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Synthesis Evaluation Using Cell Lines of Analogues Indole-Based Agents as Tubulin Inhibitors

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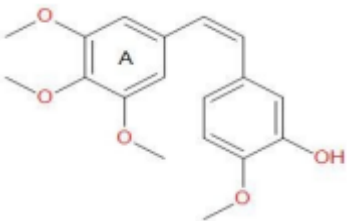
Abstract: ***Combretastatin is a natural product and atubulin inhibitor that is recognised from other synthetic, natural occurring and semi-synthetic tubulin inhibitors discovered in the past. Many pro-drugs have undergone through clinical testing as vascular targeting medicines, such as combretastatin as Vascular Disrupting Agents developed by their improved selectivity towards endothelial cells as well as their innate capacity to swiftly cause vascular shutdown and stop the tumour development. The molecular target of combretastatin and the big majority of their tubulin, synthetic variants, is well known. Disruption of the microtubule by combretastatin that targets it decreases cell growth and causes mitotic spindles in the G2-M phase of the cell cycle. Combretastatin A-4 inhibits tubulin polymerization by engaging with the microtubule's colchicine binding site and has shown significant lethal impact in a broad range of preclinical tumour types. By altering the dynamics of tubulin, such as depolymerization and polymerization combretastatin has an impact. Numerous analogues made from combretastatin have also been created due to their structural simplicity and have shown strong promise as inhibitors of tubulin polymerization. In this review, the pharmacological properties of combretastatin as a category of effective antimitotic anticancer agents are presented anddiscussed.***

Keywords: Combretastatins analogues, tubulin inhibitors, anticancer

1. Introduction

Cancer is disease in which body cells grow uncontrollably and spread to other parts of the body. These processes break down, and abnormal or damaged cells grow and multiply when they should not. These cells may form tumors, which are intissues. Tumors can be cancerous or not cancerous. Cancerous tumors spread into or invade nearby tissues and can travel to distant places in the body to form new tumors**.** This process is called metastatisis. Cancerous tumors also called malignant tumors. Combretastatins A-4, cis 1-(3, 4, 5-trimethoxy phenyl)-2-(3-hydroxy-4-methoxy phenyl) ethene, is a natural product. It was isolated by **Pettit** and co-workers in 1982 from the bark of the South African bush willow tree **Combretumcaffrum**. Among various **Stilbene derivatives,** CA-4 was the most potent. CA-4 is active in CIS form. [1, 2].

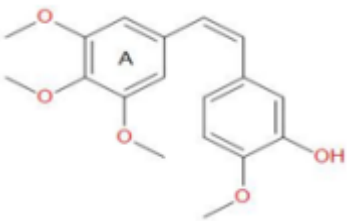


A ring is a tri methoxy ring. The B ring is connected to A by an ethenebridge. Administration of combretastatin causes a reduction in tumor blood flow resulting in hypoxia and metabolic deprivation intumors. These also alter blood flow to the heart, brain, kidney, etc. Due to alteration in the blood flow, especially in the heart and brain may cause serious harm to the patient. Another disadvantages of these combretastatin's are, that they are effective only above the maximum tolerated doses. STILBENES Stilbenes are phenolic compounds that are present in some berries. Resveratrol and its analogs have potent biological properties such as anti-inflammatory, antiaging, antiallergic, anticarcinogenic, and antimutagenic activities. Resveratrol can boost apoptosis of cancer cells by enhancing sensitivity to tumor necrosis factor α (TNF-α) and reducing NF-KB activation. [3]

Mechanism of Action of Combretastatin

CA-4 inhibits the tubulin polymerization by binding to tubulin at colchicine's binding site resulting in disruption of equilibrium which is required in the formulation of microtubules from α and β-tubulin heterodimers, it leads to abnormal mitoticspindles. This leads to cell cycle arrest in the M-phase leading to celldeath. CA-4 is not a substrate of multidrug resistance pumps (MDR), which pump out anticancer drugs by detecting it as a foreign molecule. This is the major reason for its activity against MDR-positive cancer celllines. As we have mentioned above, there is a disruption in the microtubule formation which plays a major role in maintaining cell shape. CA-4 causes microtubules to rapidly depolymerize. As a result, elongated endothelial cells round up, disrupting the endothelial cell layer surrounding the blood vessel and exposing the underlying basement membrane. This leads to blood vessel congestion and loss of blood vessels, loss of oxygen, and nutrient supply to tumor cells. Therefore, tumor cells undergone crosis. [1]

