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Recent advances in combretastatin based derivatives and prodrugs as antimitotic agents

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The dynamic and crucial role of tubulin in different cellular functions rendered it a promising target in anti-cancer drug development. Combretastatin A-4 (CA-4), an inhibitor of tubulin polymerization isolated from natural sources, is a lead molecule with significant cytotoxicity against tumour cells. Owing to its non polar nature it exhibits low solubility in natural biological fluids, thereby prompting the development of new CA-4 based derivatives. The modification of this lead molecule was mostly carried out by keeping the crucial *cis*-orientation of the double bond intact, along with a trimethoxyphenyl aromatic ring, by employing different approaches. The issue of solubility was also addressed by the development of water soluble prodrugs of CA-4. The present review highlights the investigations into the parallel development of both new CA-4 based derivatives and prodrugs in the past few years.

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1. Introduction

1.1 Microtubules: structure and function

Microtubules are dynamic, polarised, non-covalent cytoskeletal fibres which are ubiquitously found in the cytoplasm of eukaryotic cells. They perform diverse functions and are primarily involved in cell motility, cell polarization, intracellular transport, mitosis, secretion and cell shape maintenance.¹ Microtubules are mainly biopolymers composed of heterodimers, α and β tubulin, which associate together to form linear protofilaments. Each microtubule consists of 13

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protofilaments which assemble to form a hollow cylindrical shaped structure. Microtubule polarity occurs *via* the head-to-tail association of α,β -heterodimers which is a highly dynamic process. Protofilaments contribute effectively in the polarisation of microtubules *via* its two distinct ends, the faster growing plus end and the slower growing minus end. The plus end (+) consists of exposed α -subunits, whereas the minus end (–) is flanked by exposed β -subunits. Addition of dimers to the protofilament ends leads to polymerisation which predominantly occurs at a specific critical concentration of dimers. The entire polymerisation process is GTP-dependent (Fig. 1). Two GTP binding sites are present in each tubulin subunit; hence binding of two GTPs occurs. At the first site, referred to as the non-exchangeable site, binding of

GTP occurs irreversibly and is non-hydrolyzed, whereas at the second site (E site), also known as the exchangeable site, reversible binding of GTP occurs which eventually is hydrolyzed to GDP. This process leads to a continuous yet stable expansion and shrinkage of the microtubule which attains a state of dynamic equilibrium. The microtubule is generally stabilized due to the presence of a GTP cap at the plus end which when lost leads to depolymerisation of the microtubule structure due to the splaying of protofilaments. Microtubule growth occurs uninterrupted until it reaches a catastrophic point at which rapid disassembly occurs.^{2,3} Interestingly, a new cap can be added to the microtubule which subsequently guards the microtubule from shrinking, known as “rescue”. This dynamic interplay is essential for the proper functioning of the microtubule structure but also makes it vulnerable to agents which can disrupt this fine balance.^{4,5} In recent years, the microtubule and its associated structures have emerged as a viable therapeutic target in various disease conditions. Structural explorations of microtubules have revealed three distinct tubulin binding sites: vinca binding site, colchicine binding site and taxane binding site.⁶ Tubulin polymerization inhibitors bind at the vinca and colchicine binding sites and hinder cell proliferation at the metaphase during the mitosis stage, whereas the taxane binding site is a target for depolymerisation inhibitors, also referred to as microtubule stabilizers.

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1.2 Tubulin binding agents in cancer

In the past few decades, the implications of microtubule disruption have been widely explored due to the crucial role it plays in mitosis wherein the nucleus splits into two, which is followed by the formation of two daughter cells by parent cell division. This has aided in the development of cancer

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Source: *Molecular Cell Biology, Fourth Edition, H. Lodish et. al.*

Fig. 1 Stages in assembly of microtubules.

therapeutic drugs which have the potential to be explored as future drugs. Microtubules are known to form spindles which enable them to influence the segregation of chromosomes. Tubulin targeting agents selectively subdue the influence of the mitotic spindle, leading to mitotic arrest and cell death of rapidly dividing cells. Also, cell stress induction during the interphase contributes to cell death resulting *via* tubulin binding agents.

