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## Design, Synthesis, and Biological Evaluation of Water-Soluble Amino Acid Prodrug Conjugates Derived from Combretastatin, Dihydronaphthalene, and Benzosuberene-Based Parent Vascular Disrupting Agents

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### Abstract

Targeting tumor vasculature represents an intriguing therapeutic strategy in the treatment of cancer. In an effort to discover new vascular disrupting agents with improved water solubility and potentially greater bioavailability, various amino acid prodrug conjugates (AAPCs) of potent amino combretastatin, amino dihydronaphthalene, and amino benzosuberene analogues were synthesized along with their corresponding water-soluble hydrochloride salts. These compounds were evaluated for their ability to inhibit tubulin polymerization and for their cytotoxicity against selected human cancer cell lines. The amino-based parent anticancer agents **7**, **8**, **32** (also referred to as KGP05) and **33** (also referred to as KGP156) demonstrated potent cytotoxicity ( $GI_{50} = 0.11$  to 40 nM) across all evaluated cell lines, and they were strong inhibitors of tubulin polymerization

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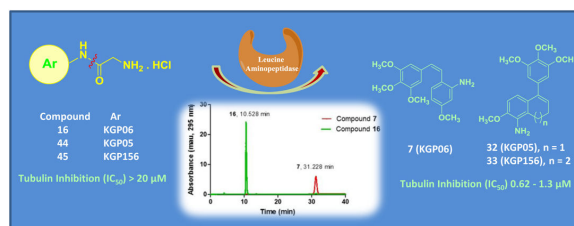
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Supplementary Data

Supplementary data including the synthesis of AVE8062, characterization data ( $^1H$  NMR,  $^{13}C$  NMR,  $^{31}P$  NMR, HPLC, HRMS) for final compounds and intermediates ( $^1H$  NMR,  $^{13}C$  NMR), HPLC chromatograms, calibration curves and rate studies related to LAP enzymatic assays, and aqueous solubility data for representative compounds associated with this article are available online.

( $IC_{50}$  = 0.62 to 1.5  $\mu$ M). The various prodrug conjugates and their corresponding salts were investigated for cleavage by the enzyme leucine aminopeptidase (LAP). Four of the glycine water-soluble AAPCs (**16**, **18**, **44** and **45**) showed quantitative cleavage by LAP, resulting in the release of the highly cytotoxic parent drug, whereas partial cleavage (<10-90%) was observed for other prodrugs (**15**, **17**, **24**, **38** and **39**). Eight of the nineteen AAPCs (**13-16**, **42-45**) showed significant cytotoxicity against selected human cancer cell lines. The previously reported CA1-diamine analogue and its corresponding hydrochloride salt (**8** and **10**, respectively) caused extensive disruption (at a concentration of 1.0  $\mu$ M) of human umbilical vein endothelial cells growing in a two-dimensional tubular network on matrigel. In addition, compound **10** exhibited pronounced reduction in bioluminescence (greater than 95% compared to saline control) in a tumor bearing (MDA-MB-231-luc) SCID mouse model 2 h post treatment (80 mg/kg), with similar results observed upon treatment (15 mg/kg) with the glycine amino-dihydronaphthalene AAPC (compound **44**). Collectively, these results support the further pre-clinical development of the most active members of this structurally diverse collection of water-soluble prodrugs as promising anticancer agents functioning through a mechanism involving vascular disruption.

## Abstract



## Keywords

Small-Molecule Synthesis; Inhibitors of Tubulin Polymerization; Vascular Disrupting Agents; Amino Acid Prodrug Salts; Anti-Cancer Agents; Combretastatin Analogues; Benzosuberene Analogues; Dihydronaphthalene Analogues

## 1. Introduction

Tumor vasculature is as an attractive target for the treatment of cancer due, in part, to its distinct characteristics, such as rapid and disorganized proliferation of endothelial cells.<sup>1-5</sup> Both small-molecules and biologics that specifically interact with tumor vasculature are referred to as vascular targeting agents (VTAs), which are further sub-classified as angiogenesis-inhibiting agents (AIAs) and vascular disrupting agents (VDAs).<sup>6,7</sup> AIAs are typically represented by compounds that target vascular endothelial growth factors to prevent the development of new tumor vasculature.<sup>7</sup> Conversely, VDAs are comprised of compounds that disrupt and directly damage established tumor vasculature, by affecting rapidly growing endothelial cells, to suppress tumor blood flow. One class of VDAs interacts with the colchicine (Fig. 1) site located on the  $\alpha$ , $\beta$ - heterodimer of tubulin, causing cell retraction, rounding, and ultimately detachment from the aggregated sheet of cells.<sup>1,4,8,9</sup>

The natural products combretastatin A-4 (CA4) and combretastatin A-1 (CA1) (Fig. 1), originally isolated by Pettit and co-workers (Arizona State University) from the South African bush-willow tree, *Combretum caffrum* Kuntze, are among the colchicine site class of tubulin binding VDAs.<sup>10–13</sup> This family of natural products, along with a number of synthetic derivatives and analogues,<sup>6,14–17</sup> significantly inhibit microtubule assembly in endothelial cells lining tumor-feeding vasculature, leading to a series of cell signaling events that ultimately result in endothelial cell morphology changes and blood flow reduction.<sup>1,7,17</sup> The inhibition of tubulin polymerization results in activation of RhoA, an intracellular coordinator of the cytoskeletal rearrangement of microtubules and actin, and leads to rapid vascular collapse.<sup>15,18,19</sup> Administration of these VDAs in amounts significantly lower than their maximum tolerated doses has resulted in tumor necrosis in treated laboratory mice.<sup>3,12</sup>

The combretastatin water-soluble phosphate prodrugs, combretastatin A-4 phosphate (CA4P also known as Zybrestat<sup>TM</sup>, Fig. 1) and combretastatin A-1 diphosphate (CA1P also known as OXi4503, Fig. 1) are among a group of VDAs which have demonstrated promising efficacy in human clinical trials.<sup>18–27</sup> They undergo enzyme-mediated dephosphorylation and, as their parent compounds (CA4 and CA1), they bind to tubulin and interfere with the tubulin-microtubule protein system. This causes pronounced morphological effects in the tumor vasculature.<sup>8,22–28</sup> Pre-clinical and pharmacokinetic evaluations of these combretastatin prodrugs in patients bearing advanced tumors indicate their efficacy as VDAs.<sup>22,29–31</sup> Interestingly, CA1P has dual mechanistic capability, functioning as both a VDA and as a cytotoxic agent based on its *in vivo* mediated conversion to a highly reactive orthoquinone.<sup>18,29–31</sup> The relative structural simplicity of the combretastatins has motivated synthetic chemists to develop libraries of structurally inspired analogues through alteration of the A-ring, the B-ring, and the ethylene bridge.<sup>6,15,16,32–35</sup>

Incorporation of the NH<sub>2</sub> substituent within either ring A or ring B in the combretastatin family resulted in new analogues that exhibited important biological activity.<sup>15,36,37</sup> In 2006, we reported the initial design and synthesis of the 2' CA4-amine (Fig. 1) and described its potent inhibition of tubulin assembly and its activity as a VDA.<sup>15</sup> Later work by others confirmed the potency of this 2' CA4-amine analogue and related compounds.<sup>34</sup> Subsequently, our studies showed that the di-amino variant of combretastatin A-1 (CA1-diamine, Fig. 1)<sup>16</sup> was strongly cytotoxic against human cancer cell lines (average GI<sub>50</sub> = 13.9 nM) and also demonstrated potent activity in regard to inhibition of tubulin assembly (IC<sub>50</sub> = 2.8 μM).<sup>16</sup> It is fairly common for compounds that interact with tubulin in the low μM range (cell free assay) to demonstrate nM cytotoxicity against human cancer cell lines; a variety of factors are postulated to influence this activity differential.<sup>38</sup> The trimethoxyphenyl moiety, the *p*-methoxyphenyl moiety, the *Z*-configuration of the two aryl rings, and the optimal 4–5 Å aryl-aryl distance all proved important for enhanced tubulin binding activity of the combretastatin analogues.<sup>17,39–41</sup> Inspired, in part, by the SAR studies associated with the combretastatins and their close structural analogues, we were the first to report the synthesis and biological activity of related dihydronaphthalene tubulin-binding agents [for example, OXi6196 and KGP05, Fig. 1],<sup>42–44</sup> followed by the discovery of a phenolic-based benzosuberene (KGP18, Fig. 1) analogue and its corresponding amino congener (KGP156, Fig. 1).<sup>40,45–48</sup> These compounds have emerged as potential pre-clinical

candidates due to their robust *in vitro* cytotoxicity (sub-nanomolar to picomolar GI<sub>50</sub> values) against selected human cancer cell lines and strong tubulin inhibitory activities.<sup>45–47</sup> Certain of these dihydronaphthalene analogues have subsequently been reported by another group.<sup>49</sup> In our previous work,<sup>46</sup> we also demonstrated robust tubule disruption [in a human umbilical vein endothelial cell (HUVEC) tube disruption assay] and cell rounding capability of KGP156, which is one of the parent compounds for several of the prodrugs designed and synthesized in this study.

The issue of limited water solubility associated with these aniline based anti-cancer agents can be addressed through prodrug strategies.<sup>50,51</sup> Amino acid prodrugs, like glycine and serine, with shorter hydrocarbon or polar side chains, are reported to be more readily water soluble and more likely to be cleaved because of their structural simplicity.<sup>50</sup> Another advantage of using amino acids is that they are capable of undergoing quantitative cleavage by hydrolytic enzymes, likely an aminopeptidase.<sup>51,52</sup> The synthesis and biological evaluation of a series of water-soluble amino acid prodrugs of amino-combretastatin were previously reported.<sup>15,35,53</sup> A water-soluble serinamide prodrug of 3' CA4-amine (Fig. 1) known as AVE8062 (Ombrabulin, synthesis<sup>36</sup> is described in Supplementary data), showed significant promise as a VDA in phase III human clinical trials<sup>54,55</sup> and enhanced antitumor activity and decreased toxicity (for normal cells) in both *in vitro* and *in vivo* models.<sup>36,53,55–57</sup> The parent drug is generated after amide bond cleavage by a hydrolytic enzyme.<sup>51,53</sup> Leucine aminopeptidase (LAP)<sup>58–60</sup> is one of a widely distributed group of aminopeptidases that exhibit broad specificity<sup>61–63</sup> and that catalyzes the hydrolysis of amino acids from the amino terminus of polypeptide chains.<sup>64</sup> There are also a number of literature reports of amino acid prodrugs cleaved by LAP.<sup>50,53</sup> In humans, LAP is found primarily in the cytosol of liver cells, which makes the serum leucine aminopeptidase a marker of hepatic disorders.<sup>65,66</sup> The increase of LAP in human sera can also be diagnostically indicative of a number of cancers such as carcinoma of the pancreas and head and neck cancer.<sup>67–69</sup>

Inspired by these developments, herein we report the synthesis of eight glycine and serine AAPCs of highly potent amino-bearing structural variants incorporated within the combretastatin, dihydronaphthalene, and benzosuberene molecular scaffolds.<sup>42,44,70,71</sup> Furthermore, in order to enhance water solubility (and potentially bioavailability)<sup>72</sup> of these newly synthesized AAPCs, their eleven hydrochloride salts were synthesized.<sup>51,53</sup> The synthesis of compounds **13**, **15**, **37**, **39**, 3' CA4-*L*-serinamide and AVE8062 were previously reported,<sup>15,36,47,53</sup> and they were re-synthesized as a part of our ongoing biological studies. Each of the parent amino-based anticancer agents and their corresponding AAPCs were evaluated for their cytotoxicity against selected human cancer cell lines and for their ability to inhibit tubulin polymerization. In addition, these amino acid prodrug hydrochloride salts and two selected serinamide prodrugs (non-salts) were evaluated for their ability to undergo amide bond cleavage by LAP.<sup>53</sup>

## 2. Results and Discussion

### 2.1 Synthesis

Twenty six compounds (including amino combretastatin, dihydronaphthalene and benzosuberene parent compounds, amino acid prodrugs, and their corresponding water soluble hydrochloride salts) were synthesized for this study. Among these, compounds **7-10**, **13**, **15**,<sup>15,16</sup> **32**,<sup>42,43</sup> **33**, **37**, **39**,<sup>46,47</sup> 3' CA4-amine, 3' CA4-L-serinamide and AVE8062<sup>36,53</sup> were re-synthesized for the purpose of further biological evaluation.

#### 2.1.1. Synthesis of Amino Acid Prodrug Conjugates of Amino Combretastatins

**1. Synthesis of Combretastatin Amines:** Our previous synthesis of 2' CA4-amine **7** involved a Wittig reaction between (4-methoxy-2-nitrobenzyl)triphenylphosphonium bromide and 3,4,5-trimethoxybenzaldehyde, followed by a reduction of the nitro group to form the corresponding amine using Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>.<sup>15,32</sup> Later, this compound was synthesized and reported by another group as well.<sup>34</sup> Switching the Wittig reaction partners to 3,4,5-trimethoxybenzyltriphenylphosphoniumbromide **4** and 4-methoxy-2-dinitrobenzaldehyde (scheme **1**), followed by the separation of *Z* and *E*-isomers (1:0.4 ratio) and reduction of *Z*-isomer **5a** to amine **6a** using zinc<sup>35</sup> in acetic acid (AcOH) increased the overall yield for compound **7** about 4-fold over two steps. The synthesis of CA1-diamine (**8**, Scheme **1**) was achieved as reported earlier,<sup>16</sup> involving a key Wittig reaction between 3,4,5-trimethoxybenzyltriphenylphosphonium bromide **4** and 4-methoxy-2,3-dinitrobenzaldehyde **1**, followed by reduction of the nitro group of *Z*-isomer **6a** to achieve the desired target compound (CA1-diamine **8**). Upon treatment with HCl (4 N), these combretastatin-based amines (**7** and **8**) were converted to their corresponding hydrochloride salts (**9** and **10**).

**2. Synthesis of Amino Acid Prodrug Conjugates:** The serine and glycine amino acid prodrug conjugates (**13** and **14**, Scheme **2**) of 2' CA4-amine **7** were prepared by coupling with Fmoc-*L*-ser(Ac)-OH and Boc-glycine-OH, respectively, in the presence of Et<sub>3</sub>N and the peptide coupling reagent propylphosphonic anhydride (T3P), followed by deprotection. This procedure is reminiscent of the synthetic strategy employed by Ohsumi and co-workers<sup>53</sup> for the synthesis of water-soluble amino acid prodrug salts of 3' CA4-amine<sup>56</sup> and our previous studies,<sup>15,16</sup> but this modified synthetic route has benefits in terms of higher yield and ease of purification from the use of T3P. Similarly, the serine and glycine amino acid prodrug conjugates (**19-21**, Scheme **3**) of CA1-diamine (**8**) were synthesized using a standard peptide coupling procedure (Scheme **2**).

