SYNTHESIS AND ANTIANGIOGENIC ACTIVITY OF SOME NOVEL ANALOGUES OF COMBRESTATIN A-4

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ABSTRACT: A series of Combretastatin A-4 analogues were synthesized in order to obtain new compounds with potential antiangiogenic activity. The structures of all synthesized compounds were confirmed by means of spectroscopical analytical techniques. All compounds were evaluated for their antiangiogenic activity by chorioallantoic membrane (CAM) assay method. The compounds showed significant antiangiogenic activity. Compound 5a showed maximum activity out of all synthesized compounds.

Keywords: Combretastatin A-4, antiangiogenic, cytotoxicity, anticancer, antitubulin.

INTRODUCTION
Angiogenesis or neovascularization is a complex process involving the activation, adhesion, proliferation, and transmigration of endothelial cells from pre-existing blood vessels. It plays a critical role in normal physiological processes such as wound healing, but also in a number of pathological processes, for instance diabetes retinopathy, arthritis, and the growth of solid tumors. Therefore angiogenesis is considered as a potential target for antitumor activity. Combretastatin A-4 is currently under investigation as an angiogenesis inhibitor (antiangiogenic). Combretastatin A-4 is a natural compound isolated by Pettit and co-workers (1982) from the bark of the South African bush willow tree Combretum caffrum.1 Out of various stilbene derivatives (termed as combretastatins) isolated from the plant, combretastatin A-4 (CA-4, Fig. 1) was found to be most potent.2 CA-4, cis-1-(3,4,5-trimethoxyphenyl)-2- (3′-hydroxy-4′-methoxy phenyl) ethene, is active in cis form.3 From the structure-activity relationship (SAR) point of view, CA-4 belongs to the class of natural compounds related to biphenyls and contains, as a key structural feature, the cis-stilbene motif. CA-4 exerts a potent cytotoxicity against a variety of human cancer cells including multi drug resistant (MDR) cancer cell lines,4-11 and also displays potent antitumor effect in a wide variety of preclinical tumor models12-16 as well as substantial antivascular (antiangiogenic) activity in tumor blood flow while causing no significant blood flow retention in normal tissues.7-22 CA-4 does not show in vivo efficacy due to its poor pharmacokinetics resulting from its high lipophilicity and low aqueous solubility24 and also due to isomerization of cis-double bond to the more thermally stable trans-isomer, which is inactive.2,25 Till today various Combretastatin analogues have been synthesized and reported to posses cytotoxic activity against various cancer cell lines.24, 26-33 CA-4 inhibits tubulin polymerization by binding to tubulin at colchicines binding site, resulting in disruption of dynamic equilibrium needed in formation of microtubules from α- and β- tubulin heterodimers, leading to formation of abnormal mitotic spindles. It results in cell cycle arrest in the M-phase, leading to apoptotic cell death.34-39 The IC50 (inhibitory concentration in 50% population) of CA-4 against tubulin polymerization is in range from 0.53 to 3.0 μM.4,10 Recently, it has been found that CA-4 induces cell death primarily through mitotic catastrophe (formation of giant, multinucleated cells). CA-4 induces mitotic catastrophe by activation of a cysteine protease, called caspase-9.40 CA-4 is not a substrate of the MDR pump, a cellular pump that rapidly transports out foreign molecules, including many anticancer drugs. This is the major reason for its superior activity against MDR positive cancer cell lines.41,42 Antivascular effect of CA-4 is related to its antitubulin activity. The cellular microtubule network plays a major role in maintaining cell shape, particularly in the case of neovascularature. CA-4 causes microtubules to rapidly depolymerize. As a result elongated endothelial cells round up, causing disruption of endothelial cell layer surrounding blood vessel and exposing of underlying basement membrane. This leads to blood
vessel congestion and loss of blood flow, loss of oxygen and nutrient supply to tumor cells. Therefore, tumor cells undergo necrosis. In view of strong anticancer/antivascular activity exhibited by CA-4, we have synthesized some novel combretastatin analogues, and tested for their antiangiogenic activity.

