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Miniperspective

Medicinal Chemistry of Combretastatin A4: Present and Future Directions

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Introduction

A growing solid tumor relies on a developing vasculature to meet its needs in terms of oxygen, nutrients, depuration, etc. This implies that if the vascular bed that has developed within the tumoral mass can be made to collapse, tumoral growth can be significantly hampered. Indeed, the first proof of principle that this could be achieved was provided more than 10 years ago when a ricin-conjugated antibody directed against an endothelial protein was able to eradicate the tumoral mass in mice.[1−4] Therapeutically, two pharmacological strategies can be foreseen that stand on this observation: (1) the development of the growing tumoral vasculature can be arrested by drugs; (2) the established vasculature perfusing the tumoral mass can be destroyed by drugs. Among the crucial questions in the field is how to specifically target the endothelial cells participating in the tumoral neovasculature without causing damage to vasculature elsewhere. A wide body of data has emerged over this issue.[3] It has now been shown that the developing vasculature and the tumoral vasculature express unique proteins and that this uniqueness can be used for selective pharmacological targeting. Indeed, if we consider a plasma membrane protein expressed solely on the undesired vasculature, we could envisage the use of specific antibodies conjugated with toxins, vaccines, etc.[3] Yet it is also possible that the neovasculature is more sensitive over normal tissues to more traditional small-molecule drugs. Indeed, this strategy has also been exploited, and a number of compounds have entered or are entering clinical trials (these drugs are cumulatively referred to as low molecular weight vasculature-disrupting agents).[4] For example, the growth of the neovasculature is dependent on activation of the vascular endothelial growth factor receptor, and therefore, a number of receptor antagonists have been devised and are currently tested or employed.[5] Disruption of tubulin polymerization also disrupts the formation of tumoral vasculature, and it is therefore no surprise that a number of agents have been brought forward into the drug pipeline that share this mechanism of action.

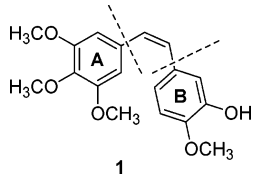

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Figure 1. Combretastatin A4.

The present review will concentrate primarily on the medicinal chemistry of one of these drugs, combretastatin A4 (CA-4[a] (**1**), Figure 1), because of space constraints and the presence of excellent reviews dealing with other aspects of this drug elsewhere.[4−9]

Yet it is difficult to limit the discussion to derivatives of one product when other products from a variety of natural sources share their capacity to inhibit tubulin polymerization binding at the same protein domain and are structurally related. Indeed, alongside CA-4, which displays a 1,2-diarylethene scaffold, other natural agents bear two polyoxygenated aromatic rings with a different molecular scaffold (e.g., biaryl for colchicine and steganacine or 1,1-diarylmethane for podophyllotoxin; Figure 2) and act on the same binding site. All these molecules have been shown to destroy neovasculature, but except for CA-4, this effect is observed close to the maximum tolerated dose. Although the reason for this is unknown, it can be hypothesized that their mode of interaction with tubulin is different. For

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[a] Abbreviations: CA-4, combretastatin A4; CA-4P, combretastatin A4 phosphate.

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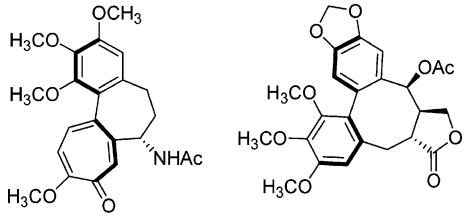

Figure 2. Natural products that inhibit tubulin and display structures similar to CA-4.

example, colchicine, unlike CA-4, binds to tubulin in a virtually irreversible manner.[10] It can also be hypothesized that, alongside tubulin, there are other targets for these drugs that determine the maximum tolerated dose.

Active compounds extracted from *Combretum caffrum* were first described just under 2 decades ago.[11−13] In the original manuscripts, CA-4 was described as a strong cell growth and tubulin inhibitor.[14] Because of its poor solubility in water, a more soluble prodrug, CA-4 phosphate (CA-4P),[7,15] has been developed as the selected lead in in vivo and in human studies. CA-4 is not the only molecule of the family to have entered clinical trials because **7** (AVE8062, a synthetic analogue bearing a different substitution on ring B; see further) has recently done so as well. Indeed, combretastatin A-1 (**10**, OXI4531; see further), which retains the same biological and structural signature, has been shown to possess features that should make it suitable for therapeutic intervention. CA-4P has completed phase I trials on advanced cancers, and the results have been published. These trials were conducted to establish the most likely treatment schedule to be successful and to establish a preliminary list of severe side effects. In brief, the side effects usually associated with cancer chemotherapy, such as bone marrow toxicity and alopecia, appear to be absent or mild.[16−18] Side effects, at least at doses that reach therapeutic levels, appear to be tolerable for an anticancer agent (for a detailed review, see ref 7). The main side effects observed were cardiovascular, with the presence of tachycardia, bradycardia, and hypertension[19] in a relevant proportion of patients. In addition, pain in the region where the tumor was located was also a very common side effect. Last, other neurological symptoms, such as neuropathy, ataxia, headache, and abdominal pain, were also observed in a small subset of patients.[17,19] Controversial evidence has been presented on the modifications of the cardiac rhythm by CA-4. A subsequent manuscript[19] analyzing one of the phase I trials[16] reported prolongation of the QTc interval in some patients (a condition that is thought to be linked to torsades de pointes, a cardiac dysrhythmia often linked to drug therapy),[19] while other studies did not observe any significant changes in the electrocardiogram.[18] Phase I studies are usually designed in a dose-escalating fashion. The maximal tolerated doses allowed for plasma membrane concentrations of CA-4 to reach micromolar range are compatible with the concentrations required for its effects on tubulin. In these trials, patients with advanced cancers[17,18] resistant to other forms of therapeutic approaches, and often with metastasis present, are recruited to evaluate safety profiles, maximal tolerated doses, and dose limiting toxicities. The real therapeutic value of a drug, therefore, is difficult to ascertain until phases II and III results are made public. Yet it is encouraging that a patient suffering from anaplastic thyroid carcinoma underwent total remission for 30 months.[16] Phase I studies often evaluate drug actions by parameters different from parameters for therapeutic success. In the case of angiostatic/angiotoxic drugs, blood flow in the tumor or metastatic area is often chosen. In the published studies, using both dynamic contrast enhanced MRI (DCE-MRI)[16−18] or positron emission tomography (PET),[20,21] it was possible to establish that a high proportion of patients did display a marked reduction in blood flow in the tumor area after administration of CA-4. These results on blood flow, together with possibility of reaching potentially therapeutic plasma membrane concentrations, have prompted further clinical trials, and CA-4 has now entered phase II.[22] Official Web sites (www.cancer.gov and www.clinicaltrials.gov) devoted to clinical studies now cite three phase II clinical trials underway, aiming at identifying the effectiveness of CA-4 in patients treated with this agent in combination with traditional chemotherapeutic agents or radiotherapy. In particular, two of these studies concentrate on advanced anaplastic thyroid cancer. The Web site of the company Oxigene also is an excellent resource for the latest information on the clinical progress of this drug (www.oxigene.com). Cancer is not the only disease in which angiogenesis plays a crucial role. In the eye, retinal neovascularization and choroidal neovascularization are important components of vision loss, and a preclinical study has suggested that CA-4 might be effective in these diseases as well,[23−25] opening new therapeutic avenues for this drug.