**3. Synthesis of Hydrochloride Salts of Amino Acid Prodrug Conjugates:** The synthesis of compound **15** is described in our earlier publication.<sup>15</sup> Our initial efforts to re-synthesize compound **15** and compound **16** (new compound) at room temperature in the presence of solvent resulted in the complete isomerization from *Z* to *E*, affording compounds **17** and **18**. These *E*-isomers were used as a model system to evaluate LAP mediated cleavage of their corresponding amino acid prodrugs. Utilizing solvent free reaction conditions,<sup>73</sup> the respective HCl salts of glycine and serine amino acid prodrug conjugates (**15** and **16**, scheme **2**) were synthesized from compounds **12** and **13**. The water-soluble hydrochloride salt of the

glycine variant of CA1-diamine (**24**, scheme **3**) was obtained using a similar synthetic strategy with only about 10% isomerization to the *E*-isomer. Our initial attempts to prepare the hydrochloride salt of compound **22** were unsuccessful. However, treatment of compound **20** (scheme **3**) with NaOH (2 M) followed by HCl (4 N) yielded the desired CA1-diamine-based water-soluble amino acid prodrug conjugate (**23**) in a single step.

### 2.1.2. Synthesis of Amino Acid Prodrug Conjugates of Amino Dihydronaphthalene and Amino Benzosuberene

**1. Synthesis of Dihydronaphthalene and Benzosuberene Amines:** The synthesis of amino dihydronaphthalene (**32**)<sup>42,44</sup> and amino benzosuberene (**33**),<sup>45–47</sup> as previously reported by our group, is illustrated in Scheme **4**. Initially, 6-methoxy-1-tetralone was nitrated to form two constitutional isomers, with one isomer being the desired product, 5-nitro-6-methoxy-1-tetralone (**25**). Compound **32** was obtained by reaction of 5-bromo-1,2,3-trimethoxybenzene with *n*-butyllithium, followed by the addition of compound **25**. The reaction of compound **30** with Zn in the presence of AcOH resulted in reduction of the nitro group, and, following a subsequent condensation reaction, the desired product (**32**) was obtained in a 53% yield (over these two steps). The synthesis of amino-benzosuberene KGP156 (**33**), initially reported by us in 2012,<sup>46</sup> utilized a sequential Wittig olefination, selective reduction with 1,4-cyclohexadiene, Eaton's reagent mediated cyclization, 1,2-addition of the appropriately functionalized aryl ring, followed by condensation and reduction.

**2. Synthesis of Hydrochloride Salt of Amino Acid Prodrug Conjugates of Amino Dihydronaphthalene and Benzosuberene:** To obtain the desired amino acid prodrugs of amino dihydronaphthalene (**32**), Fmoc-*L*-ser(Ac)-OH or Fmoc-gly-OH were reacted utilizing general peptide synthetic methodology. Compound **32** was treated with T3P, Et<sub>3</sub>N, and the appropriate Fmoc-amino acid to obtain *N*-Fmoc protected amino acid amides (**34**) and (**40**), which upon deprotection resulted in the desired amino acid prodrugs (**36**) and (**42**). The AAPCs **37** and **39** (previously reported) were re-synthesized for this study.<sup>47</sup> Similarly, the hydrochloride salts (**38**, **44**, and **45**) of amino acids (**36**, **42**, and **43**, respectively), were obtained upon treatment with a 4 N HCl in dioxane solution as shown in Scheme **5**.

## 2.2. Biological Evaluation

Each of the twenty six compounds synthesized for this study (including parent drugs, amino acid prodrugs, and their corresponding water soluble hydrochloride salts; compiled in Fig. **2**) were evaluated for their cytotoxicity against selected human cancer cell lines [SK-OV-3 (ovarian), NCI-H460 (lung), and DU-145 (prostate), Table 1]. Furthermore, these compounds were evaluated biologically for their ability to inhibit tubulin polymerization (cell free assay) and colchicine binding (Table 2). In addition, thirteen water soluble AAPCs (including two as their non-salt forms) were evaluated for enzymatic cleavage by LAP (Table **3** and Fig. **3**).<sup>37, 42–47</sup> An endothelial tube disruption assay for CA1-diamine (compound **8**, Fig. **2**) was carried out utilizing HUVECs (Fig. **4**). The hydrochloride salts of CA1-diamine (compound **10**, Fig. **5**) and dihydronaphthalene glycinamide (compound **44**, Fig. **2**) were initially evaluated for their *in vivo* ability to disrupt tumor blood flow utilizing dynamic bioluminescence imaging (BLI, Fig. **6** and **7**) studies.

**2.2.1. Cytotoxicity, Inhibition of Tubulin Polymerization, and Percent Inhibition of Colchicine Binding**—Cytotoxicity studies carried out with all twenty six compounds against selected human cancer cell lines [SK-OV-3 (ovarian), NCI-H460 (lung), and DU-145 (prostate), Table 1] indicated that each of the parent drugs (compounds 7,<sup>15</sup> 8,<sup>16</sup> 32<sup>42,43</sup> and 33<sup>46,47</sup>) were highly potent with GI<sub>50</sub> values ranging from sub-nanomolar to nanomolar. The amino dihydronaphthalene 32 was found to be especially potent (GI<sub>50</sub> = 1-3 nM across all three of the human cancer cell lines evaluated in this study) and was comparable to the clinically relevant agent CA4.<sup>35</sup> Both the serinamide and glycinamide AAPCs (compounds 15 and 16, respectively) of the 2' CA4-amine 7 demonstrated significant cytotoxicity against each of the cancer cell lines. When comparing the *E*-isomers 17 and 18, the glycinamide AAPC 18 was more cytotoxic than the corresponding serinamide AAPC 17. Negligible cytotoxicity was observed for the *bis*-substituted AAPCs (22-24). The serinamide AAPCs (36-39) of the parent compounds 32 (KGP05) and 33 (KGP156) demonstrated limited cytotoxicity against these cancer cell lines, while the glycinamide analogues (42-45) were more active.

Pronounced inhibition of tubulin polymerization and inhibition of colchicine binding were observed for amino-dihydronaphthalene 32 (assembly IC<sub>50</sub> = 0.62 μM and 92% inhibition of colchicine binding at 5 μM, respectively, Table 2). Similarly, the other parent compounds 7, 8 and 33 proved highly potent in these tubulin assays (ranging from 1.1 μM to 1.5 μM and 76% to 88% at 5 μM, respectively). Uniformly, the AAPCs (compounds 13-18, 22-24, 36-39 and 42-45) proved to be inactive (IC<sub>50</sub> > 20 μM) as inhibitors of tubulin assembly, which was anticipated in this type of cell free assay.

**2.2.2. Enzymatic Assay**—Preliminary studies were carried out to determine the ability of LAP to cleave individual amino acid prodrug conjugates (Table 3). The mono-glycinamide prodrugs 16, 18, 44, 45 were quantitatively cleaved to their parent compounds. Treatment of the *bis*-glycinamide prodrug (24) with LAP (0.12 units) resulted in disappearance of greater than 95% of the prodrug by 25 h, but only 9% of the parent CA1-diamine (compound 8) was formed. Two additional peaks, presumably the mono-glycinamide intermediates, were observed in the HPLC chromatogram. Using 1.5 and 3.7 units of LAP, the amount of CA1 diamine (compound 8) detected increased to 19% and 24% respectively. The mono-serinamide prodrugs of both the *Z* and *E*-isomers (15 and 17, respectively) of 2' CA4-amine were effectively cleaved by LAP, as was the mono-serinamide derivative of 3' CA4-amine and its hydrochloride salt (AVE8062), used as positive controls.<sup>53</sup> Only a partial release (less than 10%) was achieved with excess LAP (3.6 units, 24 h) for the mono-serinamide analogues of the amino dihydronaphthalene 38 and amino benzosuberene 39 compounds. Treatment of the *bis*-serinamide prodrug 22 of CA1-diamine or its dihydrochloride salt 23 with a large amount (1.5 units) of LAP did not result in any observable cleavage. For AAPCs that produced more than 50% of the final product upon cleavage with LAP in preliminary experiments, rate studies were conducted. The glycinamide prodrug of the *Z*-isomer 2' CA4-amine 16 was cleaved at a rate of 180 μM/min/enzyme unit (Fig. 3. A, B), 150 times faster than its corresponding serinamide analogue 15 (1.2 μM/min/enzyme unit). A similar result was observed for the glycinamide prodrug of the *E*-isomer 2' CA4-amine 18, which was cleaved at a rate more than 100 times faster than its serinamide analogue 17, as

determined by the relative rates of cleavage of each prodrug. Differences were also found in the rate of cleavage of the glycinamide prodrugs of amino dihydronaphthalene **44** (15.5  $\mu\text{M}/\text{min}/\text{enzyme unit}$ , Fig. 3. C, D) and amino benzosuberene **45** (0.12  $\mu\text{M}/\text{min}/\text{enzyme unit}$ ).

Despite a significantly faster cleavage of the glycinamide prodrug **16** versus the serinamide **15** by LAP, the cytotoxicity toward cancer cell lines over a 48 h treatment was very similar for both prodrugs and the parent *Z*-isomer 2' CA4-amine **7** (Table 1), indicating that release of compound **7** from both prodrugs in the presence of these cancer cell lines was efficient. The slower rate of cleavage for the *E*-isomer series resulted in a differential cytotoxicity for the serinamide **17** and glycinamide **18** prodrugs. The lack of cleavage by LAP of the *bis*-serinamide prodrugs **22** and **23** and the very low production of parent CA1-diamine (compound **8**) from the *bis*-glycinamide **24** was reflected in the lack of cytotoxicity for all three compounds (**22-24**). This may indicate steric hindrance in the interaction of these compounds with LAP. There is a marked difference in the LAP cleavage rates between glycinamide prodrugs (compounds **44** and **45**) that was reflected in their differential cytotoxicity in cancer cell lines, and their cytotoxicity was significantly less than that of either of the corresponding parent compounds (KGP05, KGP156, respectively).

Overall, the cancer cell line cytotoxicity mirrored the cleavage by LAP of the amino acid prodrug conjugates. In amino acid amides and peptides, an *N*-terminal L-serine residue is preferred over glycine, but there is clearly a contribution of binding interactions, steric factors and stereospecificity (L vs. D) in the position adjacent to the *N*-terminal amino acid that is observed in peptide substrates and in some non-peptidic amino acid conjugates.<sup>75,76</sup> For example, Pettit et al. reported that the serinamide prodrug of 3' CA2-amine displayed significantly less activity compared to the glycine derivative in a cancer cell line panel.<sup>35</sup>

**2.2.3. Endothelial Tube Disruption Assay**<sup>77</sup>—Rapidly growing HUVECs can be induced to form a capillary-like network of tubules, which serves as a model for evaluating VDAs. CA1-diamine (compound **8**) and its hydrochloride salt (compound **10**) demonstrated significant disruption of a capillary-like network of tubules (from HUVECs) at a concentration of 0.1  $\mu\text{M}$ . Our previous work shows a significant tubule disruption and cell rounding effect for compound **33** (KGP156) at 0.1  $\mu\text{M}$ , and this effect was greatly enhanced at a 1  $\mu\text{M}$  concentration.<sup>46</sup>

**2.2.4. Dynamic Bioluminescence Imaging**<sup>77</sup>—Bioluminescence Imaging (BLI) represents a valuable indirect *in vivo* technique for the assessment of tumor-specific vascular damage in response to treatment with VDAs.<sup>9,78,79</sup> A decrease in light flux emission at various time points post VDA administration presumably results from an inability of the injected luciferin to reach the tumor (grown from luciferase expressing transfected cancer cells) due to VDA-induced damage of the vasculature feeding the tumor. Treatment (Fig. 5) of an MDA-MB-231-luc human breast tumor induced in a SCID mouse model with the diamino-CA1 salt (compound **10**) at a dose of 80 mg/kg resulted in approximately 95% reduction in light emission [at 17 min time-point (midpoint)] at 2 h post VDA treatment relative to baseline. This reduction was still maintained (94% decrease) at the 4 h time point (fresh luciferin injection but no further VDA). There was some recovery (79% reduction,



compared to baseline) at 24 h, which is typical due to the so-called viable rim.<sup>9</sup> The glycinamide dihydronaphthalene AAPC (**44**) demonstrated (Fig. 6) similarly promising results (97% and 90% decrease in BLI signal at 4 and 24 h, respectively) when administered at a dose of 15 mg/kg, while the signal reduction at a dose of 10 mg/kg was 70% at 4 h and 55% at 24 h. In this experiment, the control mouse (saline administration) demonstrated a 22% decrease in signal at 4 h and 24% reduction at 24 h. These results, which are comparable to the clinically relevant VDA, CA4P (Fig. 7), provide further preliminary evidence suggesting that compounds **10** and **44** function as water-soluble VDAs and that glycinamide **44** is capable of being converted in vivo (presumably through the action of LAP) to its corresponding tubulin-active parent compound KGP05 (compound **32**), which then causes the tumor-specific vascular damage.

### 3. Conclusions

Eleven water-soluble amino acid prodrug conjugates (AAPCs) derived from parent amino combretastatin, dihydronaphthalene, and benzosuberene derivatives (**7**, **8**, **32** and **33**) were designed and synthesized. Each of the parent compounds (**7**, **8**, **32** and **33**) were potent inhibitors of tubulin assembly binding to the colchicine site and were strongly cytotoxic against human cancer cell lines [SK-OV-3 (ovarian), NCI-H460 (lung), and DU-145 (prostate)]. Without exception, the glycinamide AAPCs based on the dihydronaphthalene and benzosuberene molecular scaffolds demonstrated efficient cleavage by LAP and were superior in this regard to their serinamide counterparts. Cytotoxicity mirrored the relative cleavage of these agents by LAP. The story was slightly different for the AAPCs derived from the combretastatins in that both the glycinamide and serinamide AAPCs (**15-18**) were efficiently cleaved by LAP for both the 2' and 3' amino variants, but the diamine-CA1 derived glycinamide (**24**) and serinamide (**22** and **23**) were resistant to LAP mediated cleavage to regenerate the parent compound (**8**). CA1-diamine (**8**) and its corresponding hydrochloride salt (**10**) caused significant disruption to a network of HUVECs growing on matrigel, and both CA1-diamine salt **10** and dihydronaphthalene glycinamide **44** demonstrated a significant reduction in light emission in a BLI study in SCID mice bearing the luciferase expressing MDA-MB-231-luc human breast cancer cell line induced tumor. These results demonstrate that these compounds can function as water-soluble VDAs.