Structural activity relationship studies ONCA-4



C is orientation of the two benzene rings is essential. 3, 4, 5-trimethoxy substituents on A-ring of CA-4 indispensable for potent cytotoxicity, and antimitotic activity. The quantitative structure-activity relationship (QSAR) studies on CA-4 analogs. Suggest the influence of structure and substitution in the A and B rings and the bridge between them. 3, 4, 5 tri methoxy phenyl and 4-methoxy, 3-hydroxy substituent of phenyl system separated by a 2-atom bridge or essential structural characters for combretastatin's to be active. Where the hydroxy group can be replaced by hydrogen, amine, phosphate, or other derivatives for solubilization purposes. The optimal Hansch-Fujita π constant ∑πb, which is an estimation of the optimal lipophilicity of ring B should be in the range of -0.69 to -0.71 for cytotoxicity. Their structural requirements have been met in analogs such as cis-olefins, sulfonamides, sulfonates, amine or amine derivatives, ether, cyclopentane, azides, carboxamide, and heterocycles. Above, their moieties have been used to maintain the spatial arrangement of both aromatic systems, resulting in highly potent compounds whenever their substitution pattern is close to that of CA-4. The best inhibitors of tubulin polymerization are cis-stilbenes. In the chalcone series, stilbenes bearing a hydroxy group at 3 positions of the C-ring are better inhibitors than fluorine at the same position. Substitution on the bridge of stilbenes series was well tolerated (chalcones-keto CA-4 derivatives) [1]

Uses of combretastatin

It has strong antiproliferative and antiangiogenic activities. Phase II clinical trials for the treatment of advanced anaplastic thyroid cancer, pathologic myopia, and polypoidalchoroidal vasculopathy have been completed forCA-4P. CA-4 has a potent cytotoxic effect against many human cancer celllines. These are effective in multidrug resistance cancer cells in vitro while not in in-vivo because of their low solubility and in stability of cis-configuration therefore phosphate prodrugs of combretastatin have been developed to overcome low water solubility. Combretastatin is available in nanoformulations also which are effective in drug delivery at a particular site. [1]

Microtubules

Microtubules are the straight, hollow, and tubular structures of the cytoskeleton. These are arranged in a different bundle. Each tubule has a diameter of 20-30nm. Its length varies and it may be 1000 times more than the thickness. The microtubules are formed by tubulin which are the bundles of globular proteins Tubulin has 2 subunits they are α & β subunit. Microtubule's role in of cancer, the microtubules are a dynamic structure, which consists of α & β-tubulin heterodimers. The tubulin heterodimers involve in cell movement, mitosis, and intracellular trafficking. The tubulin which is a family of proteins is recognized as the target of the tubulin-binding chemotherapeutics, which suppress the process of the mitotic spindle to cause mitotic arrest and cell death. During mitosis, the spindle is formed by the microtubules to correct chromosomal segregation. Tubulin mainly binds to the agent's taxanes, vinca alkaloids, epothilones, and eribulin. These are important chemotherapeutic drugs. Which suppresses the spindle dynamics and causes mitotic arrest & cell death in rapidly dividing the cells. The tubulin in cell stress responses in cancer. Microtubules are composed of eight α-tubulinisotypes and seven β-tubulinisotypes in humans. The different tubulinisotypes possess specific tissue and developmental distributions. A high degree of structural homology can be seen in the members of the tubulin family. They can be distinguished from one another by highly divergent sequences at their carboxy-terminal (C-terminal) tail. Tubulin alterations in cancer are the diverse change in the microtubule network that have been identified and characterized in a wide variety of cancers. Tubulin inhibitors are chemotherapy drugs that interfere directly with the tubulin system, which is in contrast to those chemotherapy drugs acting on DNA microtubules that play an important role in eukaryotic cells. Tubulin polymerization inhibitors-inhibits the formation of microtubules. Ex: colchicine analogs and vinca alkaloids. They decrease the microtubule polymer mass in the cells at high concentrations and act as microtubule destabilizing agents. Tubulin depolymerization inhibitors increase the microtubule polymer mass in the cell, they act as microtubule stabilizing agents and are called depolymerization inhibitors. Ex: paclitaxel analogues. [4, 5]

2. Experimentation

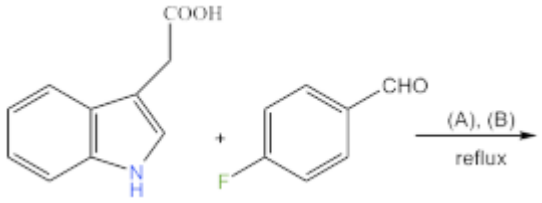
Synthesis

To a mixture of indole acetic acid and 4-fluoro benzaldehyde, add triethylamine (2ml) and acetic anhydride (4ml). Reflux it for 6-10 hours, then cool the product and add 35% of 6ml HCL to the product. Leave it for the whole night. Add ice cubes and stir vigorously to form crystals and then filtered and kept them for drying.