Medicinal Chemistry

Despite its low molecular weight and simple molecular structure, CA-4 (**1**) is one of the most powerful inhibitors of tubulin polymerization known to date. It is quite clear that such a simple structure bringing only two aromatic rings linked by a double bond in the cis configuration can lead to a limited number of analogues that will resemble its parent compound, while the possible structural modifications that **1** can undergo are obviously endless. Hundreds of combretastatin derivatives have been synthesized and described. Among the reasons for this plethora of compounds is the simplicity of the basic synthesis. Novel stilbenic compounds can be easily prepared using two main reactions: Wittig and Perkin condensations. In the Wittig reaction a mixture of the two geometrical isomers (*Z* and *E*) is always obtained, and this often leads to tedious further purification procedures. In the Perkin reaction, the *Z* isomer predominates, but a high-temperature decarboxylative process is necessary to obtain the final product.[26,27]

For the sake of simplicity, in this Miniperspective, the molecule will be subdivided into three main elements (ring A, ring B, and the double bond; Figure 1) and a synoptic SAR survey will be presented to highlight possible directions of future research. To accomplish this task, we believe it is important to evaluate these analogues not only regarding their cytotoxicity but also regarding their ability to display potent antitubulin action. Although this term might refer to a number of cellular actions, in this manuscript we will use the term antitubulin to describe the action of agents that disrupt polymerization or depolymerization of this protein by directly interacting with it. It would be intuitive to think that these two characteristics are unequivocally linked, and yet we will describe analogues that

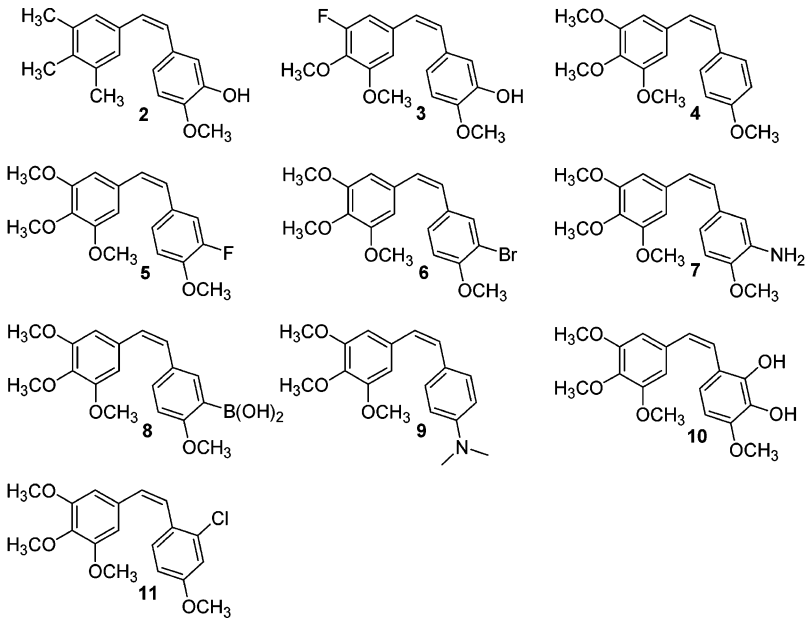

Figure 3. Selected analogues of CA-4 modified on the aromatic rings A and B.

lose just one of these properties. Which of the two is essential to obtain antivascular properties remains to be established, although it is likely that the effect on tubulin will be the most important. The review will, when possible, refer to cytotoxicity and antitubulin activity in comparison to CA-4. It needs to be said that a standardized protocol for cytotoxicity evaluation is not available, and individual laboratories report disparate values (from picomolar to micromolar) for CA-4 toxicity. It is also difficult to compare the relative loss or gain of function of compounds generated in different laboratories. Although this discrepancy might depend on the different cell lines used for the screening, our experience and that of other scientists in the field (personal communication with M. Cushman) suggest that the time of exposure is a major factor affecting the cytotoxicity of combretastatin analogues. To standardize this, we therefore propose that CA-4 analogues should be tested for cytotoxicity for 48 h, a time that has proven to be successful in many laboratories, including ours (IC_{50} for CA-4 between 1 and 3 nM in a panel of cell lines). Yet the cytotoxicity observed in this protocol might not be directly correlated with the angioselectivity of combretastatin, since shorter times of exposure have been demonstrated in vivo.[7] Therefore, for evaluation of the selectivity of analogues between endothelial cells and other cells, the time courses should be considerably shorter. Last, biological experiments should be performed with CA-4 controls alongside, which surprisingly does not always happen.