## 4. Experimental Section

### 4.1. Chemistry

**General Materials and Methods**—AcOH, acetic anhydride, acetonitrile, CH<sub>2</sub>Cl<sub>2</sub>, dimethylformamide (DMF), ethanol, methanol, HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, and tetrahydrofuran (THF) were used in their anhydrous forms or as obtained from the chemical suppliers. Reactions were performed under N<sub>2</sub>. Thin-layer chromatography (TLC) plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used to monitor reactions. Water was used to quench and wash the reaction mixture as appropriate. Purification of intermediates and products was carried out with a Biotage Isolera flash purification system using silica gel (200–400 mesh, 60 Å) or RP-18 pre-packed columns or manually in glass columns. Intermediates and products synthesized during this study were characterized on the basis of

their  $^1\text{H}$  NMR (600 or 500 MHz),  $^{13}\text{C}$  NMR (150, 125 or 90 MHz) and  $^{31}\text{P}$  NMR (240 MHz) spectroscopic data using a Varian VNMRS 500 MHz or a Bruker DRX 600 MHz or a Bruker DPX 360 MHz instrument. Melting point ranges were determined (single experiment) using a Thomas Hoover capillary melting point apparatus and are uncorrected. Spectral data were recorded in  $\text{CDCl}_3$ ,  $\text{D}_2\text{O}$ ,  $(\text{CD}_3)_2\text{CO}$ , DMSO- $d_6$ , or  $\text{CD}_3\text{OD}$ . All chemical shifts are expressed in ppm ( $\delta$ ), coupling constants ( $J$ ) are presented in Hz, and peak patterns are reported as broad singlet (bs), singlet (s), doublet (d), triplet (t), quartet (q), pentet (p), double doublet (dd), triplet of doublets (td), doublet of triplets (dt) and multiplet (m).

Purity of the final compounds was further analyzed at 25 °C using an Agilent 1200 HPLC system with a diode-array detector ( $\lambda = 190\text{--}400$  nm), a Zorbax XDB-18 HPL column (4.6 mm  $\times$  150 mm, 5  $\mu\text{m}$ ), and a Zorbax reliance cartridge guard-column; method A: solvent A, acetonitrile, solvent B, 0.1% TFA in  $\text{H}_2\text{O}$ ; or method B: solvent A, acetonitrile, solvent B,  $\text{H}_2\text{O}$ ; gradient, 10% A/90% B to 100% A/0% B over 0 to 40 min; post-time 10 min; or method C: solvent A, acetonitrile, solvent B, 0.1% TFA in  $\text{H}_2\text{O}$ ; isocratic, 10% A/90% B over 0 to 5 min, 10% A/90% B to 100% A/0% B over 5 to 25 min; post-time 5 min, flow rate 1.0 mL/min; injection volume 20  $\mu\text{L}$ ; monitored at wavelengths of 210, 254, 230, 280, and 360 nm. Mass spectrometry was carried out under positive or negative electrospray ionization (ESI) using a Thermo Scientific LTQ Orbitrap Discovery instrument. Compound numbering for the combretastatin molecular scaffold followed this protocol: the trimethoxy A ring was numbered 1-6, and the B ring was numbered 1'-6'. The ethylene bridging atoms were numbered as 1a and 1a' respectively for the carbons connected to the A ring and B ring, respectively.

## Synthesis of Amino Acid Prodrug Conjugates of Amino Combretastatins

**4.1.1. Nitration of aldehyde<sup>16</sup>:** 4-Methoxy-2-nitrobenzaldehyde (1.53 g, 8.45 mmol) was dissolved in concentrated  $\text{H}_2\text{SO}_4$  (25 mL). To this solution, a pre-cooled mixture of  $\text{HNO}_3$  and  $\text{H}_2\text{SO}_4$  (3.85 mL/2.85 mL) was added dropwise. The reaction mixture was stirred for 10 min, and then the resulting solution was added dropwise into ice-water (100 mL). After stirring for 2 h at 0 °C, the solution was filtered, and the solid containing the desired product was rinsed with ice-water (10 mL). Purification by flash column chromatography (40% EtOAc/hexanes) yielded regio-isomers **1** and **2**.

**4-methoxy-2,3-dinitrobenzaldehyde (1)<sup>16</sup>:** This compound was isolated as a yellow solid (0.879 g, 3.89 mmol, 46%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  9.95 (1H, s), 8.15 (1H, d,  $J = 8.9$  Hz), 7.40 (1H, d,  $J = 8.9$  Hz), 4.09 (3H, s),  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  184.05, 155.69, 133.11, 128.96, 121.04, 115.92, 114.53, 57.88.

**4-methoxy-2,5-dinitrobenzaldehyde (2)<sup>16</sup>:** This compound was isolated as a colorless solid (0.725 g, 3.20 mmol, 38%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  10.30 (1H, s), 8.43 (1H, s), 7.74 (1H, s), 4.15 (3H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  184.63, 155.99, 127.12, 123.78, 123.04, 109.63, 57.89.

#### 4.1.2. Synthesis of Wittig salt<sup>80</sup>

**3,4,5-Trimethoxybenzyl bromide (3)<sup>80</sup>:** The mixture of 3,4,5-trimethoxybenzyl alcohol (20.1 g, 101 mmol) and PBr<sub>3</sub> (4.80 mL, 50.7 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (100 mL) was stirred for 1 h at 0 °C. Water (10 mL) was added, and the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 × 100 mL). The combined organic phase was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and removed by evaporation under reduced pressure. After the recrystallization of the crude solid with EtOAc/hexanes, the bromide **3** (23.6 g, 90.3 mmol, 89%) was obtained as an off-white solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 6.62 (2H, s), 4.47 (2H, s), 3.87 (6H, s), 3.85 (3H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 153.3, 138.2, 133.2, 106.1, 60.9, 56.1, 34.3.

**3,4,5-Trimethoxybenzyltriphenylphosphonium bromide (4)<sup>80</sup>:** A mixture of bromide **3** (11.0 g, 42.1 mmol) and PPh<sub>3</sub> (12.1 g, 46.3 mmol) in anhydrous acetone (100 mL) was stirred for 5 h. The resulting suspension was filtered through a Buchner funnel, and the solid was washed with acetone (100 mL) followed by hexanes (50 mL) to afford an off-white solid. The solid was dried in vacuo to obtain the phosphonium salt **4** (20.3 g, 38.2 mmol, 92%) as a colorless solid. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 7.74 – 7.64 (9H, m), 7.58 – 7.50 (6H, m), 6.43 (2H, d, *J*=2.6 Hz), 5.29 (2H, d, *J*=14.1 Hz), 3.70 (3H, d, *J*=3.4 Hz), 3.43 (6H, d, *J*=3.7 Hz). <sup>13</sup>C

NMR (150 MHz, CDCl<sub>3</sub>): δ 152.91 (d, *J*=3.6 Hz), 137.49 (d, *J*=3.7 Hz), 134.84 (d, *J*=2.9 Hz), 134.58 (d, *J*=9.8 Hz), 129.99 (d, *J*=12.5 Hz), 122.44 (d, *J*=8.9 Hz), 117.76 (d, *J*=85.7 Hz), 108.76 (d, *J*=5.2 Hz), 60.83 (d, *J*=2.3 Hz), 56.16 (s), 30.72 (d, *J*=46.7 Hz). <sup>31</sup>P NMR (240 MHz, CDCl<sub>3</sub>): δ 23.2.

**4.1.3. Procedure for the synthesis of *Z*- and *E*-stilbenes (5a and 5b).<sup>15,16</sup>:** At 0 °C, a NaH suspension (0.478 g, 19.9 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was stirred for 10 min. The previously prepared solution of 3,4,5-trimethoxybenzylphosphonium bromide (1.73 g, 3.30 mmol) was added dropwise. After stirring for 20 min, 4-methoxy-2-nitrobenzaldehyde (0.501 g, 2.76 mmol) was added. The resulting mixture was stirred for 5 h. At this point, ice-water was added carefully until H<sub>2</sub> evolution stopped. Workup of the reaction mixture was carried out by extraction with CH<sub>2</sub>Cl<sub>2</sub> (2 × 25 mL), followed by washing the combined organic layers with water (twice) and brine and finally drying over Na<sub>2</sub>SO<sub>4</sub>. The *Z*- and *E*-isomers were isolated after flash column chromatography (40% EtOAc/hexanes) and recrystallization of the *Z* and *E*-isomer mixture from EtOAc and hexanes.

**(*Z*)-2-(4'-Methoxy-2'-nitrophenyl)-1-(3,4,5-trimethoxyphenyl)ethene (5a):** This compound was isolated as a yellow solid (0.690 g, 1.99 mmol, 72%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.59 (1H, d, *J*=2.7 Hz), 7.24 (1H, d, *J*=8.6 Hz), 7.01 (1H, dd, *J*=8.6, 2.6 Hz), 6.80 (1H, d, *J*=11.9 Hz), 6.62 (1H, d, *J*=11.9 Hz), 6.29 (2H, s), 3.87 (3H, s), 3.81 (3H, s), 3.62 (6H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 159.0, 152.9, 148.6, 137.4, 133.4, 131.5, 131.2, 125.9, 125.7, 120.0, 108.7, 106.3, 60.9, 55.9, 55.8.

**(*E*)-1,2,3-Trimethoxy-5-(4-methoxy-2-nitrostyryl)benzene (5b):** This compound was isolated as an orange solid (0.239 g, 0.692 mmol, 25%). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 600 MHz): δ

7.67 (1H, d,  $J=8.8$  Hz), 7.47 (1H, d,  $J=2.7$  Hz), 7.44 (1H, d,  $J=16.0$  Hz), 7.16 (1H, dd,  $J=8.7$ , 2.7 Hz), 6.92 (1H, d,  $J=16.0$  Hz), 6.74 (2H, s), 3.91 (6H, s), 3.90 (3H, s), 3.87 (3H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ):  $\delta$  159.1, 153.4, 148.3, 138.4, 132.5, 132.2, 129.1, 125.5, 122.8, 120.4, 108.9, 103.9, 61.0, 56.2, 55.9.

**4.1.4. Procedure for the synthesis of *Z*- and *E*-stilbenes (6a and 6b).<sup>16</sup>** These compounds were synthesized using the procedure as described for compounds **5a** and **5b**. Starting from NaH (1.57g, 65.4 mmol), 3,4,5-trimethoxybenzylphosphonium bromide (5.71 g, 1.09 mmol), and 4-methoxy-2,3-dinitrobenzaldehyde (2.24 g, 9.90 mmol) in  $\text{CH}_2\text{Cl}_2$  (150 mL), the desired *Z* and *E*-isomers were isolated after flash column chromatography (50% EtOAc/hexanes).

**(*Z*)-2-(4'-Methoxy-2',3'-dinitrophenyl)-1-(3,4,5-trimethoxyphenyl)ethane (6a):** This compound was isolated as a yellow solid (0.818 g, 2.09 mmol, 52%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.35 (1H, d,  $J=8.9$  Hz), 7.08 (1H, d,  $J=8.9$  Hz), 6.77 (1H, d,  $J=11.8$  Hz), 6.50 (1H, d,  $J=11.8$  Hz), 6.30 (2H, s), 3.95 (3H, s), 3.83 (3H, s), 3.69 (6H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ):  $\delta$  153.1, 150.9, 143.0, 137.9, 135.2, 134.5, 134.3, 130.7, 124.5, 121.6, 115.9, 106.0, 60.9, 57.4, 56.0.

**(*E*)-2-(4'-Methoxy-2',3'-dinitrophenyl)-1-(3,4,5-trimethoxyphenyl)ethane (6b):** This compound was isolated as an orange solid (0.630 g, 1.61 mmol, 40%).  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.84 (1H, d,  $J=9.0$  Hz), 7.25 (1H, d,  $J=9$  Hz), 6.96 (2H, dd,  $J=38.2$ , 16.0 Hz), 6.69 (2H, s), 4.01 (3H, s), 3.91 (6H, s), 3.88 (3H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  153.7, 151.0, 142.3, 139.3, 135.0, 134.6, 131.5, 130.0, 124.3, 118.8, 116.3, 104.4, 61.1, 57.5, 56.4.

#### 4.1.5. eneral procedure for the reduction of nitro groups to amines<sup>15,16</sup>

**(*Z*)-2-(2'-amino-4'-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)ethene (7)<sup>15</sup>:** Zinc (8.36 g, 128 mmol) was added slowly to a well stirred solution of *Z*-stilbene (0.884 g, 2.56 mmol) in glacial AcOH (75 mL). The resulting suspension was stirred for 3 h at room temperature. At this point, the reaction mixture was filtered through Celite<sup>®</sup>, and the Celite<sup>®</sup> was washed with ethyl acetate. The filtrate was concentrated under reduced pressure. The desired amine was purified by flash column chromatography using a pre-packed 50 g silica column [solvent A: EtOAc; solvent B: hexanes; gradient: 20%A / 80%B (1 CV), 20%A / 80%B  $\rightarrow$  75%A / 25%B (10 CV), 75%A / 25%B (2 CV); flow rate: 40 mL/min; monitored at 254 and 280 nm] to afford the amine **7** (0.615 g, 1.95 mmol, 76%, melting point range 95-98 °C) as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.04 (1H, d,  $J=8.4$  Hz), 6.52 (2H, s), 6.51 (1H, d,  $J=12$  Hz), 6.43 (1H, d,  $J=11.9$  Hz), 6.30 (1H, dd,  $J=8.4$ , 2.5 Hz), 6.26 (1H, d,  $J=2.5$  Hz), 3.81 (3H, s), 3.76 (3H, s), 3.74 (2H, b), 3.65 (6H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.1, 152.7, 144.9, 137.3, 132.3, 131.0, 130.6, 125.7, 116.1, 105.8, 104.3, 100.7, 60.8, 55.8, 55.2. HRMS:  $m/z$ . obsd 316.1544  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{18}\text{H}_{22}\text{NO}_4^+$ , 316.1543. HPLC (Method B): 13.153 min.

**(*Z*)-2-(2',3'-Diamino-4'-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)ethene (8)<sup>16</sup>:** This compound was synthesized using the procedure as described for compound **7**. Starting from zinc (13.4 g, 205 mmol) and *Z*-stilbene (0.797 g, 2.04 mmol) in glacial AcOH (72 mL), the

desired product **8** (0.403 g, 1.22 mmol, 60%) was obtained as a brown oil.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  6.66 (1H, d,  $J=8.4$  Hz), 6.52 (1H, d,  $J=12$  Hz), 6.50 (1H, d,  $J=12$  Hz), 6.48 (2H, s), 6.39 (1H, d,  $J=8.4$  Hz), 3.83 (3H, s), 3.81 (3H, s), 3.62 (6H, s), 3.49 (4H, bs).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  152.68, 147.72, 137.31, 133.00, 132.22, 131.26, 126.14, 123.31, 119.53, 117.99, 105.90, 102.23, 60.84, 55.82, 55.73. HRMS:  $m/z$ : obsd 331.1656  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{18}\text{H}_{23}\text{N}_2\text{O}_4^+$ , 331.1658. HPLC (Method B): 11.603 min.

