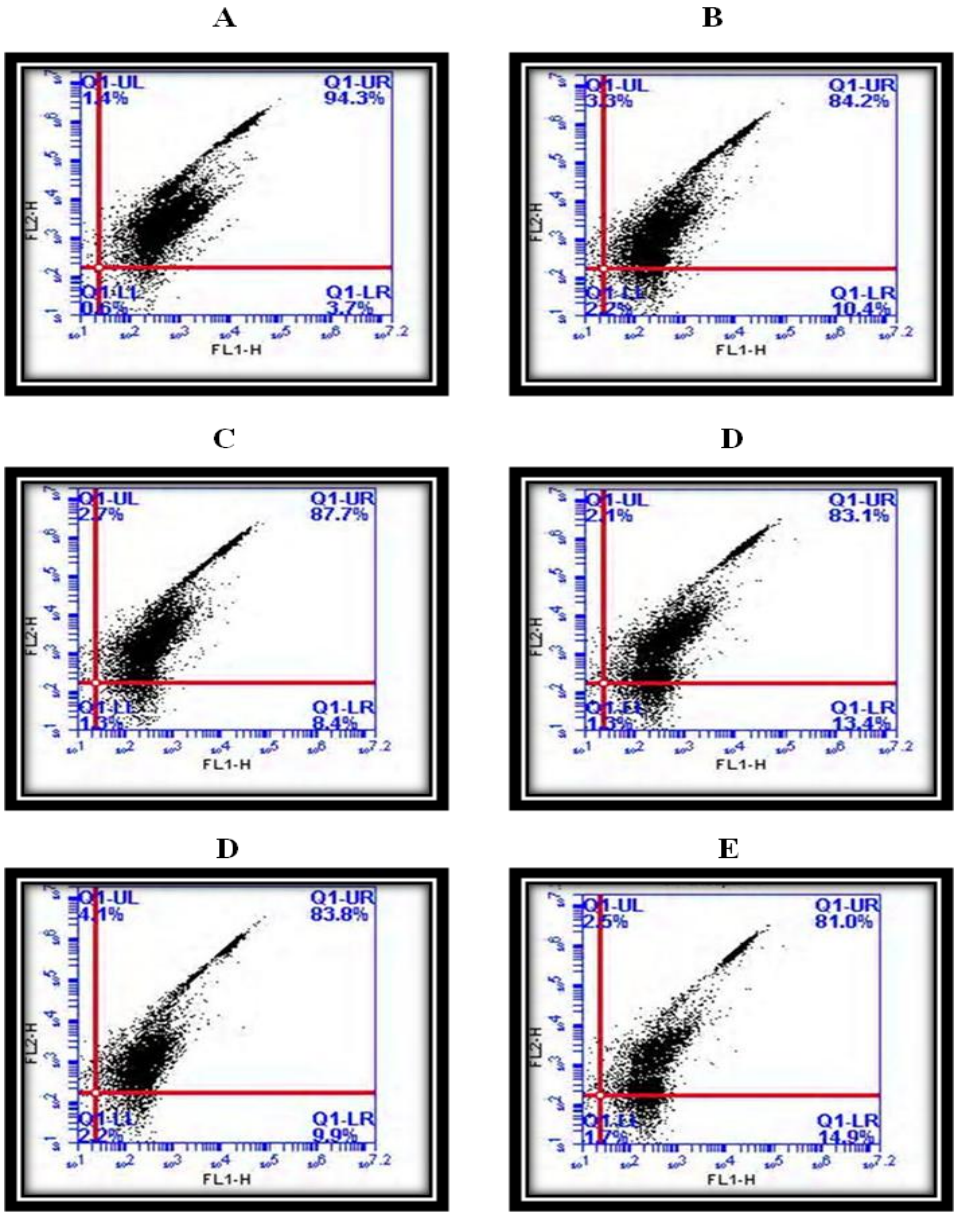
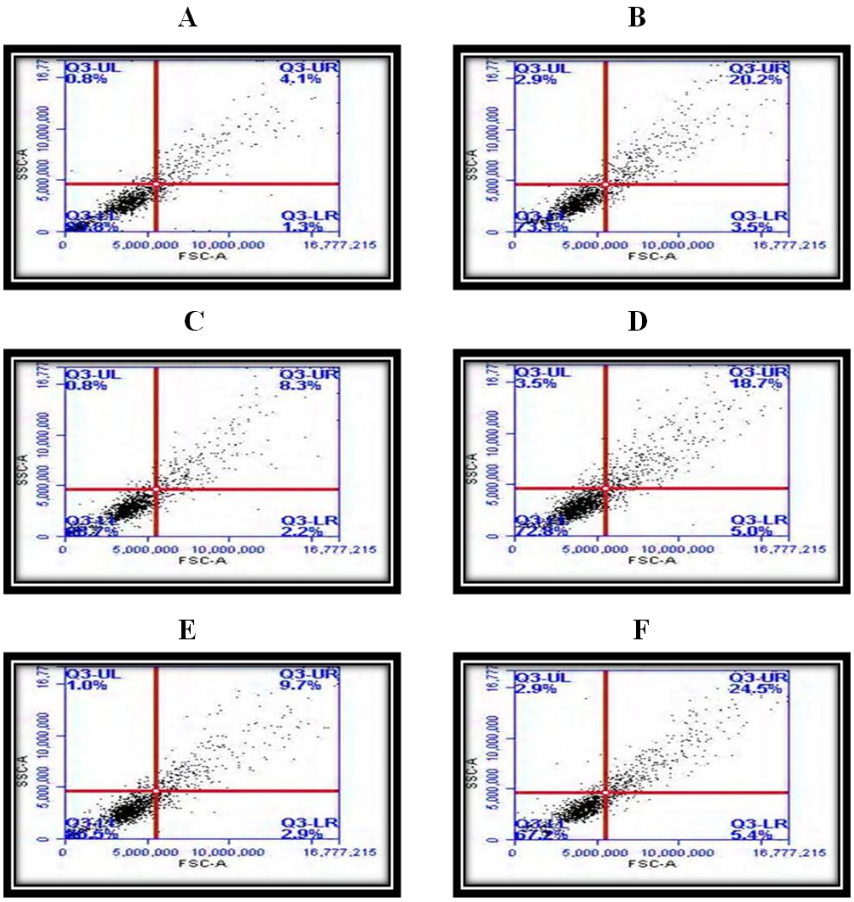


Figure 4. Compounds **5c** and **5d** triggers mitochondrial injury. Drops in membrane potential (ΔΨm) was assessed by JC-1 staining of HT-29 cells treated with test compound and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential. A: Control cells (HT-29), B: Ca-4 (10 nM), C: 5c (10 nM), D: 5c (50 nM), E: 5d (10 nM) and F: 5d (50 nM).