![Structural formula and 3D-structure of CA-4.](image)

**Fig. 1 (a) Structural formula and (b) 3D-structure of CA-4.**

**Fig. 2 Scheme of synthesis of compounds**
MATERIALS AND METHODS

Chemistry

All compounds were purified by column chromatography and recrystallization and confirmatory establishment of structure was done by melting point, TLC, UV, IR and \(^1\)H NMR. Column chromatography was performed using silica gel (Qualigens, particle size 60-120 mm). TLC was performed on silica gel TLC plates. All melting points were recorded on a DECIBEL digital melting point apparatus. IR spectra were recorded on a 8400S SHIMADZU spectrometer. \(^1\)H NMR spectra were recorded on a dpx300 spectrometer (analysis laboratory, IIT, New Delhi). Physical properties of the synthesized compounds are listed in table 1 whereas scheme of synthesis is given in fig.2.

General procedure of preparation of compounds

Procedure of preparation of compounds 3a-c

A mixture of p-nitrophenyl acetic acid 2 (2 mmol), benzaldehyde 1a or 1b or 1c (2 mmol), and triethylamine (0.5 ml) in acetic anhydride (5 ml) was refluxed for 12 hours, poured into hot saturated sodium carbonate solution (50 ml) and left overnight. The mixture was extracted with diethyl ether (2x50 ml), and the ether extracts were discarded. The aqueous solution was acidified with dilute HCl. The precipitated product was filtered with vacuum pump and dried. Product was subjected to column chromatography.

Procedure of preparation of compounds 4a-c

Concentrated H\(_2\)SO\(_4\) (0.5 ml) was added to a stirred solution of carboxylic acid 3a or 3b or 3c (0.5 mmol) in absolute methanol (20 ml), and the mixture was heated under reflux for 6 hours. About 90% of excess methanol was removed by evaporation, and the residue was poured into ice-water (300 ml). The product was extracted with diethyl ether (2x40 ml), and the combined extracts were washed with 2% NaOH solution (2x50 ml) followed by water (200 ml). Product was obtained from ether fraction.

Procedure of preparation of compounds 5a-c

A mixture of carboxylic acids 3a or 3b or 3c (0.5 mmol) and thionyl chloride (1ml) in benzene (10 ml) was refluxed for 6 hours. The excess thionyl chloride and benzene were removed under reduced pressure, and the residue was kept under vaccum for 30 minutes, and dried to give required product. Product was purified by recrystallization from EtOAc-hexane.

Procedure of preparation of compounds 6a-g

A solution of appropriate amine (0.5 mmol) in THF (5 ml) was added to a solution of acid chlorides (prepared from 3a or 3b or 3c in 0.5 mmol scale, as described above) in THF (10 ml). The mixture was stirred for 3 hours. Solvents were removed under reduced pressure, and the residue was poured onto ice (200 g). The product was extracted with diethyl ether (2x20 ml), washed with water and dried. Crude product was obtained from ether fraction. Product was purified by recrystallization from EtOAc-hexane.

Spectral data

\((2E)\)-2-(4-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)acrylic acid (3a):

FTIR (KBr) cm\(^{-1}\) 3061 and 855 (C-H), 2935 and 1456 (CH\(_3\)), 1707 (C=O), 1661 (C=C), 1598 and 1418 (COO), 1517 and 1342 (C-NO\(_2\)), 1241 and 1003 (C-O), 751 (Ar-H); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 9.98 (1H, bs), 8.26 (2H, d), 7.87 (1H, s), 7.33 (2H, d), 6.47 (2H, s), 3.78 (6H, s), 3.77 (3H, s).