#### 4.1.6. Preparation of hydrochloride salts of amino combretastatin analogues<sup>15,16</sup>

*(Z)-2-(2'-Methoxy-4'-aminophenyl)-1-(3,4,5-trimethoxyphenyl)ethene hydrochloride (9)*<sup>15</sup>: To a solution of 2' CA4-amine **7** (0.0550 g, 0.175 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (8 mL) was added a HCl solution (4 N in dioxane, 3 equiv.) at 0 °C. The reaction was monitored by TLC and stirred for 12 h. After evaporating the solvents under reduced pressure, the final hydrochloride salt **9** (0.0440 g, 0.125 mmol, 72%, melting point range 143-146 °C) was obtained as a green solid after recrystallization with ethanol/diethyl ether solution.  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.27 (1H, d,  $J=8.3$  Hz), 6.97 – 6.91 (2H, m), 6.78 (1H, d,  $J=11.9$  Hz), 6.55 (1H, d,  $J=11.8$  Hz), 6.46 (2H, s), 3.83 (3H, s), 3.71 (3H, s), 3.60 (6H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  160.11, 152.83, 152.82, 133.86, 132.04, 131.52, 130.51, 123.86, 122.08, 113.51, 108.42, 106.21, 59.66, 54.89, 54.83. HRMS:  $m/z$ : obsd 316.1544  $[\text{M}-\text{Cl}]^+$ , calcd for  $\text{C}_{18}\text{H}_{22}\text{NO}_4^+$ , 316.1543. HPLC (Method B): 13.02 min.

*(Z)-2-(2',3'-Diamine hydrochloride-4'-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)ethene (10)*: To a solution of CA1-diamine **8** (0.320 g, 0.970 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (50 mL) was added a HCl solution (4 N in dioxane, 5 equiv.), and the reaction mixture was stirred for 5 h. The resulting crystals were filtered and re-crystallized from anhydrous methanol at -20 °C. The brown crystals thus obtained were washed with dry  $\text{CH}_2\text{Cl}_2$  to obtain the salt **10** (0.130 g, 0.320 mmol, 30%, melting point range 184-186 °C).  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  6.98 (1H, d,  $J=8.5$  Hz), 6.60 (1H, d,  $J=11.9$  Hz), 6.47 (1H, d,  $J=8.6$  Hz), 6.42 (2H, s), 6.37 (1H, d,  $J=11.8$  Hz), 3.83 (3H, s), 3.62 (3H, s), 3.52 (6H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  152.7, 152.3, 137.1, 137.0, 132.6, 132.2, 129.2, 124.0, 119.0, 107.4, 106.0, 101.4, 59.7, 55.4, 54.9. HRMS:  $m/z$ : obsd 353.1476  $[\text{M}-2\text{HCl}+\text{Na}]^+$ , calcd for  $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}_4\text{Na}^+$ , 353.1472. HPLC (Method C): 13.06 min.

#### 4.1.7. Preparation of amino acid prodrug conjugates of 2' combretastatin A-4 amines<sup>15,53</sup>

*(S,Z)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-((5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)amino)-3-oxopropyl acetate (11)*<sup>15</sup>: To a solution of 2' CA4-amine **7** (0.220 g, 0.700 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (30 mL) was added  $\text{Et}_3\text{N}$  (0.110 mL, 0.760 mmol), peptide coupling reagent T3P (50% in EtOAc, 0.830 mL, 1.40 mmol) and Fmoc-*L*-ser(Ac)-OH (0.284 g, 0.760 mmol). The reaction mixture was stirred for 8 h at room temperature.  $\text{H}_2\text{O}$  (20 mL) was added, and the reaction mixture was extracted with  $\text{CH}_2\text{Cl}_2$ . The organic extract was washed with brine, dried over  $\text{Na}_2\text{SO}_4$  and concentrated under reduced pressure, and the residue was purified using flash column chromatography (50% EtOAc/hexanes) to afford the desired Fmoc-*L*-serinamide acetate **11** (0.388 g, 0.580 mmol, 83%) as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.23 (1H, b), 7.93 (2H, d,  $J=7.5$ ),

7.60 (2H, d,  $J = 7.5$ ), 7.60 (2H, d,  $J = 7.5$ ), 7.40 (2H, dd,  $J = 7.5, 7.0$ ), 7.33 (2H, dd,  $J = 7.5, 7.0$ ), 6.98 (1H, d,  $J = 8.5$ ), 6.65 (1H, d,  $J = 9.0$ ), 6.56 (1H, s), 6.00 (1H, t,  $J = 4.5$ ), 5.75 (1H, d,  $J = 6.5$ ), 4.47 (4H, m), 4.23 (1H, d,  $J = 11.5$ ), 3.89 (3H, s), 3.84 (6H, s), 3.79 (3H, s), 2.67 (2H, t,  $J = 7.5$ ), 2.30 (2H, td,  $J = 7.5, 4.5$ ), 2.11 (3H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.2, 166.6, 159.8, 153.2, 143.9, 141.7, 138.1, 135.3, 133.2, 131.5, 130.2, 128.2, 127.5, 127.5, 125.2, 125.1, 124.2, 120.4, 120.4, 111.7, 105.8, 105.8, 67.2, 63.8, 61.1, 56.43, 56.0, 55.8, 47.4, 20.8.

**(S,Z)-2-amino-3-hydroxy-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)propanamide (13)**: Fmoc-*L*-serinamide acetate **11** (0.290 g, 0.435 mmol) was dissolved in a  $\text{CH}_2\text{Cl}_2$ /MeOH mixture (10 mL, 1:1 ratio), a NaOH solution (2 M, 0.650 mL, 1.30 mmol) was added, and the mixture was stirred for 30 min. After the evaporation of solvent, the resulting oil was purified by normal phase preparative TLC (95%  $\text{CH}_2\text{Cl}_2$ /MeOH) to obtain 2' CA4-*L*-serinamide **13** (0.125 g, 0.311 mmol, 71%) as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  9.65 (1H, s), 8.04 (1H, d,  $J = 2.6$  Hz), 7.16 (1H, dd,  $J = 8.5, 0.9$  Hz), 6.69 (1H, dd,  $J = 8.5, 2.6$  Hz), 6.61 (1H, d,  $J = 12.0$  Hz), 6.50 (1H, d,  $J = 12.0$  Hz), 6.42 (2H, s), 3.82 (3H, s), 3.80 (3H, s), 3.76 (1H, dd,  $J = 10.8, 5.2$  Hz), 3.61 (7H, s), 3.37 (1H, t,  $J = 5.4$  Hz).  $^{13}\text{C}$  NMR (90 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.7, 159.5, 152.8, 137.9, 135.8, 132.6, 131.8, 130.1, 124.3, 120.1, 110.8, 106.1, 105.6, 64.8, 60.8, 56.8, 55.9, 55.4. HRMS:  $m/z$ : obsd 403.1866  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{21}\text{H}_{27}\text{N}_2\text{O}_6^+$ , 403.1864. HPLC (Method A): 12.97 min.

**(S,E)-2-amino-3-hydroxy-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)propanamide hydrochloride (17)**<sup>53</sup>: To a well stirred solution of 2' CA4-*L*-serinamide **13** (0.0950 g, 0.236 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 mL) was added a HCl solution (4 N in dioxane, 0.8 mmol) at room temperature. After completion of the reaction (monitored by TLC), diethyl ether (20 mL) was added to the reaction mixture, producing a colorless solid that was recovered by filtration through a membrane filter. After the solid was washed with diethyl ether (10 mL), the water soluble 2' CA4-*L*-serinamide **17** (0.0780 g, 0.178 mmol, 75%, melting point range 208-212 °C) was obtained as a colorless solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.72 (1H, d,  $J = 8.8$  Hz), 7.23 (1H, d,  $J = 16.2$  Hz), 7.09 (1H, d,  $J = 2.6$  Hz), 7.01 (1H, d,  $J = 16.1$  Hz), 6.92 (1H, dd,  $J = 8.7, 2.6$  Hz), 6.89 (2H, s), 4.26 (1H, t,  $J = 5.2$  Hz), 4.12 (2H, d,  $J = 5.2$  Hz), 3.91 (6H, s), 3.84 (3H, s), 3.79 (3H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  166.2, 159.5, 153.2, 137.4, 134.5, 133.9, 128.6, 126.5, 124.9, 122.4, 112.8, 111.0, 103.5, 60.5, 59.8, 55.3, 55.1, 54.5. HRMS:  $m/z$ : obsd 403.1873  $[\text{M}-\text{Cl}]^+$ , calcd for  $\text{C}_{21}\text{H}_{27}\text{N}_2\text{O}_6^+$  403.1864. HPLC (Method C): 13.23 min.

**(S,Z)-2-amino-3-hydroxy-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)propanamide hydrochloride (15)**: To 2' CA4-*L*-serinamide **13** (0.0500 g, 0.124 mmol) was added a HCl solution (4 N in dioxane, 0.36 mmol) at 0 °C. The resulting reaction mixture was stirred for 3 h, followed by evaporation of the solvent under reduced pressure at 30 °C. The resulting green oil was washed with anhydrous diethyl ether to furnish the water soluble HCl salt of 2' CA4-*L*-serinamide **15** (0.0360 g, 0.0820 mmol, 66%, melting point range 55-61 °C) as a dark green solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.37 (1H, d,  $J = 2.4$  Hz), 7.12 (1H, dd,  $J = 8.5, 2.5$  Hz), 6.75 (1H, d,  $J = 7.8$  Hz), 6.56 (1H, d,  $J = 11.8$  Hz), 6.47 (1H, d,  $J = 11.9$  Hz), 6.42 (2H, s), 3.98 (1H, t,  $J = 5.9$  Hz), 3.75 (3H, s), 3.72 (1H, d,  $J = 4.5$  Hz), 3.67 (3H, s), 3.61

(1H, m), 3.55 (6H, s). <sup>13</sup>C NMR (150 MHz, CD<sub>3</sub>OD): δ 165.35, 159.38, 152.70, 137.08, 135.26, 132.31, 131.57, 130.38, 124.80, 123.57, 111.27, 109.52, 105.93, 60.09, 59.70, 55.08, 54.87, 54.52. HRMS: *m/z*: obsd 403.1885 [M+H]<sup>+</sup>, calcd for C<sub>21</sub>H<sub>27</sub>N<sub>2</sub>O<sub>6</sub><sup>+</sup>, 403.1884. HPLC (Method C): 13.05 min.

***Tert-butyl (Z)-2-((5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)amino)-2-oxoethyl carbamate (12)***: To a solution of 2' CA4-amine **7** (0.363 g, 0.700 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (75 mL) was added Et<sub>3</sub>N (0.300 mL, 1.72 mmol), peptide coupling reagent T3P (50% in EtOAc, 1.70 mL, 2.30 mmol) and Boc-glycine-OH (0.311 g, 1.44 mmol). The reaction mixture was stirred for 3 h at room temperature. Water (25 mL) was added, and the organic solvent was removed by evaporation under reduced pressure. The resulting crude mixture was extracted with EtOAc, and the organic extract was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The residue was purified using flash column chromatography (50% EtOAc/hexanes) to afford the desired Boc-protected glycinamide **12** (0.490 g, 1.04 mmol, 90%) as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.95 (1H, s), 7.93 (1H, bs), 7.15 (1H, d, *J* = 8.5 Hz), 6.68 (1H, d, *J* = 7.7 Hz), 6.61 (1H, d, *J* = 12.0 Hz), 6.46 (1H, d, *J* = 12.0 Hz), 6.41 (2H, s), 4.80 (1H, s), 3.81 (3H, s), 3.80 (3H, s), 3.67 (1H, s), 3.61 (6H, d, *J* = 1.4 Hz), 1.46 (9H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 167.26, 159.51, 155.67, 152.86, 137.83, 135.32, 132.51, 131.43, 130.06, 124.34, 119.50, 110.91, 109.99, 105.67, 105.64, 60.91, 55.80, 55.46, 45.10, 28.29.

***(Z)-2-amino-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)acetamide (14)***<sup>81</sup>: The Boc-protected glycinamide **12** (0.128 g, 0.270 mmol) in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was reacted with TFA (0.650 mL, 1.30 mmol) and stirred for 30 min.<sup>81</sup> After evaporation of the solvent, the resulting oily residue was diluted with water (2 mL) and washed with ether (2 × 2 mL). The aqueous phase was treated with NaHCO<sub>3</sub> (1 N) until pH 7-8 and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 5 mL). The combined organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated under reduced pressure and purified using flash column chromatography (50% EtOAc/hexanes) to obtain 2' CA4 glycinamide prodrug **14** (0.0680 g, 0.183 mmol, 71%) as a brown oil. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 9.52 (1H, s), 8.08 (1H, d, *J* = 2.6 Hz), 7.14 (1H, d, *J* = 8.5 Hz), 6.65 (1H, dd, *J* = 8.6, 2.5 Hz), 6.60 (1H, d, *J* = 12.0 Hz), 6.50 (1H, d, *J* = 12.1 Hz), 6.41 (2H, s), 3.82 (3H, s), 3.80 (3H, s), 3.60 (6H, s), 3.28 (2H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 170.62, 159.52, 152.73, 137.63, 136.05, 132.44, 131.77, 130.01, 124.43, 119.59, 110.51, 105.85, 105.21, 60.89, 55.82, 55.45, 45.42. HRMS: *m/z*: obsd 373.1759 [M+H]<sup>+</sup>, calcd for C<sub>20</sub>H<sub>25</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup>, 373.1758. HPLC (Method A): 8.14 min.

***(E)-2-amino-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)acetamide hydrochloride (18)***<sup>53</sup>: To a well stirred solution of Boc-protected 2' CA4 glycinamide **12** (0.146 g, 0.310 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added a HCl solution (4 N in dioxane, 1.20 mmol) at room temperature. After completion of the reaction (monitored by TLC), diethyl ether (25 mL) was added to the reaction mixture, producing a colorless solid that was filtered through a membrane filter. After washing the product on the membrane with diethyl ether (10 mL), the HCl salt of the *E*-isomer of 2' CA4 glycinamide **18** (0.109 g, 0.226 mmol, 79%, melting point range 240-243 °C) was obtained as a colorless solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 7.66 (1H, d, *J* = 8.8 Hz), 7.20 (1H, d, *J* = 15 Hz), 7.19 (1H, d, *J* = 2.7 Hz), 6.98 (1H, d, *J* = 16.1

Hz), 6.88 (1H, d,  $J=2.5$  Hz), 6.87 (2H, s), 3.98 (2H, s), 3.88 (6H, s), 3.81 (3H, s), 3.77 (3H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  165.04, 159.47, 153.25, 137.58, 134.59, 133.83, 129.04, 126.75, 124.24, 122.22, 112.35, 110.48, 103.75, 59.76, 55.31, 54.46, 40.66. HRMS:  $m/z$ : obsd 373.1769  $[\text{M}-\text{Cl}]^+$ , calcd for  $\text{C}_{20}\text{H}_{25}\text{N}_2\text{O}_5^+$  373.1758. HPLC (Method B): 6.661 min.