1. Modifications of Ring A (Figure 3). It has been long thought that the presence of the trimethoxybenzene moiety is crucial to obtain relevant cytotoxic and antitubulin responses. This assumption is mainly based on (i) the recurrence in nature of this chemical motif in other antitubulin drugs (e.g., podophyllotoxin, colchicine, steganacine; Figure 2),[28] (ii) the potency of CA-4 over CA-3, where the meta methoxy group is replaced with a hydroxyl,[12] and (iii) pioneering work that reported a significant loss of potency when a simple aromatic ring (phenyl) was present or when deletions of the meta or para position methoxy groups were performed.[29] Similar conclusions can be drawn by the loss of cytotoxic activity when the methoxy groups are substituted with bulkier (e.g., ethoxy) groups.[30] This latter observation would also suggest that the steric factor plays a pivotal role in accommodating the ring inside the active site. Modification of the trimethoxybenzene with groups of higher lipophilic nature, to exploit hydrophobic interactions that might occur in the binding site of tubulin (trimethylbenzene, naphthalene), yields products with decreased cytotoxicity.[30,31] However, when substitutions on this ring are carefully analyzed for both cytotoxic and antitubulin activity, interesting observations can be drawn. Hadfield's group[30] demonstrated that the substitution with a trimethylbenzene group (**2**) gives a significant decrease in cytotoxicity while the selective inhibition of tubulin is maintained. Recently, Pettit et al. synthesized a series of fluorcombstatins where the methoxy group at the meta position was replaced with a fluorine group. Also in this case, the compounds synthesized (e.g., **3**) showed antitubulin activity comparable to that of CA-4, and in this instance there was only a slight loss of potency.[32] In the same study, attempts to replace fluorine with the bulkier chlorine and bromine gave compounds with reduced cytotoxicity and antitubulin activity.[32] Last, the choice to block the mobility of the methoxy groups through the formation of a dioxolane ring leads to a decrease in activity,[33] showing that it is better to maintain the conformational mobility of the three methoxy groups in order to obtain the right fit, despite the associated entropic penalty.

It is interesting to note that substitutions of the phenyl ring with heterocyclic structures have received very little attention. For instance, there is a report of replacement with furan or indole rings.[34] Unfortunately, it is not possible to reach definite conclusions regarding this modification because all these derivatives lack the olefinic counterpart.

What conclusions can be drawn by the modifications so far performed on ring A? Is the trimethoxy moiety as crucial as originally postulated? No certain answer can be given to these questions. Yet it would appear that antitubulin activity might benefit from less cumbersome functional groups, and it would be worth considering this opportunity during the drug design. Furthermore, the oxygenation of the three positions might not be as indispensable as previously thought for the antitubulin activity. Independent of this, the discrepancy between cytotoxicity and antitubulin activity poses yet another dilemma. How do compounds that display good antitubulin activity but modest or poor cytotoxicity fair in angiotoxic or angiostatic assays? The possibility exists that these agents would act on blood vessels while displaying reduced cytotoxic side effects.

2. Modifications on Ring B. Historically, it was believed that ring B was the only structured moiety amenable to modifications yielding potent compounds, and therefore, this ring has received greater attention from medicinal chemists. Modifications on this ring can be subdivided further into three main lines of research: (i) substituted phenyl rings, (ii) heterocyclic rings, and (iii) nonsubstituted aromatic rings. The last approach deserves special attention based on the impressive results obtained by Medarde's group.[35]

2.a. Substituted Phenyl Rings (Figure 3). It immediately appeared quite clear that the insertion of suitable substituents in the phenyl ring B could confer interesting pharmacological properties to CA-4. With Cushman's seminal work,[29,36] it was pointed out that the presence of a para methoxy group was fundamental while the presence of the meta hydroxyl group was not essential. Indeed, compound **4** shows minimally reduced biological activity compared to **1**. This observation led to isosteric substitution of the hydroxyl group with fluorine, and these compounds display significant cytotoxic activity (**5**).[37] The high electron-withdrawing properties of fluorine should preclude electrophilic substitutions on the phenyl ring, and this might lead to more metabolically stable compounds (with particular reference to phase II metabolism). The half-life of CA-4 is relatively short,[7,38] and therefore, generating less metabolizable compounds might be a favorable way forward. Obviously, this molecule cannot undergo a prodrug approach similar to that of CA-4 phosphate, and therefore, different strategies would be required to increase its water solubility. The substitution of the same meta hydroxyl with a bromine was also attempted (**6**),[39] but in this case a 10-fold loss of cytotoxic activity was measured with no loss in antitubulin activity, suggesting that steric factors are important. Finally, changing the position of the methoxy group from the para to the meta position[36] or inserting an electron-withdrawing group in the meta (e.g., $-NO_2$)[40] leads to a decrease in biological activity.

The meta $OH-NH_2$ isosteric substitution proved to be useful for giving a compound with a slight increase in potency compared to CA-4. Oshumi and colleagues were the first to accomplish this transformation,[41] and the resulting bioisostere **7** (AC7700 and AVE8062, the latter being the water-soluble prodrug) is currently in clinical trials. Subsequently, others have reported the same results for this compound via a novel and more efficient synthesis.[39] In this regard, a number of laboratories have observed that the amine substitution on other analogues of CA-4 generates slightly more potent compounds (about 2-fold) while the nitro substitution generates less potent compounds (about 5-fold). We therefore propose that this rank order of potency be used in the synthesis of novel products to confirm a CA-4-like mechanism of action.

Recently, a boronic acid group has been used as a mimetic of the phenolic moiety in ring B of CA-4,[42] and this compound (**8**) displayed high cytotoxic and antitubulin actions. This agent should theoretically display higher water solubility and therefore, if ever brought to in vivo studies, might not require a prodrug approach. In the same manuscript, docking studies suggested that the boronic acid gives strong hydrogen bond interactions with two pivotal amino acids present in the active site of the tubulin. Yet since the bromine substitution (**6**) did not show any loss in antitubulin activity, the significance of this interaction remains to be established.

Last, Pinney's group replaced the hydroxyl group with an azide, giving a product that maintains activity.[40] Although this substitution would not be amenable for clinical studies, the presence of an azide might be a good pharmacological tool (e.g., for affinity labeling or affinity purification of proteins that bind CA-4 and mediate the cytotoxic effect) or might allow the generation of additional analogues and chimeric compounds (including multiple-action compounds) via the fashionable click-chemistry reaction.[43] To our knowledge, there are no reports of substitutions at this position with a formyl group. Yet there is a report on CA-4-like ethers[44] that imply that compounds bearing a formyl group in the meta position would be potent antitubulin agents while lacking significant cytotoxicity (see further, compound **27**).