Methyl \((2E)\)-2-(4-nitrophenyl)-3-(3,4,5-
trimethoxyphenyl)acrylate (4a):

FTIR (KBr) cm\(^{-1}\) 3068 and 853 (C-H), 2939 and 1453 (CH\(_3\)), 1728 (C=O), 1661 (C=C), 1603 (C=O of Ar), 1519 and 1342 (C-NO\(_2\)), 1241, 1129 and 1000 (C-O), 750 (Ar-H); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.27 (2H, d), 7.73 (1H, s), 7.37 (2H, d), 6.61 (2H, s), 3.88 (3H, s), 3.79 (3H, s), 3.76 (6H, s).

\((2E)\)-2-(4-nitrophenyl)-3-(3,4,5-
trimethoxyphenyl)acryloyl chloride (5a):

FTIR (KBr) cm\(^{-1}\) 3069 and 857 (C-H), 2938 and 1459 (CH\(_3\)), 1755 (C=O), 1655 (C=C), 1604 (C=O of Ar), 1515 and 1339 (C-NO\(_2\)), 1251 and 1012 (C-O), 739 (Ar-H), 701 (C-Cl); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.23 (2H, d), 7.77 (1H, s), 7.35 (2H, d), 6.55 (2H, s), 3.81 (3H, s), 3.77 (6H, s).

\((2E)\)-N-ethyl-2-(4-nitrophenyl)-3-(3,4,5-
trimethoxyphenyl)acrylamide (6a):

FTIR (KBr) cm\(^{-1}\) 3434 and 1563 (N-H), 3068 and 852 (C-H), 2937 and 1382 (CH\(_3\)), 1691 (C=O), 1649 (C=C), 1600 (C=C of Ar), 1522 and 1342 (C-NO\(_2\)), 1458 (CH\(_2\)), 1252 and 1003 (C-O), 750 (Ar-H); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.27 (2H, d), 7.77 (1H, s), 7.30 (2H, d), 6.46 (2H, s), 5.58 (1H, bt), 3.80 (6H, s), 3.76 (3H, s), 3.36 (2H, q), 1.11 (3H, t).

\((2E)\)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-N-(2-chlorophenyl)acrylamide (6b):

FTIR (KBr) cm\(^{-1}\) 3433 (N-H), 3079 and 859 (C-H), 2924 (CH\(_3\)), 1683 (C=O), 1631 (C=C), 1600 (C=C of Ar), 1518 and 1345 (C-NO\(_2\)), 1239 and 1007 (C-O), 750 (Ar-H), 707 (C-Cl); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.24 (2H, d), 7.79 (1H, s), 7.60 (1H, d), 7.32 (4H, m), 7.15 (1H, t), 6.46 (2H, s), 9.61 (1H, bs), 3.81 (6H, s), 3.76 (3H, s).

\((2E)\)-3-(4-fluorophenyl)-2-(4-nitrophenyl)-N-(4-fluorophenyl)acrylamide (6c):

FTIR (KBr) cm\(^{-1}\) 3430 (N-H), 3075 and 851 (C-H), 2934 and 1467 (CH\(_3\)), 1689 (C=O), 1657 (C=C), 1599 (C=C of Ar), 1512 and 1339 (C-NO\(_2\)), 1231 and 1004 (C-O), 1121 (C-F), 750 (Ar-H); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.27 (2H, d), 7.89 (1H, s), 7.33 (2H, d), 7.60 (2H, d), 6.96 (2H, d), 6.47 (2H, s), 5.65 (1H, bs), 3.79 (6H, s), 3.78 (3H, s).