The maintenance of mitochondrial membrane potential (ΔΨm) is significant for mitochondrial integrity and bioenergetic function.[19] Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm), are key events that take place during drug-induced apoptosis.

Mitochondrial injury by compounds **5c** and **5d** 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 reduced mitochondrial membrane potential (ΔΨm) of HT-29 cells, assessed by JC-1 staining (Figure 4).

Annexin V-FITC for apoptosis



Sample	UL	UR	LL	LR
A: Control	0.82	4.06	93.82	1.30
B: CA-4	2.93	20.21	73.40	3.46
C: **5c** (10 nM)	0.83	8.34	88.68	2.16
D: **5c** (50 nM)	3.52	18.70	72.80	4.97
E: **5d** (10 nM)	0.98	9.67	86.47	2.88
F: **5d** (50 nM)	2.92	24.46	67.20	5.42

Figure 5: Annexin V-FITC staining. A: Control cells (HT-29), B: CA-4 (10 nM), C: **5c** (10 nM), D: **5c** (50 nM), E: **5d** (10 nM) and F: **5d** (50 nM).

The apoptotic effect of **5c** and **5d** was further evaluated by Annexin V FITC/PI (AV/PI) dual staining assay[20] to examine the occurrence of phosphatidylserine externalization and to understand whether it is due to physiological apoptosis or nonspecific necrosis. In this study, HT-29 cells were treated with these compounds for 48 h at 10 and 50 nM concentrations to examine the apoptotic effect. It was observed that these compounds showed significant apoptosis against HT-29 cells as shown in Figure 5. Results indicated that compounds **5c** and **5d** showed 10.50 and 12.55 % at 10 nM concentration, whereas they exhibited 23.67 and 29.88 % apoptosis at 50 nM concentration respectively for 48 h (Figure 5).

Molecular Docking

Here, the main objective of molecular docking was to predict the accurate binding poses of these new Phenstatin-Oxindole conjugates within the constraints of receptor binding site. Cytotoxic results of these conjugates (especially **5c** and **5d** on human colon cancer cell lines) encouraged us to perform their molecular docking studies. Docking studies revealed that these conjugates fit well in the LOC binding site present at β subunit at α subunit interface of the tubulin (Figure 6A).

The cyclic amide proton in these conjugates exhibited hydrogen bonding (red dashed line in figure 6C and 6D) with phenolic oxygen of Tyr224A (A and B represents α subunit and β subunit of tubulin) amino acid residue. The hydrogen bond length for **5c** and **5d** conjugates was1.8Å and 2.2Å respectively. Besides this, these conjugates also showed hydrophobic interactions (figure 6C and 6D) with the target protein. As similar to colchicine, the trimethoxy substituted aromatic ring in these conjugate precisely fitted into the hydrophobic pocket (figure 6B) which consists of Cys241B, Ala250B, Lys254B, Leu255B, Ala317B, Val318B, and Ala354B residues of β subunit. In addition, Thr353B is part of hydrophobic pocket for conjugate **5d**. The substituted oxyindolin-2-one rings in these conjugate showed interactions with Gln247B, Leu248B, Ser178A, Lys352B and GTP600B. Along with that,5-methoxy oxyindolin-2-one ring present in conjugate**5c** also interacted with Thr353B. 1,5-disubstituted 2-

methoxybenzylidene ring present in these conjugates interacted with Ala180A, Thr179A and Asn101A and also conjugate **5d** also interact with Lys 254B.

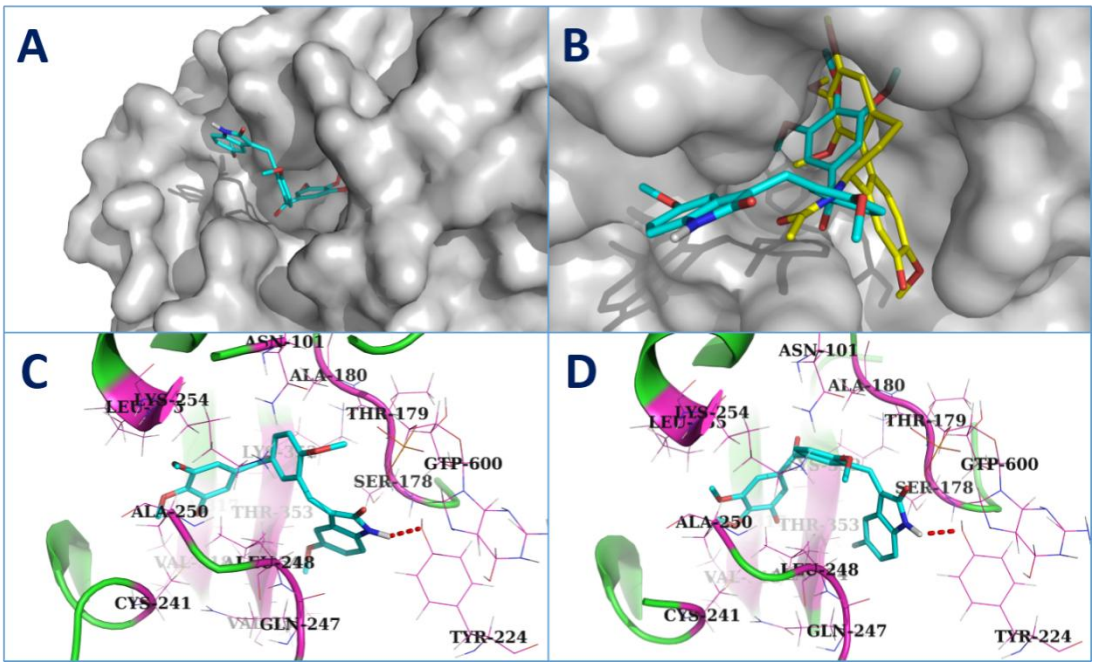


Figure 6: A) Surface binding pose of conjugate **5C** in LOC binding pocket of β subunit of tubulin; **B**) Surface binding pose of co-crystal (colchicine) ligand and conjugate **5c**; **C**) Binding pose of conjugate **5c**; **D**)Binding pose of conjugate **5d**. Conjugate **5c** and co-crystal ligand are shown in stick and coloured by the atom type (Conjugates **5c**, **5d** and co-crystal ligand are shown in stick and coloured by the atom type [carbon: cyan (**5c** and **5d**) and yellow (co-crystal ligand); oxygen: red;hydrogen: white; nitrogen: blue; fluorine: ice blue]