Reaction

Figure 20: Reagents and conditions (A) triethylamine, (B) Acetic anhydride, refluxed for 6 to 10h

3. Docking Studies



Ligand Preparation

The protein data bank (PDB id: 1SA0) has coordinates for tubulin [30, 31]. Before being prepared for analysis, the selected ligands are confirmed and the second position may need to be slightly modified. The 2D structures of ligands were developed and transformed into 3D structures using the Chem Draw Pro 12.0 application. Using the ff MMF94 force filed technique, all 3D structures were shrunk in size before the requisite file format for docking was produced using the open babel 3.1.1 application. [19]

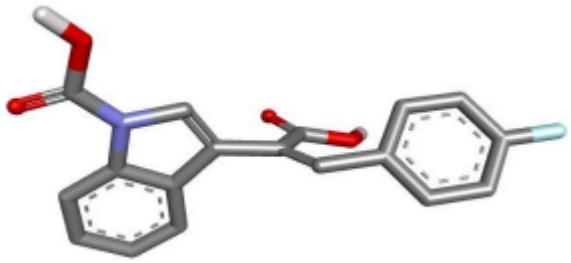
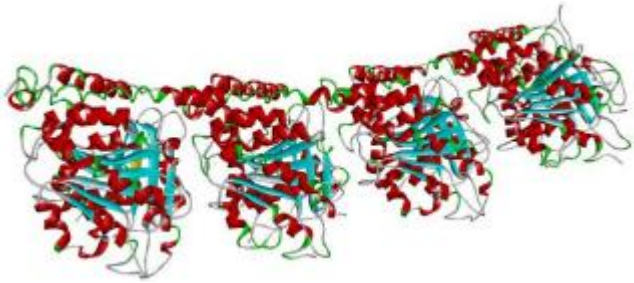


Figure 21: 3D structure of ligand

Protein Preparation

After considering all the standard factors, the [PDB: 1SA0] target for the protein data bank was selected from the RCSB database. The recovered crystal protein structure was prepared for docking by removing the native ligand, water, and hetero atoms. Software called BIOVIA Discover Visualizer 2021 was used.



Prediction of Pharmacokinetics Properties by using SWISS ADME software the Drug Likeliness (Lipinski's Parameters) has been determined. Prediction of Pharmacokinetics Properties by using SWISS ADME software the Drug Likeliness (Lipinski's Parameters) has been determined.

Cytotoxicity by Mttassay

The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. Materials Cell line-**A549 Human lung cancer cell line, NCCS, Pune.** Cell culture media - DMEM medium supplemented with 10% Foetal Bovine Serum (FBS), MP Biomedicals, Germany1X Dulbecco's Phosphate Buffered Saline (DPBS), 0.25% Trypsin-EDTA solution, MTT reagent, were all purchased from MP Biomedicals, Germany Dimethyl Sulfoxide (DMSO), cell culture grade, Merck, Germany Cell culture treated T-25 flasks from Biolite, Thermo Fisher Scientific Inc., USA.10mL serological pipettes and 96-well plates from Nunc, Thermo Fisher Scientific Inc., USA. 5mL, 2mL and 1.5mL tubes, Tarsons, India. Microscope-XDFL series, Sunny Instruments, China Analysis Software - ImageJ (Fiji) softwareV1.53j. [7]

Procedure

1) Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM-HG medium, such that 200µl of suspension contained approximately 10, 000 cells.
2) To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37^{o}C and 5% CO_2 atmosphere for 24h.
3) After 24 h, the spent medium was aspirated. 200µl of different test concentrations of test drugs were added to the respective wells. The plate was then incubated at 37^{o}C and 5% CO_2 atmosphere for **24h**.
4) The plate was removed from the incubator and the drug containing media was aspirated. 200µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and the plate was incubated at 37^{o}C and 5% CO_2 atmosphere for 3h.
5) The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan.
6) The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and the concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) was generated from the dose-response curve for the cellline. [8, 9, 10]

4. Results and Observations

Physiochemical properties of protein PDB ID: 1SA0 has been studied and tabulated.