Substitutions at the para position have also given useful information. Substitutions with an ethoxy or propoxy group give a loss in activity,[29] suggesting that steric effects can reduce the activity of these compounds. Even the replacement of the oxygen with the bulkier sulfur leads to poor activity. Therefore, the oxygen seems to be fundamental, probably acting as a hydrogen bond acceptor. In the same report, substitutions of the para methoxy group with a methyl or chlorine gave less cytotoxic compounds, although with strong antitubulin activity. This seems to indicate that the para methoxy group is fundamental for cytotoxicity but not pivotal for antitubulin action. Furthermore, substitution of the methoxy group with a dimethylamino moiety at the para position (**9**) induces at least a 10-fold loss of cytotoxic potency while minimally influencing tubulin polymerization.[36]

Insertion of substituents at the ortho position has also been attempted. Indeed, the rationale for exploiting this position is given by the presence of an ortho hydroxyl group in the natural product combretastatin A-1 (the diphosphate prodrug **10** [Oxi4503] is currently in clinical trials).[45,46] In our opinion substitutions at the ortho position might give rise to conformationally locked analogues. If the correct dihedral angle is obtained, this might have advantages for identifying more active derivatives. Insertion of a chlorine group (**11**) gave a compound over 1000 times less cytotoxic but not differing significantly in the antitubulin profile compared to its parent compound.[36] Indeed, substitution at this position with an amino group generates active products, as shown in a paper published while editing this manuscript.[47]

2.b. Heterocyclic Rings (Figure 4). It is well-known that the insertion of a heteroatom in place of a carbon atom might yield new systems with different pharmacodynamic and pharmacokinetic features. For example, the availability of a lone pair might lead to more water-soluble derivatives and to new additional interactions with biological targets. This strategy was put into practice, and substitution with a pyridine ring was carried out.[48] Results showed that this strategy could lead to active compounds but that the position of the nitrogen group plays a pivotal role. In fact, substitution at the ortho position fails to yield very active products (although the loss in cytotoxicity is far greater than the loss in antitubulin activity), while substitution at the meta position yields products (**12**) that display potent cytotoxic and antitubulin action.

2.c. Nonsubstituted Aromatic Rings (Figure 4). It has been reported that substitution of isovanillic ring B with a phenyl fails to give products with comparable biological activity.[36] Despite this fact, Medarde's group inserted a naphthalene moiety with successful results.[35] The lead compound (**13**) displayed just a 4-fold loss of cytotoxic activity and a 5-fold loss of antitubulin activity. In light of the loss in cytotoxicity described above for the substitutions on ring B (meta and para positions), this result is particularly suggestive. Recently, the same group, taking advantage of this substitution, has also attempted to replace the more lipophilic naphthalene with a quinoline (**14,**

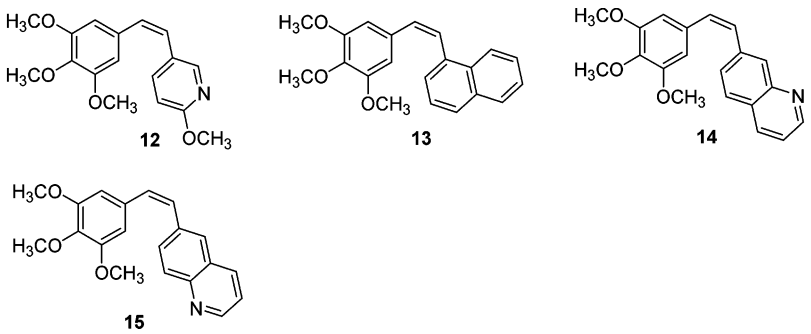

Figure 4. Analogues of CA-4 with substituted heterocyclic structures or naphthathene moiety on ring B.

15) or a quinoxaline ring system.[49] As pointed out previously, the presence of nitrogen might increase water solubility. In this case, compound **14** lost about 70-fold cytotoxic activity but retained antitubulin properties (with an approximately 10-fold compared to CA-4). Surprisingly, compound **15**, displayed a superior cytotoxic effect (10-fold loss over CA-4) but was virtually ineffective as an antitubulin agent. The strategy to replace isovanillic ring B with naphthalene has recently been reviewed by the same authors.[50]

In conclusion, the medicinal chemistry so far performed on ring B has probably taught us a few lessons. First, modifications on this ring can abrogate cytotoxicity without affecting antitubulin activity. For cytotoxicity, the para position must be occupied by a methoxy group. The meta position can undergo substitution with electron-donating groups (e.g., amino) or with electron-withdrawing groups (e.g., fluorine), but substituents cannot exceed the hydroxyl group in size. Although the substitutions at these positions do affect antitubulin activity, they do not appear to play a pivotal role.

The ortho position seems to be less characterized, although combretastatin A-1 is characterized by a hydroxyl in this position. This compound is growing in popularity because of its more potent vasculature damaging potential.[2] It is therefore likely that in the future this position will receive considerable attention. Indeed, insertion of functional groups at this position can give new binding interactions and freeze the conformation of the phenyl ring. In this case, it would be possible to generate virtually rigid analogues. Another possibility suggested to explain the actions of Oxi4503 is that it might generate reactive quinone intermediates because of the presence of a hydroxyl group in the ortho position.[51]

Two additional lines of research is warranted in this area: (i) further attempts to replace ring B with heterocyclic structures, not necessarily limited to six-membered rings and (ii) testing of the potential of compounds that in vitro have displayed low cytotoxicity but high antitubulin activity and, conversely, compounds that have displayed high cytotoxicity but low antitubulin activity (e.g., **15**), since these might display different pharmacodynamic profiles.

3. Modifications of the Double Bond. The olefinic component of CA-4 has undergone major molecular changes. Among the main reasons for this is the observation that the presence of a double bond in a *Z* configuration is fundamental to having high cytotoxic and antitubulin action, while the *E* analogue is significantly less potent, if at all active (although trans CA-4 has been shown to retain some biological activity, this could be due to isomerization to the cis form during the biological assays).[36] It is believed that the olefinic bond plays a more constitutive role than a binding role during the biological interaction; it allows placement of the benzene rings at an appropriate distance and gives the molecule the right dihedral angle to maximize the interaction with the target. The *Z* stilbenic double bonds can easily isomerize under the influence of heat, light, and protic media. Therefore, a number of groups have attempted to modify the olefinic bridge to stabilize the conformation to attempt to increase the biological effects of the compound or to minimize the possible metabolism that the olefinic group might undergo.[52] In particular, two main lines of research can be identified: (i) modification on the double bond and (ii) replacement of olefinic bridge with a ring.

3.a. Modification on the Double Bond (Figure 5). Hydrogenation of the double bond led to surrogates that were less potent. This observation can be easily explained considering the entropic penalty associated with conformationally free derivatives (**16**).[29] Nonetheless, the observation that activity could be retained to a certain extent led to the investigation of the optimal and permissive length of the bridge.[29,53] Biphenyl structures were virtually inactive. Extending the bridge via methylene, ethylene, propylene, and butylene bridges revealed that two carbon linkers were the most active and one and four carbon linkers were the least active both in cytotoxicity and in tubulin assays. Nonetheless, the olefinic bridge remained the most potent structure.