Conclusion

In the present study, phenstatin/isocombretasatin-oxindole conjugates were designed and synthesized via Knoevenagel reaction and they were investigated for their cytotoxic activity against five human cancer cells such as prostate (DU-145), lung (A549), colon (HT-29), breast (MCF-7), liver (HepG2) cancer cells with IC_{50} values ranging from 0.045-38.90 μM. Among them, two conjugates like **5c** and **5d** displayed remarkable antiproliferative activity towards lung (79 nM and 93 nM respectively) and colon (45 nM and 49 nM respectively) cancer cells in nanomolar range. The flow cytometric analysis revealed that these conjugates cause cell cycle arrest at G2/M phase. Interestingly, the mitochondrial membrane potential and Annexin V FITC

assay suggested that these compounds (**5c** and **5d**) induced cell death by apoptosis. The tubulin polymerization assay analysis results suggest that these conjugates like **5c** and **5d** exhibit significant inhibitory effect on the tubulin assembly with an IC_{50} value of 1.23 μM and 1.01 μM respectively. The binding of these compounds (**5c** and **5d**) was at the colchicine binding site of tubulin, as indicated by molecular docking studies. Thus, these conjugates could be considered as potential scaffolds that are useful newer leads for the treatment of prostate cancer.

Experimental Section

I. Chemistry

All chemicals and reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA), Lancaster (Alfa Aesar, Johnson Matthey Company, Ward Hill, MA, USA) or Spectrochem Pvt. Ltd. (Mumbai, India), and were used without further purification. Reactions were monitored by TLC performed on silica gel coated glass plates containing 60 GF254 with visualisation achieved by UV light or iodine indicator. Column chromatography was performed with Merck 60–120 mesh silica gel. ^{1}H and ^{13}C spectra were recorded on Bruker UXNMR/ XWIN-NMR (300 MHz) or Inova Varian-VXR-unity (400, 500 MHz) instruments. Chemical shifts (δ) are reported in ppm downfield from an internal TMS standard. ESI spectra were recorded on a 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 a QSTAR XL Hybrid MS–MS mass spectrometer. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected.

Preparation of methyl 5-iodo-2-methoxybenzoate (8):

To a solution of iodo salicilic acid (**7**,5.0 g, 0.019 mol) in dry acetone (30 ml), was added anhydrous K_2CO_3 (7.85 g, 0.056 mol), dimethyl sulphate (7.16 g, 0.056 mol) at 0 °C. The reaction mixture was stirred at reflux temperature for 6 h. The reaction was monitored by TLC using Ethylacetate:hexane (1:19). After completion of the reaction as indicated by the TLC, after completion of the reaction as indicated by the TLC, K_2CO_3 was removed by filtration and the solvent was concentrated under the vacuum, diluted with water and extracted with ethyl acetate. The combined organic phases were dried (Na_2SO_4) and evaporated under vacuum and the residue was purified by column chromatography (40% EtOAc-hexane) to afford compound **8** as white solid (5.1 g, 92 %); mp: 47-50 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.06 (d, J = 2.28

Hz, 1H), 7.72 (dd, *J* = 2.28 Hz, 8.85 Hz, 1H), 6.73 (d, *J* = 8.85 Hz, 1H), 3.88 (s, 3H), 3.87 (s, 3H); ESI-MS: m/z 292 $[M+1]^+$.

Preparation of (5-iodo-2-methoxyphenyl)methanol (9):

To a solution of methyl 5-iodo-2-methoxybenzoate (**8,** 5.0 g, 0.017 mol) in dry CH_2Cl_2 (50 ml) was added drop wise DIBAL (34 mL, 1.0M in hexane, 0.034 mol) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The reaction was monitored by TLC using ethyl acetate-hexane (4:6). After completion of the reaction as indicated by the TLC, Saturated ammonium chloride solution was added to the reaction mixture. Salts were removed by filtration, the solvent was dried (using Na_2SO_4) and evaporated under vacuum, and it was taken as such for the next step without further purification.

Preparation of tert-butyl (5-iodo-2-methoxybenzyloxy)dimethylsilane (10):

To a solution of (5-iodo-2-methoxyphenyl)methanol (**9**, 4.0 g, 0.015 mol) in dry CH_2Cl_2(30 ml) was added imidazole (1.16 g, 0.017 mol) and TBDMSCl (2.56 g, 0.017 mol) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. The reaction was monitored by TLC using ethyl acetate-hexane (1:10). After completion of the reaction as indicated by the TLC, The water was added to the reaction mixture and separates the organic layer. The solvent was dried (Na_2SO_4) and evaporated under vacuum and the residue was purified by column chromatography (5% concentrated under the vacuum, the combined organic phases EtOAc-Hexane) to afford compound **10** as colourless oil (5.2 g, 91%); bp: 287-289 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.72 (d, *J* = 2.26 Hz, 1H), 7.48 (dd, *J* = 2.26 Hz, 8.31 Hz, 1H), 6.56 (d, *J* = 8.31 Hz, 1H), 4.68 (s, 2H), 3.78 (s, 3H), 0.95 (s, 9H), 0.11 (s, 6H); ESI-MS: m/z 379 $[M+1]^+$.