\((2E)\)-3-(4-chlorophenyl)-2-(4-nitrophenyl)-N-(2-methylphenyl)acrylamide (6d):

FTIR (KBr) cm\(^{-1}\) 3432 (N-H), 3065 and 851 (C-H), 2934 and 1461 (CH\(_3\)), 1683 (C=O), 1663 (C=C), 1599 (C=C of Ar), 1519 and 1345 (C-NO\(_2\)), 1244 and 1004 (C-O), 750 (Ar-H); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 8.28 (2H, d), 7.77 (1H, s), 7.60 (1H, d), 7.30 (4H, m), 7.16 (1H, t), 6.44 (2H, s), 9.47 (1H, bs), 3.78 (6H, s), 3.77 (3H, s), 2.34 (3H, s).
**Pharmacology**

CAM assay is routinely used as a preliminary method to determine antiangiogenic effect of a compound. This assay is based upon the formation of a chorioallantoic membrane, in which neovascularization takes place, in fertilized chicken eggs at a certain stage of the development of the embryo. Agarose pellets impregnated with the test compound are placed onto the vascular membrane of opened eggs, and the influence on angiogenesis is evaluated. For assay purpose the fertile chicken eggs were procured from Kelchana hatchery, Morinagur.

**Antiangiogenesis study by chorioallantoic membrane (CAM) assay**

Twelve eggs were used per experiment to test one compound as a given dose. The eggs were fertilized at 37°C and 80% relative humidity in ideal conditions. The shells of eggs were cleaned with 70% EtOH to avoid infections. After 72 hrs 8-10 ml of albumin was removed with a syringe at the lower side of the egg, and the hole was sealed with tape. Subsequently the upper part of the shell was removed, and the eggs were covered with a plastic film and incubated for another 72 hrs. At this point of time, when the diameter of CAM is between 1.8 and 2.6 cm, the pellets containing the test substances were placed on the CAM. Test substances were dissolved or suspended in a 2.5% agarose solution. After gel formation, the volume of agarose gel corresponding to the dose of the test compound to be applied to the CAM was taken by means of a micropipette for viscous solutions. Therefore the agarose pellets do not have a uniform size. The half-cone-shaped agarose pellets are fixed because they slightly sink into the CAM. After 24 hrs the antiangiogenic effect was measured after addition of cream as a contrast fluid, by means of a stereomicroscope, by observing the avascular zone of cream as a contrast fluid, and an agarose pellet as a blank.

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**RESULTS AND DISCUSSION**

The antiangiogenic activity of the test compounds is listed in table 2. All the compounds were tested at a dose of 10 µg/pellet, corresponding to 30 nmol/ pellet approximately, because at higher dose most of compounds showed a toxic effect. Compound 3a, 5a, 5b and 5c showed an antiangiogenic score of more than 1. Compound 5a was found to be most potent with a score
of 1.6±0.1. The results of present study shows that synthesized compounds have significant antiangiogenic activity. The most active analogues 3a, 5a, 5b and 5c have smaller groups like COOH, COOCH₃ or COCl as bridge substituents while the least active analogues 6e, 6f and 6g comparatively large groups as bridge substituents. Present study concludes that size of bridge substituents affect the antiangiogenic activity. Furthermore, most active analogues, of present study, are potential candidate for treatment of diseases related with angiogenesis.