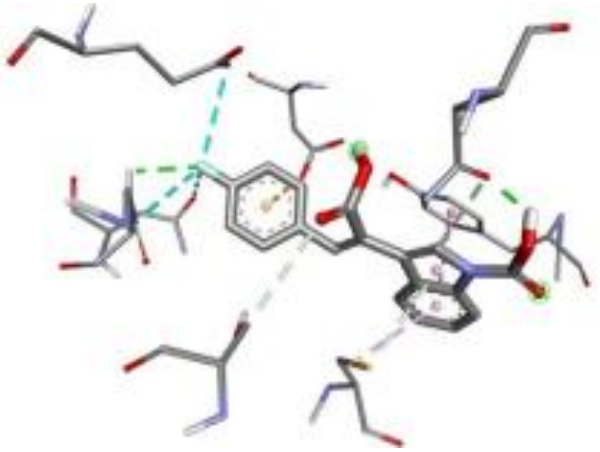
S. No	Physiochemical properties	PDB ID: 1SA0
1	Molecular formula	C142H2222N300O225S13
2	Molecular weight	1098.29
3	Total no atoms	4202
4	Total no of amino acids	9
5	Theoretical PI	3.80
6	Aliphatic index	86.67
7	Instability index	-0.54
8	GRAVY	-0.467
9	+R (Arg +Lys)	0
10	-R (Asp +Glu)	1
11	R-value work	0.249
12	R-Value free	0.232
13	R-value observed	0.233

Pharmacokinetic parameters of synthesized compounds

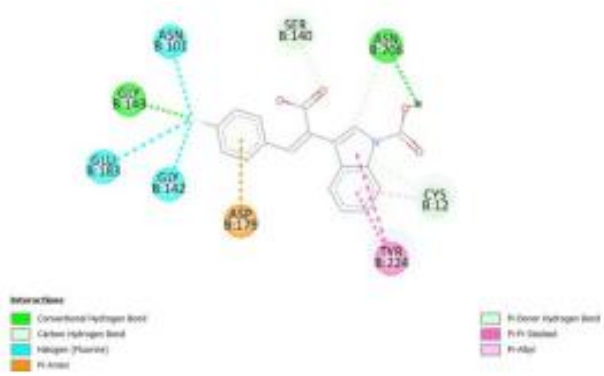
Compounds	MW (g/mol)	HBA	HBD	LogP	LogS	GI Abs	CYP1A2	BBB	LogKp
AD01	323.32	4	1	3.47	-4.29	High	No	Yes	-5.74
Adriamycin	579.96	12	6	0.36	-4.63	Low	No	No	-8.73
Mitomycin	334.33	6	3	-0.71	-1.40	Low	No	No	-8.62

Binding affinity

Compounds	Binding energy
AD01	-7.93
Adriamycin	-5.0
Mitomycin	-7.01



3D interactions of synthesized compound



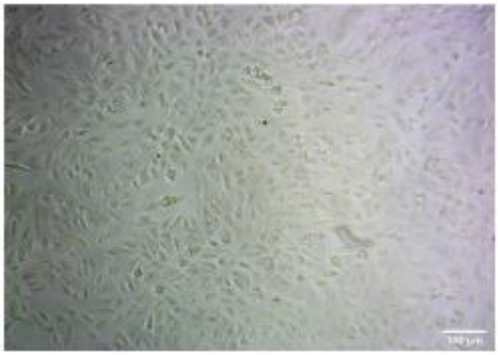
2D interactions of synthesized compound

Cytotoxicity by MTT Assay of Synthesised Compound

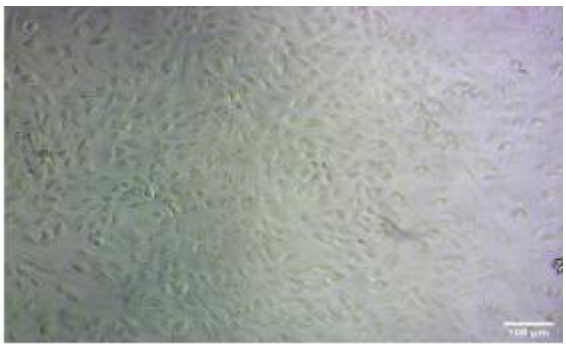
COMPOUND ADO1 IC_{50} (A-549)	Blank	Untreated	31.25	62.5	125	250	500
Reading 1	0.004	0.616	0.625	0.606	0.537	0.239	0.152
Reading 2	0.002	0.622	0.657	0.598	0.572	0.241	0.136
Reading 3	0.008	0.638	0.615	0.631	0.562	0.293	0.128
Mean OD	0.005	0.625	0.632	0.612	0.557	0.258	0.139
Mean OD-Mean Blank		0.6207	0.6277	0.6070	0.5523	0.2530	0.1340
Standard deviation		0.0114	0.0219	0.0172	0.0180	0.0306	0.0122
Standard error		0.0066	0.0127	0.0099	0.0104	0.0177	0.0071
% Standard error		1.0579	2.0408	1.6013	1.6770	2.8479	1.1367
% Viability		100	101.13	97.80	88.99	40.76	21.59