The increased burden of cancer, toxicity of chemotherapeutic agents and resistance to anticancer drugs have driven the scientific community to develop newer and more potent cancer therapeutic drugs.⁷ In this regard, leads obtained

from natural products have inspired medicinal chemists to develop both natural product hybrids and synthetic molecules in the area of drug discovery. Some of the natural tubulin depolymerisation inhibitors include paclitaxel 1, epithilone 2, and discodermolide 3 which bind to the taxane domain of tubulin. On the other hand, natural tubulin polymerisation inhibitors such as vincristine 4 and vinblastine 5 bind to the vinca domain. Colchicine 6 and combretastatins (like CA-4) 7 are also natural tubulin polymerisation inhibitors; however they bind to the colchicine binding site (Fig. 2).^{6,8} There has been a surge in the development of newer tubulin

Fig. 2 Naturally occurring inhibitors of tubulin polymerization.

binding agents in terms of increasing its safety and efficacy. Computational and molecular modelling approaches have also guided in understanding the intricate mechanisms by which the molecules bind to the active site and in synthesising newer small-molecule inhibitors targeting tubulin.⁹ Many of the tubulin binding agents have successfully entered clinical investigations and some of them have been approved as drug candidates.^{10,11}

Combretastatin has been a highly exploited scaffold because of its unique yet simple structure and has been successfully utilized in developing newer and potent tubulin polymerisation inhibitors.^{11–16} Its structural features and reactivity distinguishes it from other heterocyclic and non-heterocyclic scaffolds, making it one of the most emerging molecules in recent decades. Substantial research endeavours have been undertaken by different research groups on this interesting lead molecule and it is beyond the scope of this review to cover all of them. In the present review, we focus on some recent advances in the development of new combretastatin based tubulin polymerisation inhibitors with parallel progress in the development of a prodrug approach.

2. Combretastatins as tubulin polymerisation inhibitors

Combretastatins are a potent class of natural products which were first isolated by Pettit and collaborators from the bark of the African bush willow, *Combretum caffrum*.¹⁷ Chemically, they are phenolic stilbenes which bind to the colchicine binding site of tubulin and lead to microtubule disruption and mitotic arrest by preventing spindle formation. They have structural similarity to colchicine and hence are also referred to as 'colchicinoids'. Historically, combretastatins were known to be a traditional remedy for the treatment of scorpion sting, cardiovascular disorders and worm related disorders.¹⁸ Combretastatin has contributed several lead molecules in which the most prominent one has been combretastatin A-4 (CA-4, 7). CA-4 is a *cis*-stilbene which is active against various cancer cell lines and shows high potency. Moreover, CA-4 phosphate disodium, which is its water-soluble prodrug form, has also shown promising results and is under clinical investigation for the treatment of patients with advanced solid tumours.¹⁹ SAR studies conducted on CA-4 reveal three key features required for it to show optimum cytotoxic activity which includes *cis*-orientation of both the aromatic rings, the 3,4,5-trimethoxy moiety on ring A, and the *para*-methoxy moiety present on ring B. However, CA-4 and some of its analogues in storage are vulnerable to undergo transformation to the *trans* form which is inactive. Therefore, an effective strategy has been developed to replace the *cis*-oriented double bond by a heterocyclic moiety to conformationally hinder the transformation to the *trans* form. This has inspired several groups to design and prepare various CA-4 based analogues to improve upon its potency and efficacy.²⁰ Many of the compounds have shown a micromolar to nanomolar range of activity in vari-

ous cancer cells and as such enhanced the scope of utilizing the molecules based on a combretastatin scaffold for future drug discovery and development.

3. Combretastatins as vascular disrupting agents

In recent years there has been an enhanced understanding of the architecture and environment of tumor tissue accompanied by more useful insights into cellular processes such as metastasis and angiogenesis. These developments have paved the way for targeted cancer therapies, which exploit the indirect killing of tumor cells by targeting the tumor vasculature. This is done by two approaches, either by inhibiting the formation of new blood vessels (angiogenesis) or by targeting the vasculature which is already present. This is a promising strategy as it is well established that a solid tumor cannot grow without blood vessels (maximum of up to 2 mm³) as the source of oxygen and nutrients is cut off. Vascular disrupting agents (VDAs) are used to target the existing complex vascular architecture leading to collapse of the tumor vasculature, resulting in cell death.²¹ VDAs are classified into two types, namely, ligand directed VDAs and small molecules. The small molecules include flavonoids and tubulin depolymerizing agents such as colchicine and combretastatin A-4. In an investigation it was found that a member of the combretastatin class targets tumor vasculature and this led to the discovery of CA-4 and its prodrug form (CA-4P) for inhibition of blood flow in tumors at low concentrations. The vascular disrupting property of CA-4 is not completely elucidated, though CA-4 is well known to inhibit tubulin protein.^{22–24}