**(Z)-2-amino-N-(5-methoxy-2-(3,4,5-trimethoxystyryl)phenyl)acetamide hydrochloride (16)**<sup>73</sup>: To Boc-protected 2' CA4 glycinamide **12** (0.0898 g, 0.190 mmol) was added a HCl solution (4 N in dioxane, 0.270 mmol) at 0 °C. The resulting reaction mixture was stirred for 30 min., followed by evaporation of the solvent under reduced pressure at 30 °C. The resulting brownish oil, on washing with anhydrous diethyl ether, furnished the water soluble HCl salt of 2' CA4 glycinamide **16** (0.0691 g, 0.171 mmol, 90%, melting point range 59-63 °C) as a colorless solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.37 (1H, d,  $J=2.6$  Hz), 7.12 (1H, d,  $J=8.4$  Hz), 6.73 (1H, dd,  $J=8.3, 2.4$  Hz), 6.57 (1H, d,  $J=11.8$  Hz), 6.48 (1H, d,  $J=11.9$  Hz), 6.43 (2H, s), 3.75 (3H, s), 3.67 (3H, d,  $J=1.6$  Hz), 3.63 (2H, s), 3.56 (6H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  164.38, 159.37, 152.65, 136.96, 135.29, 132.57, 131.37, 130.58, 124.93, 123.22, 111.06, 109.45, 105.84, 59.75, 54.89, 54.51, 40.59. HRMS:  $m/z$ : obsd 373.1759  $[\text{M}-\text{Cl}]^+$ , calcd for  $\text{C}_{20}\text{H}_{25}\text{N}_2\text{O}_5^+$ , 373.1759. HPLC (Method C): 12.94 min.

#### 4.1.8. Preparation of amino acid prodrug conjugates of combretastatin A-1 diamines<sup>15,16,53</sup>

**(Z)-2-(2',3'-Diamino-4'-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)ethene-Fmoc-L-serinamide (19)**: To a solution of CA1-diamine **8** (0.780 g, 2.37 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (150 mL) was added Fmoc-L-serine (Ac)-OH (2.40 g, 7.11 mmol),  $\text{Et}_3\text{N}$  (1.00 mL, 7.11 mmol), and T3P (50% in EtOAc, 5.62 mL, 9.48 mmol). The resulting solution was stirred for 8 h at room temperature. At this point, the reaction mixture was washed with water (3  $\times$  50 mL) and brine and dried over  $\text{Na}_2\text{SO}_4$ . The organic solvent was removed under reduced pressure, and the crude product was purified by flash column chromatography (50% EtOAc/hexanes) to afford the Fmoc-L-diserinamide acetate **19** (1.31 g, 1.30 mmol, 55%) as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.35 (1H, s), 8.05 (1H, s), 7.71 (2H, d,  $J=6.6$  Hz), 7.66 (2H, s), 7.49 (4H, d,  $J=6.0$  Hz), 7.33 (4H, m), 7.23 – 7.15 (4H, m), 7.18 (1H, d,  $J=9.0$  Hz), 6.78 (1H, d,  $J=8.0$  Hz), 6.46 (1H, d,  $J=12.0$  Hz), 6.43 (2H, s), 6.39 (1H, d,  $J=12.0$  Hz), 6.09 (1H, s), 5.84 (1H, s), 4.68 – 4.02 (12H, m), 3.79 (6H, s), 3.62 (6H, s), 2.04 (6H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.0, 171.0, 168.1, 167.5, 156.6, 156.4, 152.9, 152.5, 143.6, 143.6, 143.5, 143.5, 141.3, 141.2, 137.3, 132.1, 131.0, 130.4, 128.8, 127.8, 127.8, 127.1, 127.1, 125.9, 125.1, 125.1, 125.0, 120.0, 109.6, 105.9, 67.6, 67.5, 63.7, 63.6, 60.9, 56.2, 55.9, 54.8, 46.9, 34.7, 29.1, 25.3, 20.8.

**(2S, 2'S)-N, N'-(3-methoxy-6-((Z)-3,4,5-trimethoxystyryl)-1,2-phenylene)bis(2-amino-3-hydroxypropanamide) (22)**: Fmoc-L-diserinamide acetate **19** (0.501 g, 0.485 mmol) was dissolved in a  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  mixture (20 mL, 1:1 ratio) to which a NaOH solution (2 M, 0.970 mL, 1.98 mmol) was added, followed by stirring (30 min). After the evaporation of solvent, the resulting oil was purified by normal phase preparative TLC (90%  $\text{CH}_2\text{Cl}_2/\text{MeOH}$ . 0.2%  $\text{Et}_3\text{N}$ ) to obtain CA1-L-diserinamide prodrug **22** (0.0650 g, 0.128 mmol, 27%, melting point range 75-80 °C) as a colorless solid.  $^1\text{H}$  NMR (600 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.17



(1H, d,  $J=8.6$  Hz), 6.98 (1H, d,  $J=8.7$  Hz), 6.54 (1H, d,  $J=12$  Hz), 6.49 (2H, s), 6.48 (1H, d,  $J=13.3$  Hz), 3.90 (2H, d,  $J=5.7$  Hz), 3.89 – 3.87 (1H, m), 3.86 (3H, s), 3.85 – 3.80 (2H, m), 3.76 (1H, m), 3.71 (3H, s), 3.61 (6H, s), 1.95 (4H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  180.70, 173.77, 155.83, 154.95, 139.05, 134.89, 133.67, 132.82, 130.62, 130.45, 128.38, 124.07, 111.92, 108.28, 65.55, 65.50, 61.94, 58.85, 58.54, 57.51, 57.20. HRMS:  $m/z$ : obsd 505.2289  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{24}\text{H}_{33}\text{N}_4\text{O}_8^+$ , 505.2293. HPLC (Method C): 10.94 min.

**(2*S*, 2'*S*)-((3-methoxy-6-((*Z*)-3,4,5-trimethoxystyryl)-1,2-phenylene)bis(azanediy))bis(2-((tert-butoxycarbonyl)amino)-3-oxopropane-3,1-diyl) diacetate (20)**: To a solution of CA1 diamine **8** (0.270 g, 0.817 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (50 mL) was added Boc-serine (Ac)-OH·DCHA (0.875 g, 2.04 mmol),  $\text{Et}_3\text{N}$  (0.290 mL, 2.04 mmol), and T3P (50% in EtOAc, 2.10 mL, 3.27 mmol). The resulting solution was stirred for 8 h at room temperature. At this point, the reaction mixture was washed with water ( $3 \times 20$  mL) and brine and dried over  $\text{Na}_2\text{SO}_4$ . The organic solvent was removed under reduced pressure, and the crude reaction mixture was purified by flash column chromatography (50% EtOAc/hexanes) to afford the Boc-protected-*L*-serinamide acetate **20** (0.251 g, 0.318 mmol, 39%) as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.40 (1H, s), 8.21 (1H, s), 7.14 (1H, d,  $J=8.6$  Hz), 6.75 (1H, d,  $J=8.6$  Hz), 6.45 (1H, d,  $J=12.0$  Hz), 6.41 (2H, s), 6.38 (1H, d,  $J=11.9$  Hz), 5.66 (1H, s), 5.48 – 5.41 (1H, m), 4.62 (1H, s), 4.48 (2H, d,  $J=5.2$  Hz), 4.40 (2H, d,  $J=7.5$  Hz), 4.30 (1H, dd,  $J=7.0$  Hz), 3.80 (3H, s, 1 Hz), 3.79 (3H, s), 3.62 (6H, s), 2.05 (3H, s), 2.02 (3H, s), 1.46 (9H, s), 1.43 (9H, s).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  170.97, 170.80, 168.40, 167.67, 155.56, 155.33, 152.82, 152.40, 137.27, 132.00, 130.91, 130.42, 128.50, 128.36, 125.95, 121.24, 109.53, 105.98, 64.00, 63.92, 60.80, 60.38, 56.13, 55.83, 54.05, 28.31, 28.28, 20.66, 20.65.

**(2*S*, 2'*S*)-*N*, *N*'-(3-methoxy-6-((*Z*)-3,4,5-trimethoxystyryl)-1,2-phenylene)bis(2-amino-3-hydroxypropanamide) dihydrochloride (23)**: Boc-*L*-serinamide acetate **20** (0.203 g, 0.258 mmol) was dissolved in a  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  mixture (20 mL, 1:1 ratio) to which a NaOH solution (2 M, 0.650 mL, 1.30 mmol) was added. The reaction mixture was stirred for 30 min. After the evaporation of solvent, the resulting colorless solid (0.130 g, 0.184 mmol) in  $\text{CH}_2\text{Cl}_2$  (15 mL) was reacted with a HCl solution (4 N in dioxane, 2.27 mmol) at room temperature for 2 h. Following evaporation of solvent under reduced pressure, the solid was purified by reversed phase column chromatography (RP-18 silica column, acetonitrile/water) to afford compound **20** (0.0590 g, 0.102 mmol, 40%) as an off-white solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.16 (1H, d,  $J=8.7$  Hz), 6.99 (1H, d,  $J=8.7$  Hz), 6.55 (1H, d,  $J=12.0$  Hz), 6.49 (2H, s), 6.48 (1H, d,  $J=12.0$  Hz), 4.32 (1H, dd,  $J=5.5, 4.3$  Hz), 4.27 (1H, dd,  $J=6.1, 4.8$  Hz), 4.12 (1H, dd,  $J=11.5, 4.5$  Hz), 4.03 (1H, dd,  $J=11.5, 4.5$  Hz), 4.00 (1H, dd,  $J=12.0, 8.5$  Hz), 3.89 (1H, dd,  $J=12.0, 8.5$  Hz), 3.85 (3H, s), 3.71 (3H, s), 3.61 (6H, s).  $^{13}\text{C}$  NMR (150 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  166.1, 165.6, 153.9, 152.6, 136.8, 132.4, 131.4, 131.0, 129.0, 128.7, 125.9, 121.7, 110.3, 106.2, 60.4, 59.7, 55.4, 55.1, 55.1, 55.0, 55.0. HRMS:  $m/z$ : obsd 527.2102  $[\text{M}-\text{HCl}+\text{Na}]^+$ , calcd for  $\text{C}_{24}\text{H}_{32}\text{N}_4\text{O}_8\text{Na}^+$ , 527.2112. HPLC (Method C): 11.17 min.

**Di-tert-butyl (((3-methoxy-6-(3,4,5-trimethoxystyryl)-1,2-phenylene)bis(azanediy))bis(2-oxoethane-2,1-diyl))(Z)-dicarbamate (21)**: To a solution of CA1-diamine **8** (0.300 g, 0.908 mmol) in anhydrous  $\text{CH}_2\text{Cl}_2$  (50 mL) was added Boc-glycine-OH (0.398 g, 2.27 mmol),

Et<sub>3</sub>N (0.270 mL, 1.91 mmol), and T3P (50% in EtOAc, 2.25 mL, 3.63 mmol). The resulting solution was stirred for 3 h at room temperature. At this point, the reaction mixture was washed with water (3 × 20 mL) and brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The organic solvent was removed under reduced pressure, and the crude product was purified by flash column chromatography (50% EtOAc/hexanes) to afford the Boc-protected CA1-diglycinamide **21** (0.503 g, 0.780 mmol, 86%) as a colorless solid. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 8.21 (1H, bs), 7.96 (1H, bs), 7.14 (1H, d, *J*=8.6 Hz), 6.74 (1H, d, *J*=8.6 Hz), 6.49 (1H, d, *J*=12.0 Hz), 6.44 (2H, s), 6.43 (1H, d, *J*=11.6 Hz), 5.54 (1H, bs), 5.38 (1H, bs), 3.96 (2H, d, *J*=5.6 Hz), 3.83 (2H, d, *J*=5.8 Hz), 3.80 (6H, s), 3.63 (6H, s), 1.47 (9H, s), 1.45 (9H, s). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): δ 169.2, 168.8, 156.6, 156.4, 153.1, 152.8, 137.4, 132.6, 131.2, 128.9, 128.7, 126.5, 121.7, 109.8, 106.2, 61.2, 56.4, 56.2, 45.2, 44.8, 28.7, 28.7.

**(Z)-N,N'-(3-methoxy-6-(3,4,5-trimethoxystyryl)-1,2-phenylene)bis(2-aminoacetamide)**

**(24):** To a solution of Boc-protected CA1-diglycinamide **21** (0.355 g, 0.550 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) was added a HCl solution (4 N in dioxane, 2.75 mmol) at room temperature, and the mixture was stirred for 3 h. On adding diethyl ether (25 mL) to the reaction mixture, a colorless solid formed, and it was filtered through a membrane filter. After washing the solid with diethyl ether (20 mL), CA1-diglycinamide **24** (0.142 g, 0.275 mmol, 50%, melting point range 81-84 °C) was obtained as a colorless solid. <sup>1</sup>H NMR (600 MHz, CD<sub>3</sub>OD): δ 7.18 (1H, d, *J*=8.3 Hz), 7.01 (1H, d, *J*=8.4 Hz), 6.59 (1H, d, *J*=11.8 Hz), 6.53 (1H, d, *J*=12 Hz), 6.51 (2H, s), 4.02 (4H, bs, NH<sub>2</sub>, CH<sub>2</sub>), 3.93 (2H, bs, NH<sub>2</sub>), 3.92 (2H, bs, CH<sub>2</sub>), 3.87 (3H, s), 3.73 (3H, s), 3.63 (6H, s). <sup>13</sup>C NMR (150 MHz, CD<sub>3</sub>OD): δ 164.9, 164.7, 153.9, 152.6, 136.8, 132.5, 131.4, 131.0, 128.9, 128.7, 126.0, 121.7, 110.2, 106.1, 59.7, 55.3, 55.1, 40.7, 40.6. HRMS: *m/z*. obsd 467.1897 [M -HCl+ Na]<sup>+</sup>, calcd for C<sub>22</sub>H<sub>28</sub>N<sub>4</sub>O<sub>6</sub>Na<sup>+</sup>, 467.1901. HPLC (Method A): 6.06 min.

**4.1.9. Synthesis of Amino Acid Prodrug Conjugates of Amino Dihydronaphthalene and Amino Benzosuberene<sup>42,43,46</sup>**

**6-Methoxy-5-nitro-1-tetralone (25):** To a well-stirred solution of 6-methoxy-1-tetralone (20.0 g, 114 mmol) in acetic anhydride (200 mL), a solution of HNO<sub>3</sub> (10 mL) and AcOH (10 mL) was slowly added over the course of 1 h at 0 °C. The reaction was stirred for another 20 h and was allowed to reach room temperature. Water (20 mL) was added to the reaction mixture, and the crude product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 50 mL). The organic phase was dried with Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. The residue was purified by flash column chromatography (20% EtOAc/hexanes) to give the desired 6-methoxy-5-nitro-tetralone **25** (8.28 g, 37.5 mmol, 33%) as a light yellow solid. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 8.18 (1H, d, *J*= 8.8 Hz), 7.02 (1H, d, *J*= 8.8 Hz), 3.97 (3H, s), 2.86 (2H, t, *J*= 6.5 Hz), 2.65 (2H, t, *J*= 6.5 Hz), 2.15 (2H, p, *J*= 6.5 Hz). <sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): δ 195.5, 154.4, 140.3, 137.3, 131.2, 126.1, 110.7, 56.8, 38.2, 24.7, 22.3.