Cytotoxicity by MTT assay of standard compound

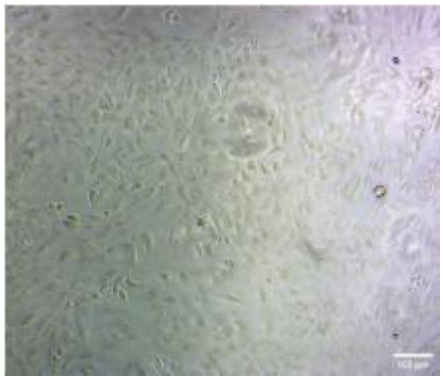
Compounds	%Viability
Adriamycin	0.11
Mitomycin	0.19



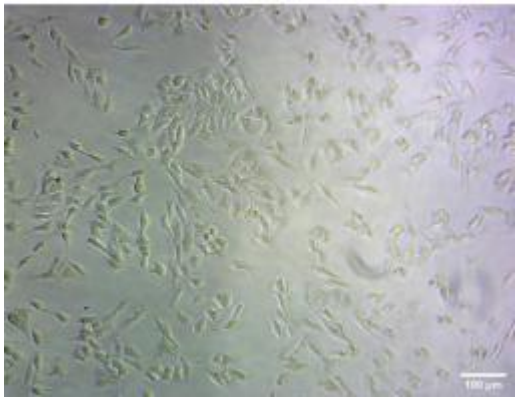
31.25µg/ml



62.5µg/ml



125µg/ml



250µg/ml
Viability of cells at different concentrations of AD01 compound

The decrease in % viability was observed with an increase in the concentration of synthesis compound AD01. Invitro exposure of A-549 cells with various concentrations of this compound suppressed the A-549 cancer cell growth.
NMR Spectra of Synthesized compound

^{1}H-NMR (500MHz, DMSO, TMS=0): 2.5 (3H, s, OAc), 3.76 (1H, dd, J=4.3, 3.6Hz, H-4), 4.1 (1H, d, J=4.5 Hz, H-6), 4.16 (1H, dd, J=4.3, 4.5Hz, H-5), 4.27 (1H, d, J=12.5Hz, αH-7), 4.69 (1H, d, J=12.5Hz, β H-7), 4.98 (1H, dd, J=3.6, 3.7Hz, H-3), 5.83 (1H, d, J=6.0 Hz, H-2), 3.2, 3.4, 5.2, 5.3, 5.5 (5H, OH, D_2O exchangeable), 7.57 (2H, d, J=7.0, 7.3, aromatic), 7.7 (1H, d, J=7.3, aromatic), 7.9 (2H, d, j=7.0, aromatic).

5. Conclusions

In conclusion, the work is being presented in two parts one is synthesis and the second is evaluation using cell lines. Part A ensures the synthesis of combretastatin's analogues with substituted benzaldehyde and the synthesized compound is new, this type of modification didn't synthesize in any research work rationality of the work was based on a butterfly model with two wings represented by aryl groups and connecting carbon chain as the body binding to the colchicine's binding site or tubulin inhibitor binding site.

Confirmation of the synthesized compound was done by chromatographic technique i.e. TLC by using a suitable solvent system and spectroscopy techniques like IR, NMR, and other possible chemical reactions. All the synthesized compounds i.e. substituted aldehydecombretastatinan alogue were tested for anticancer activity against cell lines A-549 (Lung cell line). The new synthesis compound was found to have marked anticancer activity in the concentration range of 250 to 500µ g/ml. Tubulin binding site was established by in silico docking studies of potent compounds using the Auto Dock suite and SWISS-ADME and the activity of synthesized compounds indicates that they can be promising anticancer molecules to conform their potency in the future we can do in-vivo experiments will be required to address the anticancer property in cancer chemotherapy with the reduced pain.

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