4. Recent developments in CA-4 based analogues

Most of the recent developments in the CA-4 pharmacophore have revolved around the modifications on the *cis*-olefinic bond, which included restriction of *cis* configuration by a heterocyclic moiety and replacement or substitution of an olefinic bond with a different linker. In addition, the modification of the olefinic bond has been carried out along with modification of either ring A or ring B. In some studies, the *cis*-olefinic bond has been retained and modifications in ring A or B have been investigated. This review highlights such developments that have taken place in the last few years.

4.1 Modification at the *cis*-olefinic bond

4.1.1 Replacement of the *cis*-olefinic bond with a heterocyclic moiety. Heterocycles play a pivotal role in the design of drugs and are extensively used in tweaking the properties of the lead molecules for a desired outcome. To retain the *cis*-configuration of the olefinic bond and overcome the solubility issues of CA-4, the bond has been replaced with a heterocycle by various research groups. In most of the cases, a five-

membered heterocycle has been incorporated compared to six-membered or fused heterocycles. Pyrroles, imidazoles, thiazoles, thiophenes, azetidinones, lactams and other heterocycles have been investigated. Romagnoli and coworkers reported the replacement of the olefinic bond with an imidazole ring and studied the effect of various substitutions on the phenyl ring attached to imidazole. Amongst the various 1,2-diaryl imidazoles, compound **8** with a chloro and an ethoxy group was the most active and exhibited excellent antiproliferative activity with IC_{50} values in the range of 0.4–3.8 nM against a panel of seven different cancer cell lines (Fig. 3). Mechanistic studies revealed that **8** disrupted the microtubular network and inhibited tubulin polymerization (IC_{50} 0.87 μ M). *In vivo* studies on a mouse model also demonstrated the potential of **8** for further preclinical evaluation.²⁵ In an earlier report, 1,2,5-selenadiazole was incorporated in CA-4 in place of the olefinic bond; the analogue **9** exhibited potent activity on the nanomolar level in three cancer cell lines and its mechanistic action was similar to that of CA-4.²⁶ Xu and coworkers reported substituted 1,5-diaryl pyrazoles as CA-4 analogues with significant antiproliferative and tubulin inhibition properties. The active compound **10** exhibited an IC_{50} value of less than 0.158 μ M and 1.7 μ M cytotoxic activity as well as tubulin polymerization inhibition.²⁷ In another report, new CA-4 derivatives based on a pyrazole moiety were synthesized through cycloaddition reactions of syndones in a regioselective manner and the compounds exhibited good potency against HUVEC proliferation by binding with tubulin.²⁸

In recent decades triazoles have emerged as an important element in drug design. Madadi and coworkers synthesised a

series of 4,5-disubstituted 2*H*-1,2,3-triazoles as *cis*-constrained combretastatin A-4 analogues and evaluated their cytotoxic activity against a panel of 60 cancer cell lines. Among the synthesised compounds, the analogue **11** exhibited the most potent anti-cancer activity in the screening studies, with GI_{50} values of <10 nM against almost all the cell lines in the tested panel. *In silico* studies revealed that compound **11** would have a better affinity for the colchicine binding site on tubulin when compared to the selected compounds. It was further evaluated for its cytotoxic activity by a colony formation assay against 9LSF rat gliosarcoma cells and demonstrated an LD_{50} of 7.5 nM.²⁹