Preparation of (3-((tert-butyldimethylsilyloxy)methyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanol (12)

To a solution of tert-butyl(5-iodo-2-methoxybenzyloxy)dimethylsilane (**10**, 4.0 g, 0.011 mol) in dry THF (20 ml) was added dropwise n-BuLi (7.5 mL, 1.6 Min hexane, 0.012 mol) at -78 °C. After 1 h, a solution of 3,4,5 trimethoxybenzaldehyde (**11**, 1.96 g, 0.01 mol) in dry THF (4 mL) was added drop wise at -78 °C. After 1 h stirring at rt, The reaction was monitored by TLC using ethyl acetate-hexane (4:6). After completion of the reaction as indicated by the TLC, Saturated ammonium chloride solution (10 mL) was added to the reaction mixture. The reaction mass solvent was evaporated under the vacuum, diluted with water and extracted with ethyl acetate. The combined organic layers were washed with 1 N HCl and then brine water (20 mL), dried

over Na_2SO_4, filtered, and concentrated in vacuum. Purification of the residue by column chromatography on silica gel (hexane/EtOAc, 7:3) afforded the desired product **12** as a white colour solid (5.8 g, 63%); mp: 41-43 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.41 (d, *J* = 1.98 Hz, 1H), 7.25 (dd, *J* = 1.98 Hz, 8.39 Hz, 1H), 6.79 (d, *J* = 8.39 Hz, 1H), 6.62 (s, 2H), 5.75 (s, 1H), 4.72 (s, 2H), 3.82 (s, 6H), 3.81 (s, 3H), 3.80 (s, 3H), 0.91 (s, 9H), 0.05 (d, *J* = 1.67 Hz, 6H); ESI-MS: m/z 449 $[M+1]^+$.

Preparation of (3-((tert-butyldimethylsilyloxy)methyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (13)

To a solution of (3-((tert-butyldimethylsilyloxy)methyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanol (**12**, 1.0 g, 2.2 mmol) in dry DMSO (5 ml) was added, a solution of 2-iodoxy-benzoic acid (IBX) (0.686 g, 2.45 mmol) in dimethyl sulfoxide (DMSO) (10 mL) at 10-15 °C. The reaction mixture was stirred at room temperature for 2 h. The reaction was monitored by TLC using ethyl acetate-hexane (1:4). After completion of the reaction as indicated by the TLC, Appropriate amount water was added to the reaction mixture and filtered through the celite bead. The aqueous layer was extracted with ethyl acetate. The organic layer was washed with water, brine, dried over anhydrous Na_2SO_4, and evaporated by using vacuum to get crude compounds. The residue was purified by column chromatography (10% concentrated under the vacuum, the combined organic phases EtOAc-hexane) to afford compound **13** as light yellow solid (820 mg, 82%); mp: 48-49 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.97 (d, *J* = 2.3 Hz, 1H), 7.80 (dd, *J* = 2.3 Hz, 8.3 Hz, 1H), 7.02 (s, 2H), 6.90 (d, *J* = 8.3 Hz, 1H), 4.76 (s, 2H), 3.92 (s, 3H), 3.91 (s, 3H), 3.87 (s, 6H), 0.90 (s, 9H), 0.09 (s, 6H); ESI-MS: m/z 447 $[M+1]^+$.

Preparation of tert-butyl(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzyloxy) dimethylsilane (14)

A solution of PPh_3CH_3Br (725.8 mg, 2.02 mmol) in dry THF (15 mL) was added, potassium *tert*-butoxide (188.4 mg, 1.68 mmol) at 10-15 °C under an argon atmosphere. The yellow colour mixture was stirred for 4 h. The THF of (3-((tert-butyldimethylsilyloxy)methyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone **(13**, 500 mg, 1.12 mmol**)** was added drop wise at 10-15 °C and reaction mixture was allowed to stirred for another 3 h. The reaction was monitored by TLC using ethyl acetate-hexane (1:5). After completion of the reaction as indicated by the TLC, Appropriate amount of saturated ammonium chloride solution was added to the

reaction mixture and concentrate it. The aqueous layer was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated by using vacuum to get crude compounds. The residue was purified by column chromatography (10% concentrated under the vacuum, the combined organic phases EtOAc-hexane) to afford compound **14** as light yellow solid (400 mg, 80%); mp: 60-63 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.48 (d, *J* = 2.26 Hz, 1H), 7.21 (dd, *J* = 2.26 Hz, 8.49 Hz, 1H), 6.78 (d, *J* = 8.49 Hz, 1H), 6.64 (s, 2H), 5.39 (d, *J* = 1.13 Hz, 1H), 5.28 (d, *J* = 1.32 Hz, 1H), 4.74 (s, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.81 (s, 6H), 0.88 (s, 9H), 0.07 (s, 6H); ESI-MS: m/z 445 $[M+1]^+$.

Preparation of (3-(hydroxymethyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (15a)

To a solution of (3-((tert-butyldimethylsilyloxy)methyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (**13**, 800 mg, 1.79 mmol) in dry THF (10 ml) was added, a solution of tetra butyl ammonium fluoride (2.15 mL, 1.0 M in THF, 2.15 mmol) at 10-15 °C. The reaction mixture was stirred at room temperature for 3 h. The reaction was monitored by TLC using ethyl acetate-hexane (4:6). After completion of the reaction as indicated by the TLC, Appropriate amount of saturated ammonium chloride solution was added to the reaction mixture and concentrate it. The aqueous layer was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated by using vacuum to get crude compounds. The residue was purified by column chromatography (30% concentrated under the vacuum, the combined organic phases EtOAc-hexane) to afford compound **15a** as light yellow solid (450 mg, 76%); mp: 45-47 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.91 (d, *J* = 2.26 Hz, 1H), 7.77 (dd, *J* = 2.26 Hz, 8.31 Hz, 1H), 6.97 (s, 2H), 6.85 (d, *J* = 8.31 Hz, 1H), 4.71 (s, 2H), 3.89 (s, 3H), 3.86 (s, 3H), 3.82 (s, 6H); ESI-MS: m/z 333$[M+1]^+$.

Preparation of (2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)phenyl)methanol (15b)

This compound was prepared according to the method described for compound **15a**, employing tert-butyl(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzyloxy)dimethyl silane **(14**, 500 mg, 1.124 mmol**)** to obtain the pure product **15b** as light yellow solid (300 mg, 81%); mp: 59-61 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.32 (d, *J* = 2.26 Hz, 1H), 7.23 (d, *J* = 2.26 Hz, 1H), 6.86 (d, *J* = 8.49 Hz, 1H), 6.54 (s, 2H), 5.35 (dd, *J* = 1.13 Hz, 12.8 Hz, 2H), 4.69 (s, 2H), 3.89 (s, 3H), 3.88 (s, 3H), 3.81 (s, 6H); ESI-MS: m/z 331 $[M+1]^+$.