**2-Methoxy-1-nitro-5-(3,4,5-trimethoxyphenyl)-6,7,8,9-tetrahydro-5H-benzo[7]annulen-5-ol (30):** To a solution of 5-bromo-1,2,3-trimethoxybenzene (1.11 g, 4.49 mmol) in THF (20 mL) at -78 °C, *n*-BuLi (1.80 mL, 2.50 M) was added, and the reaction mixture was stirred for 1 h. Compound **25** (0.500 g, 2.30 mmol) in THF (3 mL) was added to the reaction mixture dropwise. The reaction mixture was stirred for 18 h and was allowed to warm to

room temperature. The reaction mixture was quenched with H<sub>2</sub>O (20 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 × 25 mL). The organic extract was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure, and the residue was purified by flash column chromatography (40% EtOAc/hexanes) to yield alcohol **30** (0.521 g, 1.34 mmol, 59% yield) as a light yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.18 (1H, d, *J*=8.8 Hz), 6.85 (1H, d, *J*=8.8 Hz), 6.54 (2H, s), 3.87 (3H, s), 3.85 (3H, s), 3.81 (6H, s), 2.79 (2H, t, *J*=7.4 Hz), 2.12 (1H, s), 2.10 (2H, m), 2.04 (1H, m), 1.86 (1H, m). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 152.9, 150.0, 143.7, 137.0, 135.3, 132.1, 130.6, 110.8, 103.8, 74.9, 61.0, 56.5, 56.4, 40.6, 24.6, 18.6.

**2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-amine (32):** Alcohol **30** (0.521 g, 1.34 mmol) was dissolved in AcOH (18 mL) in a round-bottom reaction flask, followed by the addition of Zn (1.75 g, 26.8 mmol). The reaction mixture was stirred for 6 h at room temperature and then filtered through Celite<sup>®</sup>, which was washed with CH<sub>2</sub>Cl<sub>2</sub>. The filtrate was concentrated under reduced pressure and the residue was purified by flash column chromatography (30% EtOAc/hexanes) to give the desired amino-dihydronaphthalene (**32**) (0.410 g, 1.27 mmol, 89% yield, melting point range 183-185 °C) as a dark brown solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 6.59 (1H, d, *J*= 8.5 Hz), δ 6.56 (2H, s), δ 6.51 (1H, d, *J*= 8.5 Hz), δ 5.92 (1H, t, *J*= 4.5 Hz), δ 3.89 (3H, s), δ 3.85 (3H, s), δ 3.83 (6H, s), δ 2.67 (2H, t, *J*= 7.5 Hz), δ 2.41 (2H, td, *J*= 7.5, 4.5 Hz). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): δ 152.9, 146.7, 140.0, 137.1, 136.9, 134.0, 127.8, 124.2, 120.3, 115.1, 107.6, 106.2, 60.4, 56.2, 55.7, 23.0, 21.7. HRMS: *m/z*: obsd 342.1698 [M+H]<sup>+</sup>, calcd for C<sub>20</sub>H<sub>24</sub>NO<sub>4</sub><sup>+</sup>, 342.1705. HPLC (Method B): 14.18 min.

**5-(3'-Methoxy-2'-nitrophenyl)pent-4-enoic acid (27):** To a well-stirred solution of 3-(carboxypropyl)triphenylphosphonium bromide (21.5 g, 50.1 mmol) in THF (400 mL), potassium *tert*-butoxide (13.4 g, 119 mmol) was added, and the reaction mixture was stirred for 1 h at ambient temperature. The reaction mixture was cooled to 0 °C, and 3-methoxy-2-nitrobenzaldehyde (9.00 g, 47.7 mmol) in THF (100 mL) was added dropwise. After the reaction mixture was stirred for 16 h, warming from 0 °C to room temperature, the reaction was quenched with HCl (2 M, 200 mL). The organic solvent was removed by evaporation under reduced pressure, and the residue was extracted with EtOAc (4 × 300 mL). The combined organic phase was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated under reduced pressure. The resulting organic crude product was purified by flash column chromatography using a pre-packed 100 g silica column [solvent A: EtOAc; solvent B: hexanes; gradient: 12% A / 88% B (1 V), 12% A / 88% B → 100% A / 0% B (15 V), 100% A / 0% B (4 CV); flow rate: 40 mL/min; monitored at 254 and 280 nm] affording a mixture of *E* and *Z*-isomers **27** (8.78 g, 34.9 mmol, 73%) as a red solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.38 (1H, t, *J*=8.1 Hz), 7.33 (1H, t, *J*=8.2 Hz), 7.12 (1H, d, *J*=7.8 Hz), 6.95 (1H, d, *J*=8.3 Hz), 6.89 (2H, d, *J*=7.7 Hz), 6.37 (1H, d, *J*=11.3 Hz), 6.31 (2H, m), 5.83 (1H, dt, *J*=11.5, 7.0 Hz), 3.89 (3H, s), 3.87 (3H, s), 2.53 (4H, m), 2.45 (4H, m). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 178.5, 178.4, 150.9, 150.9, 134.9, 134.5, 130.8, 130.7, 123.7, 123.6, 121.7, 118.2, 111.4, 111.0, 56.5, 56.5, 33.6, 33.3, 28.1, 23.9.

**5-(3'-Methoxy-2'-nitrophenyl)pentanoic acid (28):** Anhydrous ethanol (18 mL) was added to a flask containing pentenoic acid **27** (0.750 g, 2.98 mmol) and Pd/C (10%, 0.830 g), and

the solution was stirred for 10 min at ambient temperature. 1,4-Cyclohexadiene (8.90 mL, 93.8 mmol) was added to the solution, which was then stirred for another 3 h. The reaction mixture was filtered through Celite<sup>®</sup>, and the Celite<sup>®</sup> was washed with EtOAc (3 × 20 mL). The combined filtrate was removed by evaporation under reduced pressure, and the residue was purified by flash column chromatography using a pre-packed 100 g silica column [solvent A: Et Ac; solvent B: hexanes; gradient: 12% A / 88% B (1 V), 12% A / 88% B → 100% A / 0% B (12 CV), 100% A / 0% B (2 CV); flow rate: 40 mL/min; monitored at 254 and 280 nm] to afford pentanoic acid analogue **28** (0.490 g, 1.93 mmol, 65%) as a tan solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.33 (1H, t, *J*=8.1 z), 6.87 (2H, dd, *J*=8.1, 3.7 Hz), 3.87 (3H, s), 2.58 (2H, m), 2.36 (2H, m), 1.67 (4H, m). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 179.3, 150.8, 142.0, 134.8, 130.8, 121.7, 110.3, 56.5, 33.7, 30.8, 29.9, 24.4.

**2-Methoxy-1-nitro-benzosuber-5-one (29):** Pentanoic acid analogue **28** (0.490 g, 1.93 mmol) was dissolved in Eaton's reagent (14.5 mL, 7.7% P<sub>2</sub>O<sub>5</sub> in CH<sub>3</sub>SO<sub>3</sub>H) under N<sub>2</sub>, and the reaction mixture was stirred for 16 h at ambient temperature. To the solution was added ice, which allowed to melt, and the reaction mixture was neutralized slowly with NaHCO<sub>3</sub> (aq.) and extracted with EtOAc (3 × 20 mL). The combined organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and removed by evaporation under reduced pressure, and the residue was purified by flash chromatography using a pre-packed 50 g silica column [solvent A: Et Ac; solvent B: hexanes; gradient: 7% A / 93% B (1 V), 7% A / 93% B → 60% A / 40% B (13 CV), 60% A / 40% B (1 CV); flow rate: 25 mL/min; monitored at 254 and 280 nm] to afford benzosuberone analogue **29** (0.340 g, 1.45 mmol, 75%) as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.84 (1H, d, *J*=8.8 Hz), 6.98 (1H, d, *J*=8.8 Hz), 3.95 (3H, s), 2.78 (2H, m), 2.71 (2H, m), 1.91 (2H, m), 1.81 (2H, m). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 203.2, 153.4, 141.5, 134.1, 132.3, 131.9, 110.4, 56.7, 40.4, 26.3, 24.5, 20.3.

**2-Methoxy-1-nitro-5-(3',4',5'-trimethoxyphenyl)-benzosuber-5-ol (31):** To a well-stirred solution of 3,4,5-trimethoxyphenyl bromide (0.680 g, 2.75 mmol) in THF (25 mL) at -78 °C, *n*-BuLi (1.00 mL, 2.50 M in hexanes) was added, and the reaction mixture was stirred for 1 h. Benzosuberone analogue **29** (0.390 g, 1.66 mmol) in THF (5 mL) was added dropwise into the reaction mixture, which was then stirred for 18 h and allowed to warm from -78 °C to room temperature. The reaction was quenched with H<sub>2</sub>O (20 mL), and the solvent was removed under reduced pressure. The mixture was extracted with EtOAc (3 × 25 mL), and the combined organic phase was washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography using a pre-packed 50 g silica column [solvent A: EtOAc; solvent B: hexanes; gradient: 5% A / 95% B (1 CV), 5% A / 95% B → 100% A / 0% B (13 CV), 100% A / 0% B (2 CV); flow rate: 40 mL/min; monitored at 254 and 280 nm] to afford alcohol **31** (0.468 g, 1.16 mmol, 70%) as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.68 (1H, d, *J*=8.7 Hz), 6.88 (1H, d, *J*=8.7 Hz), 6.46 (2H, s), 3.90 (3H, s), 3.84 (3H, s), 3.76 (6H, s), 2.60 (2H, m), 2.41 (1H, m), 2.37 (1H, s), 2.11 (1H, m), 1.95 (1H, m), 1.79 (2H, m), 1.53 (1H, m). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 153.3, 149.4, 142.4, 140.5, 138.6, 137.6, 133.6, 129.4, 109.3, 104.1, 79.7, 60.9, 56.3, 56.2, 40.9, 28.7, 26.4, 26.0.

**1-Amino-2-methoxy-5-(3',4',5'-trimethoxyphenyl)-benzosuber-5-ene (33):** To a solution of alcohol **31** (0.498 g, 1.16 mmol) in AcOH (15 mL) was added Zn dust (1.52 g, 23.2 mmol), and the reaction was stirred for 6 h at ambient temperature. The reaction mixture was filtered through Celite<sup>®</sup>, which was washed with CH<sub>2</sub>Cl<sub>2</sub>, and the filtrate was concentrated under reduced pressure. The concentrated residue was neutralized with NaHCO<sub>3</sub> and extracted with EtOAc (3 × 20 mL). The combined extracts were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and removed by evaporation under reduced pressure. The crude product was purified by flash column chromatography using a pre-packed 25 g silica column [solvent A: EtOAc; solvent B: hexanes; gradient: 5% A / 95% B (1 V), 5% A / 95% B → 60% A / 40% B (13 CV), 60% A / 40% B (2 CV); flow rate: 40 mL/min; monitored at 254 and 280 nm] to afford benzosuberene **33** (KGP156, 0.350 g, 0.980 mmol, 85%, melting point range 140-145 °C) as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 6.67 (1H, d, *J*=8.4 Hz), 6.52 (2H, s), 6.49 (1H, d, *J*=8.4 Hz), 6.30 (1H, t, *J*=7.3 Hz), 3.88 (3H, s), 3.86 (3H, s), 3.80 (6H, s), 2.59 (2H, t, *J*=6.9 Hz), 2.12 (2H, p, *J*=7.0 Hz), 1.95 (2H, q, *J*=7.2 Hz). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 152.9, 146.5, 143.7, 138.7, 137.4, 133.7, 132.6, 126.9, 126.4, 120.0, 107.7, 105.4, 61.1, 56.3, 55.7, 33.4, 25.8, 25.4. HRMS: *m/z*: obsd 356.1863 [M+H]<sup>+</sup>, calcd for C<sub>21</sub>H<sub>26</sub>NO<sub>4</sub><sup>+</sup>, 356.1862. HPLC (Method B): 15.34 min.

**(S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-((2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)amino)-3-oxopropyl acetate (34):** To a well-stirred solution of amino compound **32** (0.500 g, 1.45 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (30 mL), Fmoc-*L*-ser(Ac)-OH (0.648 g, 2.18 mmol), T3P (50% in EtOAc, 2.60 mL, 4.35 mmol), and Et<sub>3</sub>N (0.31 mL, 2.18 mmol) were added, and the reaction mixture was stirred for 17 h at room temperature. Water (30 mL) was added, and the reaction mixture was extracted with EtOAc (3 × 30 mL). The organic phase was rinsed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated under reduced pressure. The residue was purified by flash column chromatography (35% EtOAc/hexanes) to afford the desired Fmoc-*L*-serinamide acetate **34** (0.695 g, 1.12 mmol, 77%) as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.77 (2H, d, *J* = 7.5 Hz), 7.60 (2H, d, *J* = 7.5 Hz), 7.60 (1H, s), 7.40 (2H, dd, *J* = 7.5, 7.0 Hz), 7.33 (2H, dd, *J* = 7.5, 7.0 Hz), 6.98 (1H, d, *J* = 8.5 Hz), 6.65 (1H, d, *J* = 9.0 Hz), 6.56 (2H, s), 6.00 (1H, t, *J* = 4.5 Hz), 5.75 (1H, d, *J* = 6.5 Hz), 4.75 (1H, t, *J* = 11.5 Hz), 4.47 (4H, m), 4.23 (1H, t, *J* = 11.5 Hz), 3.89 (3H, s), 3.84 (6H, s), 3.79 (3H, s), 2.67 (2H, t, *J* = 7.5 Hz), 2.30 (2H, td, *J* = 7.5, 4.5 Hz), 2.11 (3H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 170.77, 167.42, 152.96, 143.62, 143.55, 141.32, 139.47, 137.11, 136.55, 135.32, 128.81, 127.80, 127.11, 125.30, 125.16, 124.98, 121.79, 120.03, 120.01, 107.62, 105.78, 67.27, 64.47, 60.92, 56.15, 55.67, 54.36, 47.15, 23.99, 22.92, 20.78. HRMS: *m/z*: obsd 715.2625 [M+Na]<sup>+</sup>, calcd for C<sub>40</sub>H<sub>40</sub>N<sub>2</sub>O<sub>9</sub>Na<sup>+</sup>, 715.2630.

**(S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-((3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)amino)-3-oxopropyl acetate (35)<sup>47</sup>:** This compound was synthesized using the procedure as described for compound **34**. Starting from amino compound **33** (0.355 g, 1.00 mmol), Fmoc-*L*-ser(Ac)-OH (0.553 g, 1.50 mmol), T3P (50% in EtOAc, 1.50 mL, 2.50 mmol), and Et<sub>3</sub>N (0.210 mL, 1.50 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (25 mL), the desired product **35** (0.393 g, 0.556 mmol, 56%) was obtained as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.74 (2H, d, *J* = 7 Hz), 7.65 (1H, s), 7.57

(2H, d,  $J = 7.5$  Hz), 7.38 (2H, dd,  $J = 7.5, 7.0$  Hz), 7.27 (2H, dd,  $J = 7.5, 7.0$  Hz), 6.97 (1H, d,  $J = 8.5$  Hz), 6.73 (1H, d,  $J = 8.5$  Hz), 6.49 (2H, s), 6.36 (1H, t,  $J = 7.5$  Hz), 5.94 (1H, d,  $J = 6.5$  Hz), 4.82 (1H, bs), 4.49 (4H, m), 4.23 (1H, t,  $J = 6.5$  Hz), 3.86 (3H, s), 3.79 (6H, s), 3.74 (3H, s), 2.59 (2H, t,  $J = 7.5$  Hz), 2.14 (2H, m), 2.10 (3H, s), 1.95 (2H, m).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  170.90, 168.51, 156.24, 153.31, 152.94, 143.69, 143.60, 142.46, 141.32, 140.74, 138.38, 137.42, 133.53, 129.45, 127.84, 127.15, 125.04, 122.19, 120.04, 108.42, 105.36, 67.32, 64.61, 60.95, 56.20, 55.71, 54.26, 47.14, 33.91, 27.09, 25.63, 20.82. [Note: A portion of the experimental data for this compound was reproduced from reference 47 with permission from Elsevier.]