In an interesting report, 1,2-diaryl pyrroles were investigated as the structural analogue of CA-4 with a pyrrole ring mimicking the *cis*-olefinic bond. A series of 1,2-diaryl pyrroles were synthesized and evaluated against three different cancer cell lines, and most of them displayed activity. The pyrrole derivative **12** effectively inhibited three cell lines with IC_{50} values of 0.390, 0.070 and 0.045 μ M against SGC-7901, HT-1080 and KB cell lines, respectively. Moreover, it showed lower toxicity towards the normal cell line L929 with an IC_{50} value of 30.08 μ M. Compound **12** acted by inhibiting tubulin polymerization, resulting in microtubule destabilization.³⁰ Similarly, based on the rigidity of the thiophene moiety, Wang and coworkers synthesized a series of 2,3-diarylthiophenes containing CA-4 analogues as promising antiproliferative agents. Compound **13** demonstrated high potency against three tumor cell lines with IC_{50} values in the range of 0.52–2.21 μ M. Further studies on compound **13** revealed that it acted *via* the inhibition of tubulin

Fig. 3 CA-4 derivatives: *cis*-olefinic bond replaced with heterocycles.

polymerization leading to microtubule destabilization.³¹ The replacement of the olefinic bond with a thiazole ring was also explored and the research group of Wang reported diarylthiazole derivatives as antimitotic agents with good cytotoxicity against some cancer cell lines. The CA-4 analogue **14** with a 2-amino substituted thiazole ring exhibited very significant activity with an IC_{50} of 8.4–26.4 nM against five human cancer cell lines and mechanistic studies revealed that the mode of action was similar to that of CA-4.³² In earlier studies, Salehi and co-workers investigated the incorporation of a sulphide bond on the 2-position of the thiazole.^{33,34} Zheng and co-workers designed and synthesized a series of new pyridine bridged analogues of CA-4 as antitubulin agents. Compounds **15** and **16** were the most potent from the series, exhibiting an IC_{50} value of <0.09 μ M against some cancer cell lines, and the activity from mechanistic studies was comparable of that of CA-4.³⁵ In another study, fused triazolopyridazine as a mimic of the olefinic bond of CA-4 was also investigated, and the most potent compound showed activity comparable to that of CA-4.³⁶

Very recently, Malebari and coworkers reported β -lactam analogues of CA-4 to overcome the drug resistance associated with glucuronidation in chemoresistant HT-29 colon cancer cells. Analogue **17** was the most potent inhibitor of tumour growth with an IC_{50} value of 15 nM independent of the status of 5-diphosphoglucuronosyl transferase (UGT) enzyme (Fig. 4). Similar to CA-4, compound **17** was disruptive to the microtubular assembly in MCF-7 and HT-29 cells, causing G_2/M arrest and apoptosis.³⁷ In other investigations, trisubstituted azetidinone derivatives were designed as *cis*-restricted CA-4 analogues and evaluated for antitumour activity under *in vitro* and *in vivo* conditions.^{38,39} Thiazolidinones were also reported to mimic the *cis*-olefinic bond of CA-4 and amongst them compound **18** was the most potent with IC_{50} values of 1.21 and 2.12 μ M against tumor cell proliferation and tubulin polymerization inhibition, respectively.⁴⁰ Guan and co-workers synthesised a series of CA-4 analogues containing a maleic anhydride/*N*-substituted maleimide moiety by employing a microwave-assisted process and further evaluated them for antiproliferative activities against three tumour cell lines. Amongst them, **19** was the most promising compound, showing excellent IC_{50} values of 1.3, 0.65 and 1.1 μ M against SGC-7901, HT-1080 and KB cell lines, respectively.⁴¹ In another study, the *cis*-olefinic bond was also replaced by NH attached imidazo-pyridine/pyrazine rings and four of

such compounds significantly inhibited tubulin polymerization and were found to be potent against kidney (HEK 293T), breast (MCF 7) and cervical cancer (SiHa and HeLa) cell lines.⁴²