This carbon chain length rule, though, would appear to be in contrast with the observations on phenstatin (**17**), discovered serendipitously by Petitt and co-workers.[54] This molecule, where the methylene bridge is replaced by a carbonyl group, shows antitubulin activity similar to that of CA-4 and a modest loss of potency in cytotoxicity assays. Other phenstatin analogues have been prepared replacing isovanillic ring B with an indole (**18**) or an aniline derivative (**19**). Also in this case, active compounds were obtained, suggesting that the SAR of (**1**) is maintained for phenstatins.[55−57] This observation that the length of the bridge can be varied while maintaining significant biological properties is strengthened by two additional series of compounds: (i) vinylogous analogues of CA-4 (**20**),[58] characterized by four carbon atoms and a linker distance far from the optimal value, which retain activity as both cytotoxic and antitubulin agents and (ii) chalcones. Although the observation on **20** is interesting conceptually, the fact that only one of the four geometrical isomers of the double bonds is active limits the suitability of these compounds for further development.[58] In contrast, chalcones, which can be prepared with a one-pot procedure, might be more suited for further exploitation (e.g., modifications of the remaining double bond, rigid analogues, etc.). These structures, bearing an α,β unsaturated carbonyl linker, also give potent cytotoxic inhibitors of tubulin polymerization (**21**).[59] It is quite remarkable that a growing number of papers where the two phenyl rings are separated by three atoms are appearing in the literature. These derivatives can be considered rigid cis analogues of chalcones where the double bond is substituted with various heterocyclic rings. The presence

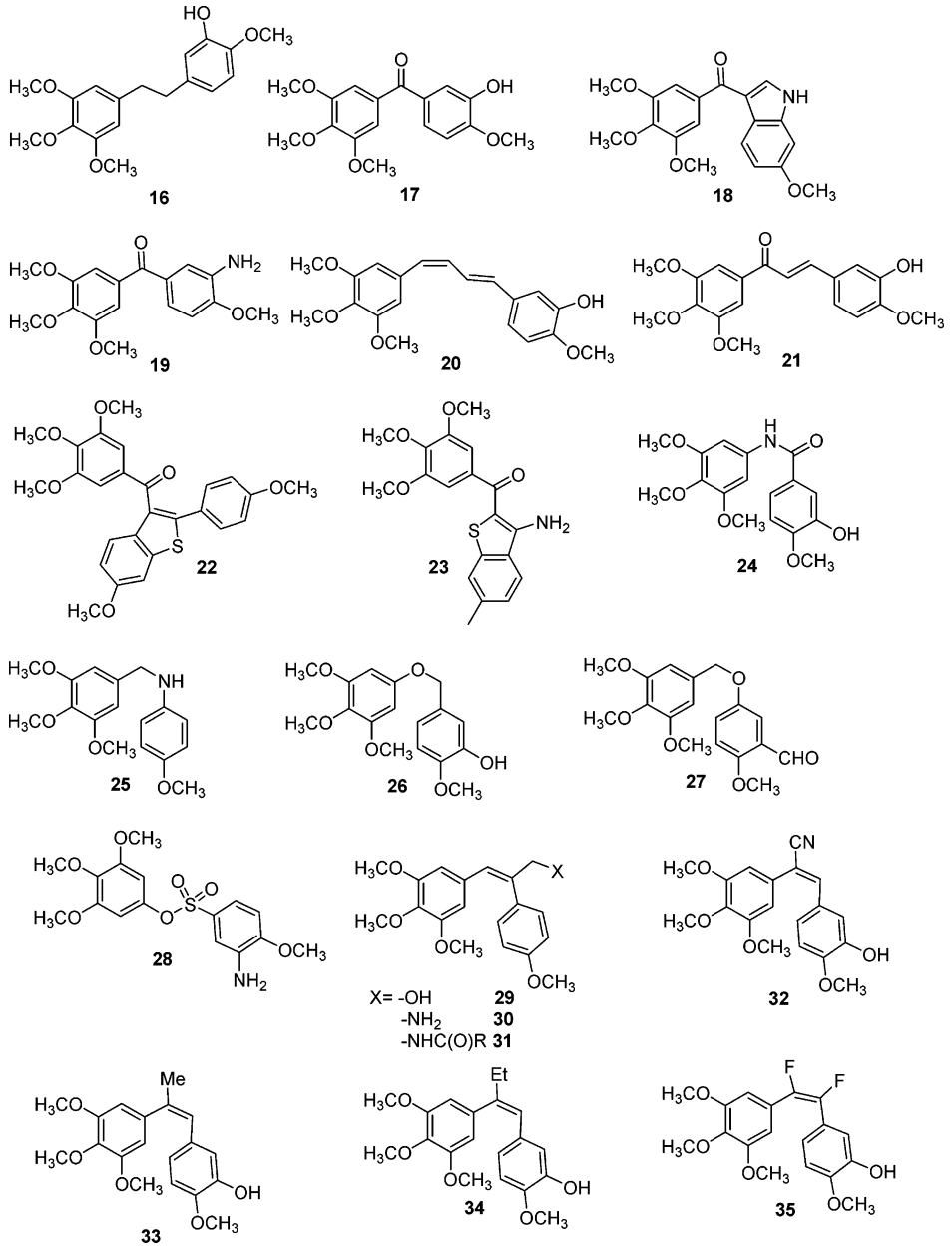

Figure 5. Selected analogues of CA-4 with modifications on the double bond.

in nature of cis chalcones is rare, but it is well-known that *E*-chalcones can isomerize in solution.[60] Interestingly, these compounds can be prepared in few synthetic steps: Flynn's group reported the synthesis of potent analogues of combretastatins through a palladium-mediated multicomponent reaction (e.g., **22**).[61] Chalcones have been recently reviewed elsewhere and will not be discussed further in this Miniperspective.[62] Compound **23**, a compound that resembles phenstatin, is another significant example of a highly cytotoxic and antitubulin agent with a different spacing between the phenyls.[63]

Although linkage via a single oxygen or nitrogen atom led to virtually inactive compounds in regard to tubulin[29,44,53] (an expected result due to the short distance between the rings), a few attempts have been made to substitute the carbon atoms participating in the olefinic bridge. Replacement of the double bond with an amide group, which has a double bond character, appeared to induce a significant loss in both cytotoxicity and antitubulin activity (**24**). Surprisingly, though, the amine substitution (**25**) did not induce such a dramatic change (in both parameters) compared to CA-4.[36,64] Similarly, insertion of an ethoxyl linker between the two aromatic rings gives products with some biological activity. Interestingly, when oxygen is nearer the trimethoxybenzene moiety (**26**), the compounds retain more cytotoxic and antitubulin effects, although there is still a significant loss compared to CA-4. Strikingly, a compound (**27**) bearing the oxygen further from the trimethoxybenzene moiety and with a formyl group in the meta position displayed impressive antitubulin activity while losing cytotoxic activity.[44]

Introduction of a sulfonate group in place of a double bond[65] gave products with nanomolar cytotoxicity and potent antitubulin action (e.g., **28**). This substitution appears quite promising, but stability studies should be performed before reaching firm conclusions.