**(S)-2-Amino-3-hydroxy-N-(2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)propanamide (36):** To a stirred solution of  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (6 mL, 1:1 ratio) were added Fmoc-*L*-serinamide acetate **34** (0.30 g, 0.43 mmol) and a NaOH solution (2 M, 0.48 mL, 0.97 mmol). After stirring for 18 h at room temperature, the solvent was evaporated under reduced pressure, and water (6 mL) was added. The solution was extracted with EtOAc ( $3 \times 10$  mL), and then the combined organic phase were rinsed with brine, dried with  $\text{Na}_2\text{SO}_4$ , and concentrated under reduced pressure. The residue was purified by flash column chromatography (5% MeOH/  $\text{CH}_2\text{Cl}_2$ ) to afford the desired serinamide **36** (0.12 g, 0.28 mmol, 65%, melting point range 176-178 °C) as a yellow solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.91 (1H, s), 6.98 (1H, d,  $J = 8.5$  Hz), 6.65 (1H, d,  $J = 9.0$  Hz), 6.56 (2H, s), 6.00 (1H, t,  $J = 4.5$  Hz), 4.00 (1H, dd,  $J = 11, 4.5$  Hz), 3.88 (3H, s), 3.84 (6H, s), 3.81 (3H, s), 3.81 (1H, dd,  $J = 11, 4.5$  Hz), 3.69 (1H, t,  $J = 4.5$  Hz), 2.67 (2H, t,  $J = 7.5$  Hz), 2.30 (2H, td,  $J = 7.5, 4.5$  Hz).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  172.6, 152.9, 152.8, 139.4, 137.0, 136.6, 135.2, 128.7, 125.1, 125.0, 122.3, 107.7, 105.7, 65.3, 60.8, 56.6, 56.1, 55.7, 23.9, 22.9. HRMS:  $m/z$ : obsd 429.2041  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_6^+$ , 429.2026. HPLC (Method A): 7.80 min.

**(S)-2-Amino-3-hydroxy-N-(3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)propanamide (37)<sup>47</sup>:** This compound was synthesized using the procedure as described for compound **36**. Starting from Fmoc-*L*-serinamide acetate **35** (0.393 g, 0.556 mmol) and a NaOH solution (2 N, 0.630 mL, 1.26 mmol) in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (6 mL, 1:1 ratio), the desired product **37** (0.185 g, 0.418 mmol, 75%, melting point range 234-235 °C) was obtained as a colorless solid.  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.73 (1H, s), 6.97 (1H, d,  $J = 8.5$  Hz), 6.78 (1H, d,  $J = 8.5$  Hz), 6.49 (2H, s), 6.36 (1H, t,  $J = 7.5$  Hz), 4.04 (1H, dd,  $J = 11, 4.5$  Hz), 3.86 (3H, s), 3.84 (6H, s), 3.80 (3H, s), 3.80 (1H, dd,  $J = 11, 4.5$  Hz), 3.71 (1H, t,  $J = 4.5$  Hz), 2.62 (2H, t,  $J = 6.5$  Hz), 2.16 (2H, m), 1.98 (2H, q,  $J = 7.0$  Hz).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ ):  $\delta$  173.5, 153.1, 152.8, 142.5, 140.6, 138.3, 133.5, 129.1, 127.6, 122.5, 109.9, 108.4, 105.3, 65.9, 60.9, 56.3, 56.1, 55.8, 34.0, 27.0, 25.5. HRMS:  $m/z$ : obsd 443.2190  $[\text{M}+\text{H}]^+$ , calcd for  $\text{C}_{24}\text{H}_{31}\text{N}_2\text{O}_6^+$ , 443.2177. HPLC (Method A): 8.23 min. [Note: A portion of the experimental data for this compound was reproduced from reference 47 with permission from Elsevier.]

**(S)-2-Amino-3-hydroxy-N-(2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)propanamide hydrochloride (38):** Serinamide **36** (0.055 g, 0.13 mmol) was dissolved in  $\text{CH}_2\text{Cl}_2$  (0.8 mL), and HCl (4 N in dioxane, 0.080 mL, 0.32 mmol)

was added to the solution. After stirring for 1 h, the mixture was removed by evaporation to dryness, and the crude solid was re-crystallized using EtOAc/MeOH to afford desired serinamide salt **38** (0.015 g, 0.032 mmol, 25%, melting point range 241-242 °C) as a light yellow solid <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub> D): δ 6.98 (1H, d, *J* = 8.5 Hz), 6.83 (1H, d, *J* = 8.5 Hz), 6.58 (2H, s), 6.01 (1H, t, *J* = 4.5 Hz), 4.20 (1H, dd, *J* = 7.0, 4.0 Hz), 4.15 (1H, dd, *J* = 11, 4.0 Hz), 3.97 (1H, dd, *J* = 11, 4.0 Hz), 3.82 (3H, s), 3.80 (6H, s), 3.79 (3H, s), 2.67 (2H, m), 2.30 (2H, m). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub> D): δ 166.1, 153.7, 152.9, 139.4, 136.9, 136.8, 135.6, 128.3, 125.3, 124.6, 121.6, 107.8, 105.7, 60.6, 59.7, 55.1, 55.1, 54.8, 23.2, 22.4. HRMS: *m/z*: obsd 429.2063 [M-Cl]<sup>+</sup>, calcd for C<sub>23</sub>H<sub>29</sub>N<sub>2</sub>O<sup>+</sup>, 429.2026. HPLC (Method A): 7.94 min.

**(S)-2-Amino-3-hydroxy-N-(3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)propanamide hydrochloride (39)**<sup>47</sup>: This compound was synthesized using the procedure as described for compound **38**. Starting from serinamide **37** (0.10 g, 0.23 mmol) and HCl (4 N in dioxane, 0.28 mL, 1.1 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (6 mL, 1:1 ratio), the desired product **39** (0.074 g, 0.15 mmol, 67%, melting point range 260-263 °C) was obtained as a colorless solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub> D): δ 6.96 (1H, d, *J* = 8.5 Hz), 6.92 (1H, d, *J* = 8.5 Hz), 6.53 (2H, s), 6.39 (1H, t, *J* = 7.5 Hz), 4.26 (1H, dd, *J* = 7.5, 4.5 Hz), 4.19 (1H, dd, *J* = 11, 4.0 Hz), 3.99 (1H, dd, *J* = 11, 7.5 Hz), 3.82 (3H, s), 3.76 (6H, s), 3.75 (3H, s), 2.62 (2H, m), 2.16 (2H, m), 1.92 (2H, m). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub> D): δ 166.9, 153.9, 152.8, 142.5, 140.7, 138.4, 137.1, 133.3, 129.3, 126.9, 121.7, 108.5, 105.1, 60.7, 59.7, 55.1, 55.1, 54.8, 33.5, 26.2, 24.9. HRMS: *m/z*: obsd 443.2214 [M-Cl]<sup>+</sup>, calcd for C<sub>24</sub>H<sub>31</sub>N<sub>2</sub>O<sub>6</sub><sup>+</sup>, 443.2177. HPLC (Method A): 8.32 min. [Note: A portion of the experimental data for this compound was reproduced from reference 47 with permission from Elsevier.]

**(9H-fluoren-9-yl)methyl 2-((2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)amino)-2-oxoethylcarbamate (40)**: This compound was synthesized using the procedure as described for compound **34**. Starting from amino compound **32** (0.500 g, 1.45 mmol), Fmoc-glycine-OH (0.648 g, 2.18 mmol), T3P (50% in EtOAc, 2.60 mL, 4.35 mmol), and Et<sub>3</sub>N (0.310 mL, 2.18 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (30 mL) with 12 h stirring, the desired product **40** (0.678 g, 1.10 mmol, 75%) was obtained as a yellow solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.75 (2H, d, *J* = 7.5 Hz), 7.60 (2H, d, *J* = 7.5 Hz), 7.46 (1H, s), 7.38 (2H, t, *J* = 7.5 Hz), 7.29 (2H, t, *J* = 7.5 Hz), 6.97 (1H, d, *J* = 8.5 Hz), 6.65 (1H, d, *J* = 9.0 Hz), 6.57 (2H, s), 5.99 (1H, t, *J* = 4.5 Hz), 5.61 (1H, s), 4.48 (2H, d, *J* = 7 Hz), 4.24 (1H, t, *J* = 7 Hz), 4.12 (2H, s), 3.88 (3H, s), 3.84 (6H, s), 3.76 (3H, s), 2.67 (2H, t, *J* = 7.5 Hz), 2.30 (2H, td, *J* = 7.5, 4.5 Hz). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 167.71, 156.57, 152.94, 152.63, 143.84, 143.66, 141.31, 139.47, 137.09, 136.61, 135.38, 128.80, 127.76, 127.07, 125.19, 125.00, 121.92, 120.00, 107.57, 105.80, 67.19, 60.92, 56.16, 55.65, 47.17, 44.86, 24.05, 22.94.

**(9H-fluoren-9-yl)methyl 2-((3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)amino)-2-oxoethylcarbamate (41)**: This compound was synthesized using the procedure as described for compound **34**. Starting from amino compound **33** (0.200 g, 0.563 mmol), Fmoc-glycine-OH (0.250 g, 0.841 mmol), T3P (50% in EtOAc, 1.07

mL, 1.68 mmol), and Et<sub>3</sub>N (0.118 mL, 0.841 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) with 12 h stirring, the desired product **41** (0.350 g, 0.552 mmol, 98%) was obtained as a colorless solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.73 (2H, d, *J* = 7.04 Hz), 7.58 (2H, d, *J* = 7.04 Hz), 7.42 (1H, s), 7.35 (2H, t, *J* = 6.80 Hz), 7.27 (2H, t, *J* = 6.80), 6.96 (1H, d, *J* = 8.20 Hz), 6.73 (1H, d, *J* = 7.87 Hz), 6.49 (2H, s), 6.35 (1H, t, *J* = 6.45 Hz), 5.80 (1H, s), 4.45 (2H, m), 4.22 (1H, t, *J* = 6.93 Hz), 4.13 (2H, s), 3.86 (3H, s), 3.79 (6H, s), 3.76 (3H, s), 2.58 (2H, t, *J* = 6.93 Hz), 2.14 (2H, td, *J* = 6.93, 7.32 Hz), 1.95 (2H, q, *J* = 7.32 Hz). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 168.93, 156.84, 153.43, 152.97, 143.76, 142.50, 141.36, 140.86, 138.45, 137.46, 133.53, 129.40, 127.89, 127.82, 127.14, 125.10, 122.34, 120.05, 108.44, 105.41, 67.26, 60.99, 56.26, 55.75, 47.23, 44.93, 33.97, 27.18, 25.69.

**2-Amino-N-(2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)acetamide (42):** This compound was synthesized using the procedure as described for compound **36**. Starting from Fmoc-glycinamide **40** (0.477 g, 0.768 mmol) and a NaOH solution (2 M, 0.770 mL, 1.54 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (9 mL/ 1:1 ratio) with 13 h stirring, the desired product **42** (0.285 g, 0.715 mmol, 93%, melting point range 180-181 °C) was obtained as a tan solid. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 8.84 (1H, s), 6.93 (1H, d, *J* = 8.5 Hz), 6.63 (1H, d, *J* = 9.0 Hz), 6.55 (2H, s Hz), 5.95 (1H, t, *J* = 4.5 Hz), 3.85 (3H, s), 3.80 (6H, s), 3.77 (3H, s), 3.55 (2H, s), 2.67 (2H, t, *J* = 7.5 Hz), 2.26 (2H, td, *J* = 7.5, 4.5 Hz), 2.02 (2H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 171.5, 152.8, 152.8, 139.5, 136.9, 136.7, 135.1, 128.6, 125.0, 124.7, 122.5, 107.6, 105.7, 60.8, 56.1, 55.6, 45.0, 24.1, 22.9. HRMS: *m/z*: obsd 399.1930 [M+H]<sup>+</sup>, calcd for C<sub>22</sub>H<sub>27</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup>, 399.1914. HPLC (Method A): 8.10 min.

**2-Amino-N-(3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzof[7]annulen-4-yl)acetamide (43):** This compound was synthesized using the procedure as described for compound **36**. Starting from Fmoc-glycinamide **41** (0.350 g, 0.552 mmol) and a NaOH solution (2 M, 0.560 mL, 1.12 mmol) in CH<sub>2</sub>Cl<sub>2</sub>/MeOH (6 mL, 1:1 ratio) with 13 h stirring, the desired product **43** (0.193 g, 0.468 mmol, 83%, melting point range 123-128 °C) was obtained as a colorless solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 6.92 (1H, d, *J* = 8.58 Hz), 6.88 (1H, d, *J* = 8.61 Hz), 6.53 (2H, s), 6.37 (1H, t, *J* = 7.31 Hz), 3.80 (3H, s), 3.76 (3H, s), 3.74 (6H, s), 3.53 (2H, s), 2.60 (2H, t, *J* = 6.81 Hz), 2.13 (2H, p, *J* = 6.88 Hz), 1.93 (2H, q, *J* = 7.11 Hz). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD): δ 175.20, 155.54, 154.20, 144.12, 142.12, 139.97, 138.54, 134.61, 130.37, 128.23, 123.91, 109.79, 106.61, 61.15, 56.57, 56.19, 45.06, 34.96, 27.69, 26.38. HRMS: *m/z*: obsd 413.2074 [M+H]<sup>+</sup>, calcd for C<sub>23</sub>H<sub>29</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup>, 413.2071. HPLC (Method A): 8.45 min.

**2-Amino-N-(2-methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)acetamide hydrochloride (44):** This compound was synthesized using the procedure as described for compound **38**. Starting from glycinamide **42** (0.274 g, 0.688 mmol) and HCl (4 N in dioxane, 0.510 mL, 2.04 mmol) in MeOH (5 mL), the desired product **44** (0.171 g, 0.393 mmol, 57%, melting point range 238-239 °C) was obtained as a light yellow solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub> D): δ 6.97 (1H, d, *J* = 8.5 Hz), 6.82 (1H, d, *J* = 8.5 Hz), 6.58 (2H, s), 6.01 (1H, t, *J* = 4.5 Hz), 3.97 (2H, s), 3.81 (3H, s), 3.80 (6H, s), 3.79 (3H, s), 2.69 (2H, m), 2.31 (2H, m). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub> D): δ 165.1, 153.7, 152.9, 139.5, 136.9, 136.8,



135.6, 128.3, 125.3, 124.6, 121.6, 107.8, 105.7, 59.7, 55.1, 54.7, 40.2, 33.2, 22.47. HRMS: *m/z*: obsd 399.1919 [M–Cl]<sup>+</sup>, calcd for C<sub>22</sub>H<sub>27</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup>, 399.1914. HPLC (Method A): 8.03 min.