Preparation of 2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzaldehyde (16a)

A solution of DMSO (0.258 g, 3.3 mmol) in dry CH_2Cl_2(3 mL) was added drop wise to a magnetically stirred solution of oxalyl chloride (209 mg, 1.65 mmol) in dry CH_2Cl_2(5.0 mL) kept at -78 °C under an argon atmosphere. The mixture was stirred for another 15 min. The CH_2Cl_2 of (3-(hydroxymethyl)-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methanone (**15a**, 500 mg, 1.5 mmol) was added drop wise and reaction mixture was allowed to stirred for 3 h. Triethylamine (607 mg, 6.0 mmol) was added drop wise over 5 min, and the stirred solution was allowed to warm to room temperature. The appropriate amount of water was added to reaction mixture and organic layer was separated and the aqueous phase extracted with CH_2Cl_2 (3 ×20 mL). The combined organic layer were washed with brine (10 mL), dried over anhydrous Na2SO4 and evaporated by using vacuum to get crude compound. The residue was purified by column chromatography (30% concentrated under the vacuum, the combined organic phases EtOAc-hexane) to afford compound **16a** as light yellow solid (420 mg, 84%); mp: 74-76 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 10.49 (s, 1H), 8.29 (d, *J* = 2.26 Hz, 1H), 8.13 (dd, *J* = 2.26 Hz, 8.87 Hz, 1H), 7.15 (d, *J* = 8.87 Hz, 1H), 7.01 (s, 2H), 4.05 (s, 3H), 3.95 (s, 3H), 3.87 (s, 6H); ESI-MS: m/z 331 $[M+1]^+$.

Preparation of 2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzaldehyde (16b)

This compound was prepared according to the method described for compound **15a**, employing (2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)phenyl)methanol (**15b**, 500 mg, 1.51 mmol)to obtain the pure product **16b** as light yellow solid **(**419 mg, 84%); mp: 89-92 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 10.48 (s, 1H), 7.88 (d, *J* = 2.26 Hz, 1H), 7.52 (dd, *J* = 2.26 Hz, 8.49 Hz, 1H), 6.98 (d, *J* = 8.49 Hz, 1H), 6.50 (s, 2H), 5.41 (dd, *J* = 0.92 Hz, 14.3 Hz, 2H), 3.96 (s, 3H), 3.88 (s, 3H), 3.81 (s, 6H); ESI-MS: m/z 329 $[M+1]^+$.

General Preparation of phenstatin/isocombretatstatin-oxindoles (5a-h and **6a-h)**

To the phenstatin-3-aldehyde/isocombretastatin-3-aldehyde (**16a-b)** (0.303mmol/0.304 mmol) prepared in the above step was added corresponding substituted oxindoles (**17a-h**) (0.303mmol) and catalytic amount of piperidine (1.0 ml) in ethanol. Heated the reaction mixture to reflux for 4 h at 85 °C. The solid compounds obtained in the reaction vessel were filtered and washed with ethanol for 4-5 times. After complete air drying the final compounds phenstatin/isocombretastatin-oxindole analogues **(5a-h** and **6a-h)** were obtained as pure solids (yield 71-90%).

Synthesis of (*E*)-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5a)

(*E*)-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5a**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the oxindole (**17a**, 40.3 mg, 0.303 mmol) in ethanol as yellow solid (120 mg, yield: (89%); mp: 190-192 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 300 MHz) δ (ppm): 8.81 (s, 1H), 8.06-8.02 (m, 1H), 7.96 (d, *J* = 8.12 Hz, 1H), 7.61 (d, *J* = 7.36 Hz, 1H), 7.30 (d, *J* = 7.55 Hz, 1H), 7.13 (d, *J* = 7.55 Hz, 1H), 7.10-7.06 (m, 3H), 6.98 (d, *J* = 7.74 Hz, 1H), 4.03 (s, 3H), 3.98 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 194.3, 161.7, 152,6, 149.3, 144.3, 142.6, 139.6, 137.6, 131,2, 129.7, 127.4, 124.5, 123.3, 121.6, 121.4, 119.9, 119.4, 111.1, 109.7, 107.8,60.9, 56.4, 56.1; ESI-MS: m/z 446 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{24}O_6N$ calcd: 446.15981, found: 446.15833 $[M+ H]^+$.

(*E*)-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)-5-nitroindolin-2-one (5b)

(*E*)-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)-5-nitroindolin-2-one (**5d**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 5-nitro oxindole (**17b**) (49.4 mg, 0.303 mmol) in ethanol as light yellow solid (105 mg, yield 72%); mp: 204-205 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 300 MHz) δ (ppm): 9.04 (d, *J* = 0.75 Hz, 1H), 8.52 (d, *J* = 1.5 Hz, 1H), 8.31 (s, 1H), 8.27 (dd, *J* = 1.5 Hz, 8.3 Hz, 1H), 8.02 (d, *J* = 9.06 Hz, 1H), 7.12 (d, *J* = 8.3 Hz, 1H), 7.09-7.07 (m, 3H), 4.11 (s, 3H), 4.01 (s, 3H), 3.89 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 196.6, 162.8, 152.5, 145.2, 143.5, 141.8, 138.3, 136.5, 136.6, 132.8, 128.8, 126.3, 125.6, 122.7, 121.4, 118.8, 115.7, 110.6, 108.1, 61.4, 56.5, 56.3; ESI-MS: m/z 491 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{23}O_8N_2$ calcd: 491.14489, found: 491.14338 $[M+ H]^+$.

(*E*)-5-methoxy-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5c):

(*E*)-5-methoxy-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5c**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 5-methoxy oxindole (**17c**) (53.9 mg, 0.303 mmol) in ethanol as light red solid (112 mg, yield 78%); mp: 189-191 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.32 (d, *J* = 1.2 Hz, 1H), 8.03 (s, 1H), 7.95 (dd, *J* = 1.8 Hz, 8.69 Hz, 1H), 7.13-7.15 (m, 2H), 7.02 (s, 2H), 6.95-6.93 (m, 1H), 6.85 (dd, *J* = 2.1 Hz, 8.5 Hz, 1H), 4.01 (s, 3H), 3.98 (s, 3H), 3.87 (s, 6H), 3.70 (s, 3H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm):195.8, 161.8, 152.8, 146.9, 141.8, 135.4, 135.1, 132.3, 132.1, 129.4, 128.1,

123.4, 123.1, 122.0, 115.8, 113.4, 111.7, 110.5, 109.5, 107.6, 61.1, 56.2, 55.9; ESI-MS: m/z 476 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{26}O_7N$ calcd: 476.17038, found: 476.16928 $[M+ H]^+$.