A few attempts have been made to replace the hydrogen atom on the double bond with other groups. Substitutions with hydroxylmethyl (**29**), aminomethyl (**30**), or amidomethyl (**31**) groups led to a significant decrease in activity.[29,41,66,67] Yet the rigid nitrile functional group appears to retain functional activity (**32**).[41] Interestingly, substitution of the hydrogen closer to the trimethoxyphenyl ring with a methyl (**33**) or an ethyl group (**34**)

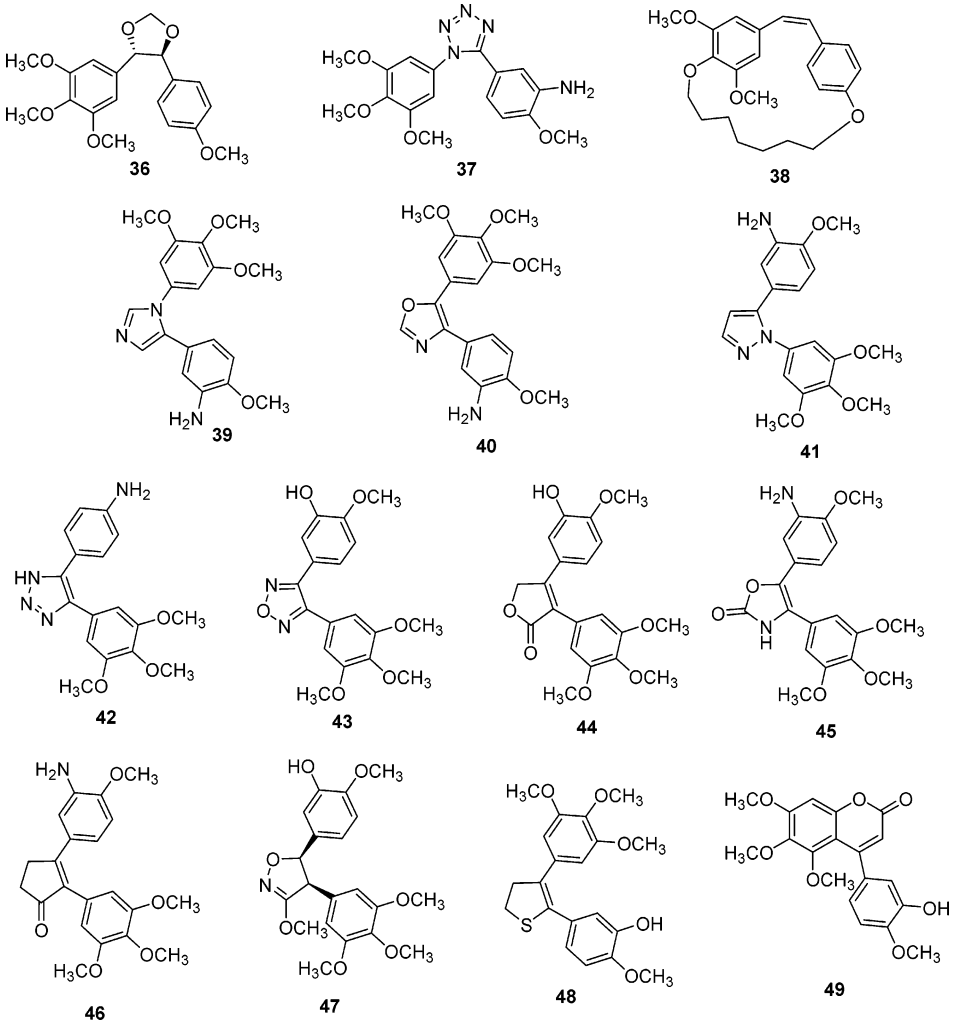

Figure 6. Selected analogues of cis-restricted CA-4.

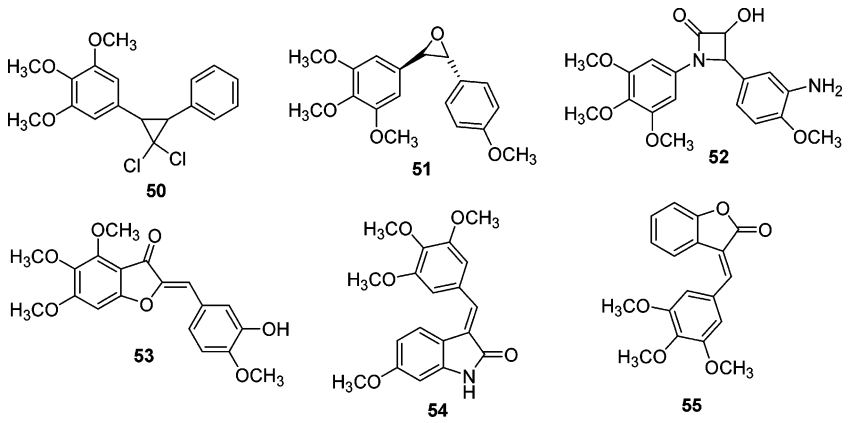

Figure 7. Selected analogues of cis-restricted CA-4.

did not induce any significant loss of antitubulin activity while it significantly decreased cytotoxicity (approximately 100-fold). Furthermore, preliminary data suggested that colchicine binding was also unaffected.[67] Hydroxyl substitutions gave products devoid of activity,[68] even in enantiomeric pure form.[69] In contrast, a patent has recently appeared claiming that 1,2-difluorostilbenes possess antitubulin activity similar or superior to that of CA-4 (**35**).[70]

It is difficult to reconcile these modifications to create a unifying SAR. Some modifications, such as those on compounds **19**, **21**, and **28**, can be seen as bioisosteres of the double bond, and indeed, they retain both cytotoxicity and antitubulin properties. It is also possible that the steric hindrance plays a major role in modifying the cytotoxic role while playing a lesser role in preventing tubulin polymerization (e.g., **33**, **34**). It is nonetheless clear that ample space is present to generate compounds with further substituents to increase the activity of these compounds.