4.1.2 Other replacements and substitutions of the *cis*-olefinic bond. As a replacement of the *cis*-olefinic bond of CA-4, Amaral and collaborators reported a series of *N*-acylhydrazone derivatives as antiproliferative agents. Several compounds exhibited moderate to high antiproliferative potency with IC_{50} values ranging between 18 μ M and 4 nM. Among them, compound **20** was identified as the most potent compound which can inhibit microtubule polymerization and exhibit excellent *in vitro* and *in vivo* antiproliferative activity with an improved selectivity index when compared to CA-4 (Fig. 5). The antiproliferative profile was also evaluated against human lymphocytes to determine the selectivity index of compounds, which were better than that of CA-4.⁴³ In another report, Zhu and coworkers reported an acylhydrazone derivative with ring B replaced with an indole ring as an antiproliferative agent (IC_{50} values 0.08 to 35.6 μ M) and tubulin inhibitor.⁴⁴ In an interesting investigation, Engdahl and co-workers reported azo-combretastatin A4 **21** as a photoisomerizable tubulin polymerization inhibitor. The synthesis of azo-CA4 provided a *trans* isomer which on irradiation with a 400 nm LED flashlight resulted in *ca.* 90% isomerisation to the *cis* isomer. On evaluation of the effect of azo-CA4 on tubulin polymerization little activity was observed; however in the presence of an appropriate light source **21** exhibited good inhibition of tubulin polymerization. Similar results were obtained on the evaluation of cytotoxicity against HeLa cancer cell line in the presence or absence of isomerizing light. Compound **21** resulted in complete cell death at 500 nM with a 200-fold increase in bioactivity compared to its *trans* counterpart.⁴⁵ In an independent report, Borowiak and coworkers reported **21** as a photostatin with 250 times more activity of the *cis* form which is formed by isomerization of the *trans* form under an appropriate light source.⁴⁶ In an interesting report, cyclopropylamide analogues of CA4 were developed and found to exhibit cytotoxic properties through a microtubule stabilizing mechanism.⁴⁷

Substitutions at the *cis*-olefinic bond were also investigated by tethering CA-4 to different types of heterocycles. Kamal and co-workers linked the CA-4 scaffold to 1,3,4 oxadiazoles and investigated their antiproliferative activity against five cancer cell lines. Hydrid **22** was the most potent,

Fig. 4 CA-4 derivatives: *cis*-olefinic bond replaced with heterocycles (continued).

Fig. 5 Chemical structures with replacements and substitutions at *cis*-olefinic bond.

exhibiting an IC_{50} value of 0.1 μM against the DU-145 cell line and showed a significant inhibitory effect on tubulin assembly.⁴⁸ In a similar report, Reddy and collaborators synthesized a series of new benzoxazoles containing combretastatin A-4 derivatives and evaluated their antiproliferative activity. Among them 23 demonstrated enhanced potency, when compared to the control drug (Adriamycin), specifically against A549 and MCF-7 cell lines with an IC_{50} of $<0.1 \mu M$.⁴⁹

4.2 Modification of both *cis*-olefinic bond and aromatic ring

A number of studies have been carried out to explore the antitubulin properties of CA-4 derivatives on modification of both the *cis*-olefinic bond and the aromatic ring. Kamal and coworkers designed and synthesized new mimics of CA-4 by replacement of the *cis*-double bond with a conformationally restricted pyridine and replacement of ring B with either a benzimidazole or a benzothiazole ring. The incorporation of the pyridine ring was envisaged by the structural resemblance to another tubulin inhibitor, E7010. The mimic with the benzothiazole moiety (24) was the most potent compound that exhibited antiproliferative activity superior to that of CA-4 (GI_{50} up to 40 nM) and significant tubulin inhibition (IC_{50} 0.91 μM) (Fig. 6). Mechanistic studies revealed the mode of action to be similar to that of CA-4 and docking studies suggested binding of the compound at the colchicine site.⁵⁰ Encouraged by the results, the same group recently reported triazole and tetrazole mimics of CA-4 and the mimic 25 with a benzothiazole moiety was the most potent amongst the series.⁵¹ In an earlier report, they reported arylpyrazole linked benzimidazole conjugates as a potent disruptor of the microtubular assembly.⁵² Mahal and coworkers investigated the effect of imidazole and indole rings as the replacement of the *cis*-olefinic bond and ring B, respectively. The derivative 26 was more active than CA-4 with IC_{50} values in the range of 19–31 nM against drug resistant cell lines. However, 26 did not interfere significantly with tubulin polymerization; it disrupted the microtubular system and was less toxic than CA-4 to normal cells.⁵³ Chaudhary and coworkers synthesized