Synthesis of (*E*)-5-fluoro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5d)

(*E*)-5-fluoro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one **(5d)** was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 5-fluoro oxindole (**17d**, 45.7 mg, 0.303 mmol) in ethanol as yellow solid (118 mg, yield: (84%); mp: 201-203 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 300 MHz) δ (ppm): 8.81-8.79 (m, 1H), 8.16 (s, 1H), 8.01 (d, *J* = 8.3 Hz, 1H), 7.72 (d, *J* = 8.3 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 8.1 Hz, 1H), 7.09-7.07 (m, 3H), 4.03 (s, 3H), 3.98 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 194.9, 164.1, 160.9, 159.7, 159.1, 152.8, 142.2, 140.1, 134.4, 133.7, 131.6, 130.7, 122.5, 120.4, 116.6, 116.4, 112.8, 112.5, 112.1, 107.7, 61.1, 57.0, 56.9; ESI-MS: m/z 464 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{23}O_6NF$ calcd: 464.15039, found: 464.14934 $[M+ H]^+$.

(*E*)-5-chloro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5e)

(*E*)-5-chloro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5e**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 5-chloro oxindole (**17e**) (50.6 mg, 0.303 mmol) in ethanol as light red solid (109 mg, yield 75%); mp: 188-190 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.89 (d, *J* = 0.9 Hz, 1H), 8.07 (s, 1H), 7.98 (dd, *J* = 1.4 Hz, 8.2 Hz, 1H), 7.57 (d, *J* = 1.86 Hz, 1H), 7.28 (d, *J* = 1.9 Hz, 1H), 7.09-7.07 (m, 3H), 6.92 (d, *J* = 8.2 Hz, 1H), 4.05 (s, 3H), 3.99 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 198.0, 162.6, 152.5, 135.9, 135.1, 133.1, 132.7, 132.3, 130.3, 129.3, 128.7, 123.3, 122.5, 121.8, 121.7, 120.2, 111.7, 110.5, 107.8, 61.2, 56.3, 56.1; ESI-MS: m/z 480 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{23}O_6NCl$ calcd: 480.12084, found: 480.11969 $[M+ H]^+$.

(*E*)-6-chloro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5f)

(*E*)-6-chloro-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5f**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 6-chloro oxindole (**17f**) (50.6 mg, 0.303 mmol) in ethanol as light red solid (113 mg, yield 78%); mp: 179-182 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 300 MHz) δ (ppm): 8.86-8.84 (m, 1H), 8.04 (s, 1H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.52 (d, *J*

= 8.1 Hz, 1H), 7.11 (d, *J* = 7.9 Hz, 1H), 7.09-7.07 (m, 3H), 7.00 (s, 1H), 4.04 (s, 3H), 3.99 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 198.1, 161.4, 152.8, 138.8, 136.3, 134.9, 132.3, 129.8, 128.4, 124.6, 123.3, 122.6, 120.1, 113.5, 111.8, 110.5, 109.9, 107.9, 107.4, 61.0, 56.2, 56.0; ESI-MS: m/z 480 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{23}O_6NCl$ calcd: 480.12084, found: 480.11969 $[M+ H]^+$.

(*E*)-5-bromo-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5g)

(*E*)- 5-bromo-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5g**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 5-bromo oxindole (**17g**) (63.6 mg, 0.303 mmol) in ethanol as light red solid (123 mg, yield 78%); mp: 184-186 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.89-8.87 (m, 1H), 8.06 (s, 1H), 7.96 (d, *J* = 7.8 Hz, 1H), 7.65 (d, *J* = 7.8 Hz, 1H), 7.30 (d, *J* = 7.55 Hz, 1H), 7.23 (d, *J* = 7.55 Hz, 1H), 7.10-7.07 (m, 3H), 4.05 (s, 3H), 3.99 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm):197.2, 162.7, 152.5, 136.4, 136.0, 132.3, 132.1, 128.9, 128.6, 126.1, 125.4, 123.1, 121.8, 116.1, 114.5, 112.7, 112.2, 110.9, 110.5, 108.1, 107.7, 61.2, 56.8; ESI-MS: m/z 480 $[M+H]^+$; HRMS (ESI m/z) for $C_{26}H_{23}O_6NBr$ calcd: 524.07033, found: 524.06896 $[M+ H]^+$ and $C_{26}H_{23}O_6N^{81}Br$ calcd: 526.06828, found: 526.06694 $[M+ H]^+$.

(*E*)-6-bromo-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (5h)

E)- 6-bromo-3-(2-methoxy-5-(3,4,5-trimethoxybenzoyl)benzylidene)indolin-2-one (**5h**) was obtained using above described method by adding phenstatin-3-aldehyde (**16a**) (100 mg, 0.303 mmol) and catalytic amount of piperidine to the 6-bromo oxindole (**17h**) (63.6 mg, 0.303 mmol) in ethanol as light yellow solid (123 mg, yield 78%); Yield: 119 mg (75%); mp: 181-185 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 300 MHz) δ (ppm): 8.90-8.89 (m, 1H), 8.06 (s, 1H), 7.98 (d, *J* = 8.68 Hz, 1H), 7.70 (s, 1H), 7.39 (d, *J* = 8.68 Hz, 1H), 7.10-7.07 (m, 3H), 6.84 (d, *J* = 8.31 Hz, 1H), 4.05 (s, 3H), 3.99 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm):197.3, 162.3, 161.7, 161.1, 152.4, 141.3, 140.9, 136.2, 135.5, 134.8, 132.8, 126.6, 124.9, 123.2, 122.1, 121.2, 119.9, 116.1, 112.4, 108.6, 108.4, 61.4, 56.4; HRMS (ESI m/z) for $C_{26}H_{23}O_6NBr$ calcd: 524.07033, found: 524.06896 $[M+ H]^+$ and $C_{26}H_{23}O_6N^{81}Br$ calcd: 526.06828, found: 526.06694 $[M+ H]^+$.