3.b. Analogues Where the Olefinic Group Is Replaced by a Ring (Figures 6 and 7). The first group that attempted this modification replaced the olefinic moiety with dioxolane scaffolds.[68] Although cytotoxicity was not evaluated, most of these compounds were devoid of antitubulin activity. Remarkably, a dramatic loss of activity was recorded for all the

compounds except for the *S*,*S* enantiomer **36**, where the loss was not as severe.

Further attempts followed, and 1 year later, Oshumi and coworkers replaced the double bond with heterocyclic five-membered rings (1,3-thiazol-2-amine, pyrazole, 1,3-thiazole, 1,2,4-triazoles, tetrazoles, e.g., **37**).[71] The work was prompted by the observation that when colchicine and CA-4 structures were overlaid, a good match was present, showing that the double bond region was amenable to expansion. Quite remarkably, the compounds retained both cytotoxic and antitubulin activities.

The advantage of these substitutions is manifold. Indeed, these cis-locked analogues provide at least three main advantages: (a) prevention of combretastatin isomerization from cis to trans; (b) increased specificity, since the trans conformation might be recognized by other cellular targets; and (c) the possibility of using heterocyclic systems that might improve the therapeutic potential of these drugs. This is strengthened by computational analysis showing that tubulin, on its colchicine site, can host compounds with different spatial orientations and that three different binding domains can be involved in the interaction with these molecules.[72] An interesting possibility explored to rigidify combretastatins was to synthesize a paracyclophane derivative. Although the idea is quite attractive, the resulting compounds (e.g., **38**) did not lead to biological activity on tubulin, precluding their use as antivascular agents.[73] It is not surprising that to enhance the efficacy or potency of combretastatin analogues, a number of five-membered heterocyclic compounds have been prepared. Most of these, with the exception of the most recently published, have been reviewed elsewhere.[74]

Although this manuscript does not aim to review the synthetic avenues undertaken to develop new combretastatin analogues, it is obvious that the substitution of the olefinic bridge with a ring requires rather distinct procedures compared to the other analogues described. Furthermore, for some compounds new and exciting synthetic procedures have been described. For example, the furazan ring has been successfully obtained by dehydrating 1,2 diphenyloximes via the Mitsunobu reaction.[75] As interesting is the use of the van Leusen reaction to yield 1,5-substituted imidazoles via a one-pot procedure.[76] Among the other reactions used, it is worth mentioning the formation of benzo[*b*]furan and indole derivatives via a multicomponent Pd-catalyzed reaction[61] and the possibility of building coumarinic analogues using the Suzuki reaction.[77] Numerous other interesting synthetic procedures have been used, and it is our opinion that in the future a combination of the right heterocyclic compound (that possesses the optimum pharmacological profile), coupled with a procedure that will allow rapid synthesis of numerous compounds, might yield important information that will eventually be used in in vivo studies. Indeed, the challenge will be to devise a method that will easily allow substitutions in the presence of polyfunctionalized aromatic rings.

Among the five-membered rings, imizadoles (**39**),[76] 1,3-oxazole (**40**),[76] pyrazole (**41**),[76] triazoles (**42**),[78] furazan (**43**),[75] 2(5*H*)-furanone (**44**),[79] diaryloxazolones (**45**),[80] 2-cyclopenten-1-one (**46**),[81] 4,5-dihydroisoxazole (**47**),[82] 2,3-dihydrothiophene (**48**),[83] and arylcoumarin (**49**)[77] have been synthesized to date. Most of these retain both cytotoxic and antitubulin activity, and these two activities appear to correlate well. Indeed, a number of compounds were found to be slightly more potent than combretastatin itself (for example, combretafurazan).[75] Following this strategy, three-, four-, and six-membered rings were also used to replace the olefinic portion of CA-4. Pyrazine,[76] 2,3,8,8a-tetrahydro-5(1*H*)-indolizinone,[84] benzene ,and pyridine[82] were used as six-membered rings, but in all cases severe drops in activity were observed. Two reports have appeared with three-membered rings. Dichlorocyclopropyl analogues were synthesized exploiting the strong reactivity of dichlorocarbene toward the double bonds. Also in this instance, a severe reduction of cytotoxicity was noted (**50**).[85] Hadfield's group prepared the quite elusive anti epoxide of a close structural analogue derivative of CA-4 (**51**). Also in this case a significant drop in activities was noted.[67] Among the four-membered rings, azetidone analogues were prepared.[86] Biological activity can be retained (e.g., **52**), and these compounds display a 2-fold drop in antitubulin activity compared to CA-4.

In conclusion, it can be inferred that the substitution of the double bond with five-membered rings seems to be the best option for medicinal chemists. It is difficult to say if the decrease in activity, observed in some cases, is due to steric interactions or can be ascribed to an incorrect orientation of the two phenyl rings into the binding site. In any case, it is clear that to maximize the potency, the two phenyl rings must have a 1,2 relationship; indeed, 1,3 relationships give a strong reduction in activity.[82,87,88] Moreover, the presence of an aromatic character does not seem to be necessary.

The strategy of replacing the double bond with a five-membered ring does not block the free rotation of the two aromatic rings. For this reason a series of papers appeared, where the authors attempted to reduce the conformational mobility associated with the two phenyl rings of CA-4. Conformationally locked structures can be obtained through the insertion of a new ring between the double bond and the aromatic substituent. Such substructures are present in some natural products such as indanocine, aurone, and isoaurostatin. Hadfield's group synthesized rigid analogues of chalcones.[89] In this case, cytotoxicity was retained, but these analogues did not show any antitubulin action (e.g., **53**). Other compounds that appeared to retain activity have been conformationally restricted with an indolinone ring (**54**). Biological testing of these compounds demonstrated that these compounds retain cytotoxic and antitubulin activity.[90] In contrast, significant loss of potency was observed with isoaurostatin analogues (**55**) and phenanthrene-like derivatives.[29,91,92]

Pharmacological Considerations, Future Directions, and Conclusions

The present review is a synopsis of some of the analogues that have been made in the past 2 decades that have allowed an increase in our understanding of the actions of CA-4 and have hopefully created the basis for additional agents that will enter clinical trials. It is important to stress that a number of other equally interesting analogues have been synthesized and reported but, because of space constraints, have not been mentioned.