Fig. 6 Chemical structures with modification of both *cis*-olefinic bond and aromatic ring.

a series of 4,5-diaryl-2-aminoimidazoles as analogues of CA-4 with replacement of the *cis*-olefinic bond and ring B. Four of the compounds exhibited potent antiproliferative activities on the nanomolar level. Derivative 27 with a quinoline ring was more potent than CA-4 with IC_{50} values of 3 and 10 nM against MCF-7 and HeLa cancer cell lines, respectively, with a mechanism of action similar to that of CA-4.⁵⁴

Combretastatin A-4 is a simple 1,2-diaryl ethylene scaffold and therefore extensive work has been carried out towards its diversification in search of a potent drug candidate. On the other hand, isocombretastatins are 1,1-diaryl ethylene scaffolds and comparatively fewer reports are available on them. Notably, the research group of Hamze reported the synthesis of different derivatives of isocombretastatin and evaluated their antiproliferative activity against selected human cancer cell lines.^{55–57}

4.3 Modification of ring B

Exploiting the click chemistry strategy for access of 1,2,3-triazoles, Kamal and co-workers reported a series of triazole tethered aminocombretastatin hybrids as cytotoxic tubulin polymerization inhibitors and apoptosis inducers. Amongst the series, conjugates 28 and 29 were as potent as CA-4, exhibiting excellent cytotoxicity with IC_{50} values of 53 and 44 nM, respectively, against A459 lung cancer cell line (Fig. 7). The tubulin polymerization inhibition property of these conjugates were also significant with IC_{50} values of around 1.0 μM .⁵⁸ In an earlier report, Vilanova and co-workers synthesized hybrids of CA-4 and pironetin fragment by modification at ring B of CA-4. The two pharmacophoric moieties were tethered through a spacer of varying length with a triazole ring and some of the hybrids exhibited comparable cytotoxicity with that of CA-4 with lowered toxicity towards normal cells.⁵⁹ More recently Kamal and coworkers reported arylcinnamide linked CA-4 hybrids as tubulin polymerization

Fig. 7 CA-4 derivatives with modification of ring B.

inhibitors. Conjugates **30** and **31** were the most active amongst the series that display GI₅₀ values of 56 and 31 nM, respectively, against MCF-7 breast cancer cell line. SAR studies revealed that for an improved activity, the 3 and 4 position of the phenyl ring of the cinnamide moiety needs to be occupied for improved activity. The tubulin polymerization inhibition property of these conjugates was also good (IC₅₀ 1–2 μM) and interestingly they induced apoptotic cell death by caspase activation.⁶⁰ In an interesting investigation, ring B was replaced by a bioisosteric benzoxazolone ring by Gerova and co-workers and twenty eight *cis* and *trans* isomers were synthesized. On cytotoxic evaluation against selected cell lines, compound **32** exhibited IC₅₀ values of 0.19–0.73 μM against the tested cell lines, with an IC₅₀ value of 0.25 μM against the combretastatin resistant cell line HT-29.⁶¹

5. Recent developments in CA-4 based prodrugs

Most of the new chemical entities are organic frameworks which limits their solubility under water based physiological conditions, resulting in reduced availability at the site of action. The concept of prodrug addresses the issues relating to the bioavailability of potential drug candidates. A prodrug is an inactive chemical species at the pharmacological level which metabolises to generate the active drug form under specific conditions. Combretastatin A-4 suffers the limitation of water solubility and to overcome it, prodrug forms of combretastatin A-4 have been developed.⁶² A CA-4 prodrug with a phosphate group (CA-4P, fosbretabulin **33**) is in advanced clinical trials and a prodrug with an aminohydroxy group (AVE8062, ombrabulin **34**) is in phase III clinical trials (Fig. 8).^{63,64} The CA-4P prodrug has been well studied both in monotherapy and in combination therapy in advanced solid tumours, gastrointestinal adenocarcinoma, non-small cell

lung cancer, squamous cell carcinoma and epithelial ovarian or fallopian tube cancer. The response to the therapy was varied with little to partial response. CA-4P was well tolerated; however some mild to moderate side effects were observed that include nausea, headache, diarrhoea, tachycardia and hypertension.⁶⁴ In addition to the development of new CA-4 based molecules for cancer treatment, parallel progress has been under way to improve the efficacy of CA-4 with new prodrug forms. Some of the recent findings in this aspect are discussed here in this review.