(*E*)-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6a)

(E)-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6a**) was obtained using above described method by adding isocombretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the oxindole (**17a**) (40.4 mg, 0.304 mmol) in ethanol as light red solid (119 mg, yield 88%); mp: 179-180 °C;^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.10 (s, 1H), 7.73 (d, *J* = 1.7 Hz, 1H), 7.53 (d, *J* = 7.9 Hz, 1H), 7.47 (dd, *J* = 1.9 Hz, 8.7 Hz, 1H), 7.25-7.20 (m, 1H), 6.99 (t, *J* = 7.63 Hz, 16.1 Hz, 2H), 6.89 (t, *J* = 7.6 Hz, 15.2 Hz, 1H), 6.58 (s, 2H), 5.40 (d, *J* = 7.5 Hz, 1H), 5.36 (d, *J* = 7.3 Hz, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 3.83 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 170.0, 157.9, 152.9, 149.1, 141.2, 137.7, 137.6, 133.4, 131.3, 129.6, 124.5, 123.3, 122.8, 121.8, 121.5, 113.4, 110.6, 110.2, 110.0, 105.4, 60.8, 56.0, 55.6; ESI-MS: m/z 444 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{26}O_5N$ calcd: 444.18055, found: 444.17838 $[M+ H]^+$.

(*E*)-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)-5-nitroindolin-2-one (6b)

(*E*)-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)-5-nitroindolin-2-one (**6b**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 5-nitro oxindole (**17b**) (54.2 mg, 0.304 mmol) in ethanol as yellow solid (109 mg, yield 73%); mp: 181-183 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.57 (d, *J* = 1.9 Hz, 1H), 8.28 (s, 1H), 8.22 (dd, *J* = 2.1 Hz, 1H), 7.79 (d, *J* = 1.8 Hz, 1H), 7.45 (dd, *J* = 1.8 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 1H), 7.02 (d, *J* = 8.7 Hz, 1H), 6.58 (s, 2H), 5.49 (d, *J* = 7.5 Hz, 1H), 7.39 (d, *J* = 7.4 Hz, 1H), 3.95 (s, 3H), 3.90 (s, 3H), 3.83 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 158.3, 152.4, 145.1, 143.5, 140.5, 136.4, 133.9, 133.5, 129.5, 122.1, 121.8, 118.6, 114.4, 111.3, 111.1, 110.9, 110.8,, 107.7, 105.7, 61.1, 56.1, 55.8; ESI-MS: m/z 489 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_7N_2$ calcd: 489.16563, found: 489.16464 $[M+ H]^+$.

(*E*)-5-methoxy-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6c)

(*E*)-5-methoxy-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6c**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 5-methoxy oxindole (**17c**) (49.6 mg, 0.304 mmol) in ethanol as yellow solid (118 mg, yield 81%); mp: 178-180 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 7.97 (s, 1H), 7.80 (d, *J* = 2.26 Hz, 1H), 7.34 (dd, *J*

= 2.26 Hz, 9.1 Hz, 1H), 7.16 (d, *J* = 2.26 Hz, 1H), 6.95 (d, *J* = 8.3 Hz, 1H), 6.79 (d, *J* = 8.3 Hz, 1H), 6.75 (dd, *J* = 2.26 Hz, 8.3 Hz, 1H), 6.54 (s, 2H), 5.36 (d, *J* = 13.5 Hz, 2H), 3.90 (s, 3H), 3.87 (s, 3H), 3.81 (s, 6H), 3.62 (s, 3H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 170.2, 157.8, 154.4, 152.9, 149.0, 137.0, 135.3, 133.9, 133.2, 131.4, 129.4, 123.3, 122.7, 114.9, 113.2, 110.6, 110.3, 109.3, 105.3, 60.9, 56.0, 55.6, 55.7; ESI-MS: m/z 474 $[M+H]^+$; HRMS (ESI m/z) for $C_{28}H_{28}O_6N$ calcd: 474.19111, found: 474.19001 $[M+ H]^+$.

(*E*)-5-fluoro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6d)

(E)-5-fluoro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6d**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 5-fluoro oxindole (**17d**) (45.9 mg, 0.304 mmol) in ethanol as yellow solid (120 mg, yield 86%); mp: 178-179 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.13 (s, 1H), 7.70 (d, *J* = 1.13 Hz, 1H), 7.44 (dd, *J* = 2.1 Hz, 8.7 Hz, 1H), 7.25 (dd, *J* = 2.4 Hz, 9.0 Hz, 1H), 6.99 (d, *J* = 8.7 Hz, 1H), 6.94 (dd, *J* = 2.4 Hz, 8.7 Hz, 1H), 6.90-6.87 (m, 1H), 6.54 (s, 2H), 5.41 (d, *J* = 7.5 Hz, 1H), 5.38 (d, *J* = 7.5 Hz, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.82 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 170.3, 160.7, 159.6, 152.5, 138.8, 138.1, 136.4, 135.9, 133.6, 132.7, 129.3, 126.0, 122.2, 116.5, 116.1, 112.7, 111.9, 111.1, 110.1, 109.1, 105.5, 61.1, 56.1, 55.8; ESI-MS: m/z 462 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_5NF$ calcd: 462.17113, found: 462.16957 $[M+ H]^+$.

(*E*)-5-chloro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6e)

(*E*)-5-chloro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6e**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 5-chloro oxindole (**17e**) (50.9 mg, 0.304 mmol) in ethanol as yellow solid (110 mg, yield 76%); mp: 178-180 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.11 (s, 1H), 7.72 (d, *J* = 1.7 Hz, 1H), 7.48 (dd, *J* = 3.6 Hz, 8.7 Hz, 1H), 7.29 (dd, *J* = 2.1 Hz, 8.9 Hz, 1H), 6.99 (d, *J* = 8.7 Hz, 1H), 6.94 (dd, *J* = 3.5 Hz, 8.9 Hz, 1H), 6.91-6.88 (m, 1H), 6.54 (s, 2H), 5.41 (d, *J* = 7.5 Hz, 1H), 5.38 (d, *J* = 7.5 Hz, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.82 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 172.9, 156.5, 154.5, 150.2, 138.6, 138.4,133.9, 133.1, 130.1, 129.1, 128.2, 128.0, 122.8,

123.1, 122.1, 121.5, 111.9, 111.6, 110.5, 110.0, 107.4, 106.5, 60.9, 56.0, 55.6; ESI-MS: m/z 478 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_5NCl$ calcd: 478.14158, found: 478.14158 $[M+ H]^+$.