It is important to highlight a few dilemmas that CA-4 and its analogues have posed the scientific community. CA-4 displays cytotoxicity at low nanomolar concentrations. In contrast, in vitro tubulin assays and colchicine binding displacement studies indicate that these latter two effects occur at a 100-fold higher concentration. The most common assay for tubulin polymerization is represented by a rapid turbidimetric assay[36,93,94] that monitors instantaneous effects on polymerization. Indeed, the difference between long-term treatment and instant measurements can theoretically explain such concentration differences. A similar consideration can be made for colchicines binding, where the radioligand binds in an irreversible manner. In contrast, a less popular assay allows long-term cell culture of

cells in the presence of analogues followed by tubulin extraction in the presence of paclitaxel.[75,94] This then allows comparison of the global long-term effect of the analogues on the polymerized/unpolymerized tubulin ratio. Surprisingly, also in this assay (which does not have the same kinetic problems as the previous one) combretastatin requires high nanomolar/low micromolar concentrations to induce significant changes.[75] Furthermore, it will not have passed unnoticed to the careful reader that, while describing the medicinal chemistry, a number of compounds displayed obvious discrepancies between cytotoxic and antitubulin activities. The question therefore is whether the cytotoxic effect of CA-4 and its analogues is dependent on the action on tubulin or whether it is unrelated. It needs to be said that peak plasma concentrations in clinical studies, where vascular shutdown has been observed, are compatible with the low micromolar concentrations required for the activity on tubulin in vitro. Furthermore, in vitro cell cycle analysis of cells treated with CA-4 or its close analogues suggests that cells are arrested after a few hours in the G2/M phase at concentrations close to the IC_{50} values for cytotoxicity. Such effects would strongly suggest that tubulin is a relevant target for combretastatins. Yet, a cell-cycle specific agent would be expected to yield strong hematopoietic side effects, which instead are absent in CA-4 treated patients. Another dilemma is represented by the strong selectivity of this agent in vivo for the neovasculature and the contrasting nonselective cytotoxicity in vitro (virtually all cell lines tested are generally equally sensitive to CA-4). A possibility, mentioned earlier in the text, is that prolonged exposure of cells to CA-4 results in nonselective toxicity, while the selectivity on endothelial cells is apparent after short exposures. This would be compatible with the short half-life of CA-4 in vivo[7] and in vitro.[95] Furthermore, what makes the pharmacodynamic and clinical profile of CA-4 so different from that of other tubulin inhibitors, including those used clinically (paclitaxel) or unsuitable for clinical use (colchicine, podophyllotoxin)? At least four main hypotheses can be proposed to explain these discrepancies: (i) CA-4 targets, in vivo, a subpopulation of tubulin (a possibility supported by drug resistance data);[96,97] (ii) CA-4, alongside tubulin, recognizes a yet unidentified relevant target; (iii) the interaction between tubulin and combretastatin is qualitatively different. For example, it is known that colchicine binding is irreversible while combretastatin binding to tubulin is not.[10] (iv) Combretastatin, for as yet unknown reasons, rapidly and preferentially disrupts in vivo cellular processes in the endothelium that require tubulin assembly. These last two possibilities are beginning to be investigated. Two exciting findings have emerged recently: (i) CA-4 may exert its antiangiogenic effect through rapid functional inhibition of the VE-cadherin/β-catenin complex, required for endothelial cell−cell adhesion and survival during neovessel assembly and remodeling,[98] and (ii) CA-4 may interfere with Rho and Rho-kinase signaling and lead to actin reorganization.[6] Whether these are primary targets, whether the trigger remains tubulin depolymerization, or whether these mechanisms act in concert remains to be fully elucidated (Figure 8). Last, it is possible that the endothelial cells are more sensitive to tubulin disorganization, and that, in this cell type, a brief burst of CA-4 leads cells to collapse in an irreversible manner. Independently of the mechanism, it would be important to unravel the detailed mechanism of action of CA-4 to improve the primary screen used in medicinal chemistry for this class of compounds. For example, it would be interesting to exploit some of the compounds already described to generate affinity labels to pull down binding partners of CA-4 and identify them by a proteomic approach.

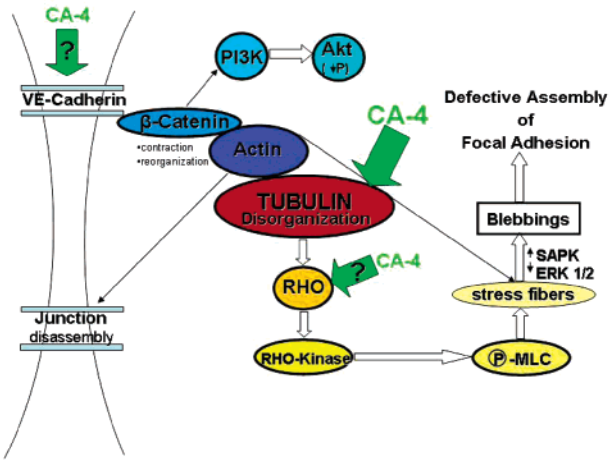

Figure 8. Schematic representation of potential targets of CA-4 in endothelial cells: P-MLC, phosphorylated myosin light chain; ERK, extracellular receptor kinase; SAPK, stress-activated protein kinase.

Last, does the simple structure of CA-4 and the concentrated medicinal chemistry effort already profused mean that most interesting modifications have been exploited? Should medicinal chemists move to more complex natural products that have been shown to elicit effects on neovasculature? We believe not, since more challenges lie ahead. The strong cytotoxic nature of CA-4, for example, would make it an excellent bullet against the tumor itself, if properly targeted. It is also possible that in the future this field will move in the direction of chimeric drugs, with CA-4's action backed up by a drug with a different mechanism of action. The first attempts to generate these chimeric drugs have been made but with little success.[99,100,101] On a similar line of thought is an exciting recent manuscript that employed advanced pharmaceutical technology to devise a nanocell that temporally releases two different drugs. In the first phase, CA-4 is released to cause vascular shutdown, while in the second phase the nanocell trapped in the tumor area releases a traditional chemotherapeutic agent.[102]

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