Suzuki and co-workers recently reported prodrug monotherapy (PMT) of ovarian cancer employing two CA-4-β-galactosyl conjugates (**35**, **36**) to address the issue of bioavailability and severe side effects in chemotherapy of ovarian cancer. In PMT, chemotherapy selective to cancer cells is realized by administration of a nontoxic prodrug which releases the active drug species under appropriate conditions, such as response to an enzymatic activity. In ovarian cancer cell lines β-galactosidase activity is enhanced and the two galactosyl conjugates (**35**, **36**) were envisaged to transform into CA-4 by releasing their β-galactose moieties in response to β-galactosidase activity (Fig. 9). On evaluation against ovarian cell lines (OVCAR3 and OVK18) conjugate **36** with a self-immolating benzyl group linking CA-4 and a β-galactose moiety was more cytotoxic than **35**, which was without a linker. Conjugate **36** exhibited EC₅₀ values of 2.47 and 2.67 nM against OVCAR3 and OVK18 cell lines, respectively, which were comparable or superior to that of the parent CA-4. To elucidate whether the cytotoxicity of **36** is caused by the disruption of intracellular microtubule assembly, intracellular α-tubulin and DNA were evaluated using suitable fluorescent dyes. At 10 nM concentration, **36** displayed disruption of microtubule assembly, cytoskeleton contraction along with nuclear contraction of some cells and fragmentation.⁶⁵ Glutathione (GSH)-mediated prodrug activation is one of the promising approaches to site-specific drug delivery as the concentration of GSH is 30–40-fold higher in cancer cells compared to healthy cells. To ensure that drug delivery is achieved at the intended site, theranostic compounds (imaging agents with fluorescent biomarkers and the drug molecule) are being widely employed. Kong and coworkers designed a novel CA-4 prodrug, YK-5-252 (**37**), containing a disulphide bond, which cleaved in the presence of GSH. In addition, **37** contains dicyanomethylene-4H-pyran (DCM), a near-infrared

Fig. 8 Prodrugs of CA-4 in clinical trials.

Fig. 9 Design of prodrugs of CA-4 for selective delivery.

(NIR) fluorophore, which allowed fluorescence monitoring of cleavage through live cell imaging. Prodrug 37 was evaluated for toxicity in normal cells (MCF10A) and TNBC cells (MDA-MB-231) and compared with CA-4. Results indicated that 37 reduced the toxicity of CA-4; however both compounds were potent in normal cells and the tubulin polymerization inhibition ability of 37 was very sluggish.⁶⁶

Nkegang and coworkers reported a light activated prodrug strategy with a tumor-targeting group to the prodrug system and folate receptor (FR)-overexpressing cancer cells were selectively targeted. The group designed folate conjugated multifunctional prodrugs (CA4-L-Pc-PEG_n-FA) and in the formulation L was a single oxygen cleavable linker, and Pc was phthalocyanine (photosensitizer) with folic acid (FA). The prodrugs with a longer PEG spacer exhibited higher and specific uptake to SC colon 26 tumours in mice *via* a folic acid mediated mechanism.⁶⁷ In an earlier report, the same group

prepared a prodrug formulation, CMP-L-CA4, where CMP was dithiopyrroline (photosensitizer) and L an aminoacrylate linker. The prodrug under appropriate irradiation (690 nm diode laser) released CA-4 because of cleavage of the aminoacrylate linker, exhibiting a five times increase in IC₅₀ value in MCF-7 breast cancer cell line.⁶⁸ Recently, Liu and co-workers proposed and synthesized a poly(L-glutamic acid)-combretastatin A conjugate (PLG-CA4) for enhanced therapeutic efficiency of CA-4. It was investigated and compared with 33 in murine colon C26 tumour and was found to distribute around tumour cells owing to low tissue penetration in the solid tumour.⁶⁹ Various water soluble amino acid prodrug conjugates of CA-4 were also synthesized and evaluated for cleavage by the enzyme leucine aminopeptidase (LAP).⁷⁰