(*E*)-6-chloro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6f)

(*E*)-6-chloro-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6f**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 6-chloro oxindole (**17f**) (50.9 mg, 0.304 mmol) in ethanol as yellow solid (116 mg, yield 79%); mp: 179-182 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.13 (s, 1H), 7.81 (d, *J* = 1.5 Hz, 1H), 7.51 (d, *J* = 8.9 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 1H), 6.89 (d, *J* = 6.58 Hz, 1H), 6.68 (d, *J* = 2.4 Hz, 1H), 6.90-6.87 (m, 1H), 6.54 (s, 2H), 5.41 (d, *J* = 7.5 Hz, 1H), 5.38 (d, *J* = 7.5 Hz, 1H), 3.92 (s, 3H), 3.88 (s, 3H), 3.82 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 173.6, 161.1, 160.5, 159.9, 151.8, 149.4, 147.5, 140.0, 134.5, 130.2, 129.5, 127.2, 123.2, 116.4, 115.4, 112.6, 111.2, 111.1, 109.0, 106.9, 61.3, 56.1, 55.7; ESI-MS: m/z 478 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_5NCl$ calcd: 478.14158, found: 478.14158 $[M+ H]^+$.

(*E*)-5-bromo-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6g)

(*E*)-5-bromo-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6g**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 5-bromo oxindole (**17g**) (64.5 mg, 0.304 mmol) in ethanol as yellow solid (119 mg, yield 75%); mp: 184-186 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm):8.09 (s, 1H), 7.77 (dd, *J* = 1.8 Hz, 8.5 Hz, 2H), 7.40-7.35 (m, 2H), 6.96 (d, *J* = 8.5 Hz, 1H), 6.55 (s, 2H), 6.48 (dd, *J* = 2.3 Hz, 18.1 Hz, 1H), 5.42 (d, *J* = 18.1 Hz, 2H), 3.92 (s, 3H), 3.88 (s, 3H), 3.82 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 176.7, 160.6, 160.0, 157.1, 151.8, 149.4, 140.3, 137.7, 136.2, 134.5, 133.5, 132.3, 131.1, 126.7, 122.8, 120.7, 116.3, 115.9, 112.5, 109.1, 106.6, 61.2, 56.0, 55.6; ESI-MS: m/z 522 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_5NBr$ calcd: 522.09106, found: 522.09033 $[M+ H]^+$ and $C_{26}H_{23}O_6N^{81}Br$ calcd: 524.08902, found: 524.08823 $[M+ H]^+$.

(*E*)-6-bromo-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (6h)

(*E*)-6-bromo-3-(2-methoxy-5-(1-(3,4,5-trimethoxyphenyl)vinyl)benzylidene)indolin-2-one (**6h**) was obtained using above described method by adding *iso*combretastatin-3-aldehyde (**16b**) (100 mg, 0.304 mmol) and catalytic amount of piperidine to the 6-bromo oxindole (**17h**) (64.5 mg, 0.304 mmol) in ethanol as yellow solid (121 mg, yield 76%); Yield: 120 mg (76%); mp: 181-185 °C; ^{1}H NMR ($CDCl_3$ + 1 drop [D]TFA, 500 MHz) δ (ppm): 8.10 (s, 1H), 7.65 (s, 1H), 7.47 (dd, *J* = 1.5 Hz, 8.9 Hz, 1H), 7.36 (d, *J* = 8.9 Hz, 1H), 7.11 (s, 1H), 7.02-6.97 (m, 2H), 6.55 (s, 2H), 5.38-5.35 (m, 2H), 3.93 (s, 3H), 3.91 (s, 3H), 3.83 (s, 6H); ^{13}C NMR ($CDCl_3$ + 1 drop [D]TFA, 75 MHz) δ (ppm): 178.1, 159.9, 157.9, 157.7, 150.7, 148.5, 139.7, 135.4, 133.1, 132.9, 132.5, 133.1, 124.1, 122.4, 119.9, 115.3, 111.9, 111.7, 109.2, 105.9, 61.1, 59.9, 55.7; ESI-MS: m/z 522 $[M+H]^+$; HRMS (ESI m/z) for $C_{27}H_{25}O_5NBr$ calcd: 522.09106, found: 522.09023 $[M+H]^+$ and $C_{26}H_{23}O_6N^{81}Br$ calcd: 524.08902, found: 524.08814 $[M+H]^+$.

II. **Biology**

Anticancer activity

The cytotoxic activity of the compounds was determined using MTT assay.[21] Cells were seeded in 100 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. After incubation cells were treated with test compounds for 48 h. 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.

Cell cycle analysis

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell-cycle phases. HT-29 cells were incubated for 48 h with compounds **5c** and **5d** at concentrations of 10 and 50 nM. 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).[22]

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. 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 reference compound in this study. To determine the IC50 values of the compounds against tubulin polymerization, the compounds were pre-incubated with tubulin at varying concentrations. Assays were performed under similar conditions as employed for polymerization assays as described above.[23]

Mitochondrial membrane potential

HT-29 cells were cultured in six-well plates after treatment with compounds **5c** and **5d** at 10 and 50 nM concentrations for 48 h. After 48 h of treatment, cells were collected by trypsinization and washed with PBS followed by resuspending in JC-1 (5 µg/ml) and incubated at 37 ^{0}C for 15 min. Cells were rinsed three times with medium and suspended in pre warmed medium. The cells were then subjected to flow cytometric analysis on a flow cytometer (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential.[24]

Annexin staining assay for apoptosis

HT-29 cells were seeded in six-well plates and allowed to grow overnight. The medium was then replaced with complete medium containing compounds **5c** and **5d** at 10 and 50 nM concentrations. After 48 h of drug treatment, cells from the supernatant and adherent monolayer cells were harvested by trypsinization, washed with PBS at 5000 rpm. Then the cells were stained with Annexin VFITC and propidium iodide using the Annexin-V-FITC apoptosis detection kit (Sigma aldrich). Flow cytometry was performed for this study as described earlier.[25]

Molecular docking

The molecular docking studies were performed at the colchicine (LOC) binding site of the tubulin (PDB ID: 3E22).[26] The co-ordinates of the crystal structure were obtained from RCSB-Protein Data Bank and suitable corrections to it were made by using Protein Preparation Wizard from Schrödinger package. In regard to the ligands, molecules were constructed using ChemBio3D Ultra 12.0 and their geometries were optimized using molecular mechanics. Finally, docking studies were performed on the most active molecules (**5c** and **5d**) by using AutoDock 4.2 docking software[27]and the results were visualized through PyMOL.[28]

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Graphical Abstract

Design, Synthesis of Phenstatin/isocombretastatin-Oxindole Conjugates as Antimitotic agents

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