Table 1 Physical properties of the synthesized compounds

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R, R₁, R₂</th>
<th>Molecular formula</th>
<th>Solvent system: [Dichloromethane:Carbontetrachloride:Methanol (10:10:1)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₁₈H₁₇NO₇</td>
<td>0.792 90</td>
</tr>
<tr>
<td>4a</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₁₉H₁₉NO₇</td>
<td>0.717 122</td>
</tr>
<tr>
<td>5a</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₁₈H₁₆ClNO₆</td>
<td>0.792 107</td>
</tr>
<tr>
<td>6a</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₁₉H₁₉N₃O₆S</td>
<td>0.700 117</td>
</tr>
<tr>
<td>6b</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₂₄H₂₁FN₂O₆</td>
<td>0.646 102</td>
</tr>
<tr>
<td>6c</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₂₀H₂₂N₂O₆</td>
<td>0.569 112</td>
</tr>
<tr>
<td>6d</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₂₅H₂₄N₂O₆</td>
<td>0.708 97</td>
</tr>
<tr>
<td>6e</td>
<td>R'=3,4,5-tri-OCH₃</td>
<td>C₂₁H₂₂ClN₂O₆</td>
<td>0.669 77</td>
</tr>
<tr>
<td>3b</td>
<td>R'=p-N(CH₃)₂</td>
<td>C₁₇H₁₆N₂O₄</td>
<td>0.792 210</td>
</tr>
<tr>
<td>4b</td>
<td>R'=p-N(CH₃)₂</td>
<td>C₁₈H₁₈N₂O₄</td>
<td>0.880 200</td>
</tr>
<tr>
<td>5b</td>
<td>R'=p-N(CH₃)₂</td>
<td>C₁₇H₁₅ClN₂O₃</td>
<td>0.804 152</td>
</tr>
<tr>
<td>6f</td>
<td>R'=p-N(CH₃)₂</td>
<td>C₁₉H₂₃N₃O₃</td>
<td>0.610 162</td>
</tr>
<tr>
<td>3c</td>
<td>R'=o-NO₂</td>
<td>C₁₅H₁₀N₂O₆</td>
<td>0.280 80</td>
</tr>
<tr>
<td>4c</td>
<td>R'=o-NO₂</td>
<td>C₁₆H₁₂N₂O₆</td>
<td>0.760 Decomposed</td>
</tr>
<tr>
<td>5c</td>
<td>R'=o-NO₂</td>
<td>C₁₅H₁₂ClN₂O₅</td>
<td>0.816 92</td>
</tr>
<tr>
<td>6g</td>
<td>R'=o-NO₂</td>
<td>C₁₇H₁₄N₃O₅</td>
<td>0.782 62</td>
</tr>
</tbody>
</table>

*aSolvent system: [Dichloromethane:Carbontetrachloride:Methanol (10:10:1)]
**Table 2** Antiangiogenic activity of compounds in the CAM assay

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Concentration (µg/pellet)</th>
<th>nmol/pellet</th>
<th>Antiangiogenic score b ± sd (n = no. of experiment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>10</td>
<td>28</td>
<td>1.4 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>4a</td>
<td>10</td>
<td>27</td>
<td>1.0 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>5a</td>
<td>10</td>
<td>26</td>
<td>1.6 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>6a</td>
<td>10</td>
<td>24</td>
<td>0.9 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>6b</td>
<td>10</td>
<td>22</td>
<td>0.4 ± 0.2 (n = 2)</td>
</tr>
<tr>
<td>6c</td>
<td>10</td>
<td>26</td>
<td>0.7 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>6d</td>
<td>10</td>
<td>22</td>
<td>0.3 ± 0.3 (n = 2)</td>
</tr>
<tr>
<td>6e</td>
<td>10</td>
<td>21</td>
<td>0.6 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>3b</td>
<td>10</td>
<td>32</td>
<td>1.0 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>4b</td>
<td>10</td>
<td>31</td>
<td>0.8 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>5b</td>
<td>10</td>
<td>30</td>
<td>1.2 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>6f</td>
<td>10</td>
<td>29</td>
<td>0.6 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>3c</td>
<td>10</td>
<td>29</td>
<td>0.8 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>4c</td>
<td>10</td>
<td>30</td>
<td>0.6 ± 0.2 (n = 2)</td>
</tr>
<tr>
<td>5c</td>
<td>10</td>
<td>30</td>
<td>1.1 ± 0.1 (n = 2)</td>
</tr>
<tr>
<td>6g</td>
<td>10</td>
<td>29</td>
<td>0.3 ± 0.4 (n = 2)</td>
</tr>
<tr>
<td>Agarose pellet</td>
<td></td>
<td></td>
<td>0.1 ± 0.1 (n = 10)</td>
</tr>
<tr>
<td>β-1,4-galactan sulphate (LuPS S5)</td>
<td>50</td>
<td>2.5</td>
<td>1.4 ± 0.1 (n = 10)</td>
</tr>
</tbody>
</table>

b0 = no or weak effect, 1 = medium effect, 2 = strong effect

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**REFERENCES**


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