Han and coworkers reported an oxazole bearing derivative of CA-4 and its prodrug (38, 39) with a phosphate group as

Fig. 10 Prodrugs of CA-4 derivatives.

an effective tubulin polymerization inhibitor (Fig. 10). The prodrug form inhibited NCI-H1975 xenograft tumor growth with little weight loss and damage to normal tissue even after 19 days of drug administration. It also inhibited the growth of H22 hepatocellular carcinoma bearing mice and exhibited enhanced activity in combination with cisplatin.⁷¹ Investigating water soluble amino acid prodrugs of CA-4 derivatives, Yu and coworkers synthesized eleven amino derivatives of CA-4 and evaluated their anticancer properties under both *in vitro* and *in vivo* conditions. The parent compound was a CA-4 derivative in which the *cis*-bond was replaced by a 2-aminothiazole ring and different amino acid derivatives based on this parent compound were designed to improve its solubility. The valine attached derivative **40** exhibited IC₅₀ values in the range of 0.2–0.4 μ M and exhibited high efficacy in human carcinoma xenografts. The mechanism of action was similar to that of CA-4 with significant tubulin inhibition by binding at the colchicine domain of tubulin.⁷² In an earlier report, O'Boyle and coworkers synthesized water soluble amino acid and phosphate derivatives of β -lactam combretastatins followed by evaluation of antiproliferative and inhibition of tubulin polymerization properties. The phosphate derivatives (**41–43**), though exhibiting good antiproliferative ability, could not disrupt the microtubule assembly in MCF-7 breast cancer cells. Another series of CA-4 derivatives, β -lactam amino acid amides (**44**, **45**) displayed both potent anticancer and tubulin polymerization inhibition properties by binding to the colchicine domain.⁷³

6. Conclusion

Microtubules and their associated proteins have emerged as viable targets in the drug discovery program, particularly in the treatment of cancer. Different lead molecules are identified that bind to one of the three binding sites of tubulin, thereby inhibiting the dynamic process of tubulin polymerization or depolymerisation. Amongst them, combretastatin A-4 is a lead molecule, which binds to the colchicine site and exerts antiproliferative activity by the inhibition of the tubulin polymerization process. Simplicity of structure and significant activity accompanied by issues of low solubility in natural biological systems catapulted CA-4 to be one of the most actively researched lead molecules in anticancer drug development programs around the world. Extensive research endeavours were undertaken to develop new derivatives of CA-4 to address the challenges in the development of anticancer drugs. Moreover, *cis*-orientation of the double bond was identified to be most crucial for activity along with a trimethoxy aromatic ring. To nullify the possible isomerisation of the *cis* orientation to an inactive *trans* orientation, different approaches have been developed. The replacement of the *cis*-bond with a heterocyclic moiety is of significant potential as it results in the desired conformational restriction as well as addition of new pharmacological properties because of incorporation of heteroatoms. Though a good number of po-

tent CA-4 derivatives have been developed in recent years, very few have been taken up for clinical studies.

To overcome the issue of solubility the prodrug concept has been successfully applied and the prodrugs of combretastatin A-4, CA-4P (with a phosphate group) and AVE8026 (with an amide bond containing amino alcohol group), are in the advanced phase of clinical trials. CA-4P is currently being investigated both in monotherapy and in combination with drugs like paclitaxel, carboplatin, and bevacizumab. It is also being investigated in combination with other therapeutics such as radio immunotherapy and radiotherapy. Most of these investigations are against advanced solid tumors, gastrointestinal adenocarcinoma, non-small cell lung cancer, squamous cell carcinoma (head and neck), prostate, ovarian, primary peritoneal, or fallopian tube cancer and have shown partial response to the treatment accompanied by some adverse effects. Recently the other prodrug of CA-4, AVE8026 (ombrabulin), was discontinued for further development by Aventis Sanofi as the phase III clinical studies were not encouraging. Therefore, a fresh impetus is required to take up a parallel concerted effort towards the development of new CA-4 based derivatives and prodrugs. In addition, nanoparticle based delivery systems/formulations and combination therapy need to be explored to successfully move combretastatin A-4 from bench to bedside.

7. Conflict of interest

The authors declare no competing interests.

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