**2-Amino-N-(3-methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)acetamide hydrochloride (45):** This compound was synthesized using the procedure as described for compound **38**. Starting from glycinamide **43** (0.150 g, 0.364 mmol) and HCl (4 N in dioxane, 0.450 mL, 1.80 mmol) in MeOH/CH<sub>2</sub>Cl<sub>2</sub> (4 mL, 1:1 ratio), the desired product **45** (0.0847 g, 0.189 mmol, 53%, melting point range 274–276 °C) was obtained as a light yellow solid. <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 6.98 (1H, d, *J* = 8.59 Hz), 6.94 (1H, d, *J* = 8.62 Hz), 6.54 (2H, s), 6.41 (1H, t, *J* = 7.31 Hz), 3.99 (2H, s), 3.84 (3H, s), 3.77 (3H, s), 3.77 (6H, s), 2.64 (2H, t, *J* = 6.81 Hz), 2.16 (2H, p, *J* = 6.72 Hz), 1.95 (2H, q, *J* = 7.13 Hz). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD): δ 167.39, 155.48, 154.25, 144.05, 142.07, 139.85, 138.63, 134.75, 130.80, 128.30, 123.13, 109.93, 106.63, 61.17, 56.59, 56.24, 41.63, 35.01, 27.70, 26.35. HRMS: *m/z*: obsd 413.2074 [M–Cl]<sup>+</sup>, calcd for C<sub>23</sub>H<sub>29</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup>, 413.2071. HPLC (Method A): 8.47 min.

## 4.2. Biological Evaluations

**4.2.1. Cell Culture and SRB Cytotoxicity Assay**<sup>16,82–84</sup>—Three cancer cell lines (DU-145, prostate; SK-OV-3, ovarian; and NCI-H460, lung cancer) were grown and passaged using DMEM media supplemented with 10% FBS (Gibco One Shot®) and 1% gentamycin sulfate (Teknova, Hollister, CA). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, up to passage 15 for use in these experiments. The sulforhodamine B (SRB) assay was used to assess inhibition of human cell line growth as previously described.<sup>16,82–84</sup> Briefly, cancer cells were plated at 7500 cells/well using DMEM supplemented with 5% FBS and 1% gentamycin sulfate in 96-well plates and incubated for 24 h. Subsequently, 10-fold serial dilutions of the compounds to be tested were then added to the wells. After 48 h, the cells were fixed with 10% trichloroacetic acid (final concentration), stained with sulforhodamine B (Acid Red 52) (TKI, Tokyo), read at 540 nm, and normalized at 630 nm with an automated Biotek Elx800 plate reader (Biotek, Winooski, VT). A growth inhibition of 50% (GI<sub>50</sub> or the drug concentration causing a 50% reduction in the net protein staining relative to controls) was calculated from optical density data with Excel software.

**4.2.2. Inhibition of Tubulin Polymerization**<sup>85</sup>—Tubulin assembly experiments were performed using 0.25 mL reaction mixtures (final volume),<sup>85</sup> which contained 1.0 mg/mL (10 μM) purified bovine brain tubulin, 0.8 M monosodium glutamate (pH 6.6 in a 2 M stock solution) 4% (v/v) dimethyl sulfoxide, 0.4 mM GTP, and varying compound concentrations. Initially, all components except GTP were preincubated for 15 min at 30 °C in 0.24 mL. After chilling the mixtures on ice, 10 μL of 10 mM GTP was added. The reaction mixtures were placed in cuvettes held at 0 °C in Beckman DU-7400 and DU-7500 spectrophotometers equipped with electronic temperature controllers. The temperature was jumped to 30 °C over about 30 s, and polymerization was followed turbidimetrically at 350 nM for 20 min. Each reaction set included a reaction mixture without compound, and the

IC<sub>50</sub> was defined as the compound concentration that inhibited extent of assembly by 50% after 20 min at 30 °C.

**4.2.3. Colchicine Binding Assay<sup>86</sup>**—Inhibition of [<sup>3</sup>H]colchicine binding to tubulin was determined using 100 µL reaction mixtures, each containing 1.0 µM tubulin, 5.0 µM [<sup>3</sup>H]colchicine (from Perkin-Elmer), 5% (v/v) dimethyl sulfoxide, potential inhibitors at 1.0 or 5.0 µM and components demonstrated to stabilize the colchicine binding activity of tubulin<sup>86</sup> (1.0 M monosodium glutamate [adjusted to pH 6.6 with HCl in a 2.0 M stock solution], 0.5 mg/mL bovine serum albumin, 0.1 M glucose-1-phosphate, 1.0 mM MgCl<sub>2</sub>, and 1.0 mM GTP). Incubation was for 10 min at 37 °C, a time point at which the binding reaction in the control is 40-60% complete. Reactions were stopped by adding 2.0 mL of ice-cold water and placing the samples on ice. Each sample was poured onto a stack of two DEAE-cellulose filters, followed immediately by 6 mL of ice-cold water, and the water was aspirated under reduced vacuum. The filters were washed three times with 2 mL of water and, following removal of excess water under a strong vacuum, placed into vials containing 5 mL of Biosafe II scintillation cocktail. Samples were counted the next day in a Beckman scintillation counter. Samples with potential inhibitors were compared to controls with no inhibitor to determine percent inhibition. All samples were corrected for the amount of colchicine that bound to the filters in the absence of tubulin.

**4.2.4. *In Vitro* LAP Cleavage Assay**—LAP (from porcine kidney microsomes) was purchased from Sigma-Aldrich or Calzyme Laboratories Inc, and its activity (enzyme units) was determined with 24 mM *L*-leucine *p*-nitroanilide as substrate in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C.<sup>5</sup> In preliminary experiments, the extent of cleavage of individual amino acid prodrug conjugates were determined. In a general procedure, LAP (appropriate amount of units) was pre-incubated in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C. The enzymatic reaction was initiated by the addition of substrate. After varying periods of incubation, a small portion of the reaction mixture was quenched by the addition of acetonitrile on ice and centrifuged. The resulting supernatant was diluted by HPLC solvent (acetonitrile/water) and filtered through a 0.2 µm nylon syringe filter and analyzed by HPLC (Agilent Technologies 1200 series) with an Agilent ZORBAX Eclipse XDB-18 column (4.6 × 150 mm, 5 µm). HPL operating conditions were: mobile phase, acetonitrile/water containing 0.05% TFA, flow rate, 1.0 mL/min; column temperature, 22 °C; diode array UV detector. For rate studies, aliquots were withdrawn from incubation mixtures at different time points (1-15 min), and they were immediately quenched with acetonitrile and analyzed by HPLC (see supplemental data for more information). Standard calibration curves were used to determine the concentration of individual amino acid prodrug conjugates and their parent compounds.<sup>87</sup>

**4.2.5. *In Vitro* HUVEC Tubule Disruption Assay**—A layer of Matrigel<sup>TM</sup> (9.5 mg/mL) was plated manually into each well of a 24-well plate, which was then placed into an incubator at 37 °C for 60 min prior to cell plating. HUVECs (124,000 cells suspended in 300 µL of M200 growth factors) were plated into 24 wells and allowed to incubate at 37 °C for 16 h. The incubated HUVECs were treated with varying concentrations of inhibitors

(0.01  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 1.0  $\mu\text{M}$ ), and a photographic record was obtained (9 fields/well, 40x objective).

**4.2.5. *In Vivo* Tumor Model<sup>78</sup>**—Human breast cancer cells, MDA-MB-231(ATCC), were transfected with a lentivirus containing a firefly luciferase reporter. Induction of tumors was carried out by injecting  $10^6$  cells mixed with 30% Matrigel<sup>TM</sup> (BD Biosciences, San Jose, CA) into the mammary fat pads of female SCID mice (UTSW breeding colony). Tumors were allowed to grow to about 5 mm in diameter, determined by calipers, before BLI and assessment of VDAs. All animal procedures were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

**4.2.6. *In Vivo* Bioluminescence Imaging (BLI)<sup>79</sup>**—BLI was performed as described previously.<sup>79</sup> Briefly, anesthetized, tumor bearing mice ( $\text{O}_2$ , 2% isoflurane, Henry Schein Inc., Melville, NY) were injected subcutaneously in the fore-back neck region with 80  $\mu\text{L}$  of a solution of luciferase substrate, *D*-luciferin (sodium salt, 120 mg/kg, in saline, Gold Biotechnology, St. Louis, MO). Mice were maintained under anesthesia (2% isoflurane in oxygen, 1  $\text{dm}^3/\text{min}$ ), while baseline BLI was performed using a Xenogen IVIS<sup>®</sup> Spectrum (Perkin-Elmer, Alameda, CA). A series of BLI images was collected over 35 min using the following settings: auto exposure time, f-stop = 2, Field of view = D, binning = 4 (medium). Light intensity-time curves obtained from these images were analyzed using Living Image<sup>®</sup> software. Mice were injected intraperitoneally with either 120  $\mu\text{L}$  of saline (vehicle), CA4P (provided by OXiGENE at 120 mg/kg in saline as used previously<sup>9</sup>) compound **10** (80 mg/kg), or compound **44** (5, 10, 15, or 20 mg/kg) in saline immediately after baseline BLI. Bioluminescence imaging was repeated, with new luciferin injections 2 and/or 4 and 24 h later. Dosing with 20 and 30 mg/kg was repeated in a separate cohort of mice. Analysis of variance was used to compare the maximum BLI light intensity observed on each occasion with respect to drug administration. Time-point and significance assessed using Fisher's PLSD (protected least squares difference) using Statview software with  $p < 0.05$  considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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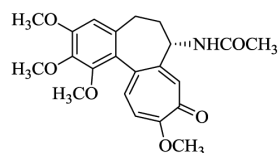
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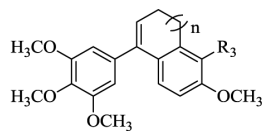
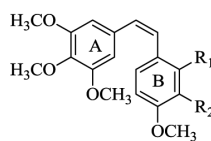
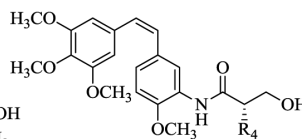
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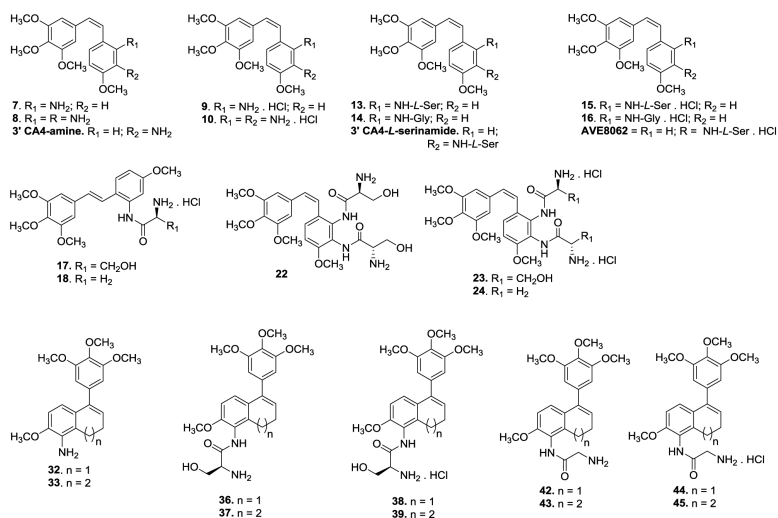


Colchicine

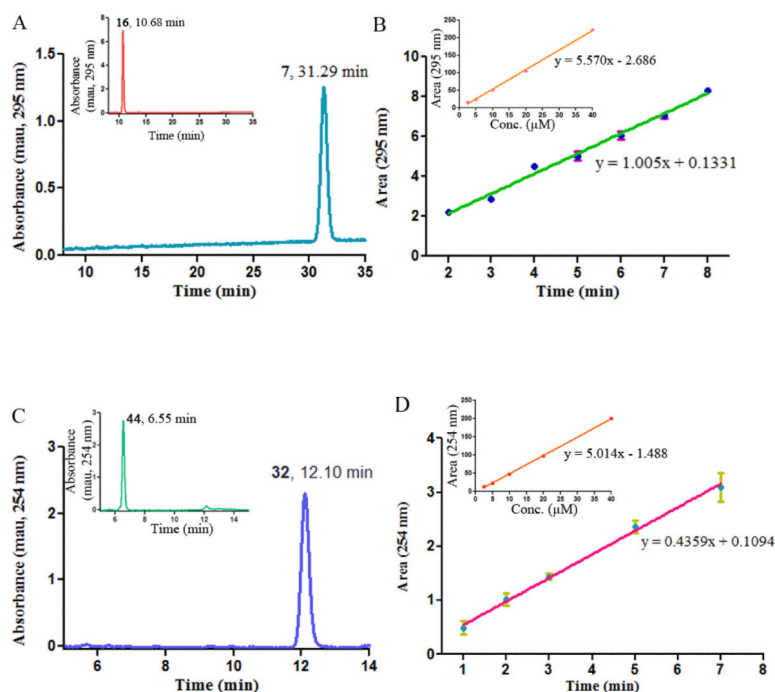
**Fused-Ring Analogues**Dihydronaphthalene phenol (OXi6196):  $n = 1$ ;  $R_3 = \text{OH}$ Dihydronaphthalene amine (KGP05):  $n = 1$ ;  $R_3 = \text{NH}_2$ Benzosuberene phenol (KGP18):  $n = 2$ ;  $R_3 = \text{OH}$ Benzosuberene amine (KGP156):  $n = 2$ ;  $R_3 = \text{NH}_2$ **Combretastatin Natural Products and Analogues**Combretastatin A-4 (CA4) :  $R_1 = \text{H}$ ;  $R_2 = \text{OH}$ Combretastatin A-4P (CA4P) :  $R_1 = \text{H}$ ;  $R_2 = \text{OPO}_3\text{Na}_2$ Combretastatin A-1 (CA1) :  $R_1 = R_2 = \text{OH}$ Combretastatin A-1P (OXi4503) :  $R_1 = R_2 = \text{OPO}_3\text{Na}_2$ 2' CA4-amine:  $R_1 = \text{NH}_2$ ;  $R_2 = \text{H}$ CA1-diamine:  $R_1 = R_2 = \text{NH}_2$ 3' CA4-amine:  $R_1 = \text{H}$ ;  $R_2 = \text{NH}_2$ 3' CA4-*L*-serinamide:  $R_4 = \text{NH}_2$ AVE8062:  $R_4 = \text{NH}_2 \cdot \text{HCl}$ 

**Figure 1.** Colchicine and natural and synthetic combretastatin, dihydronaphthalene, and benzosuberene analogues



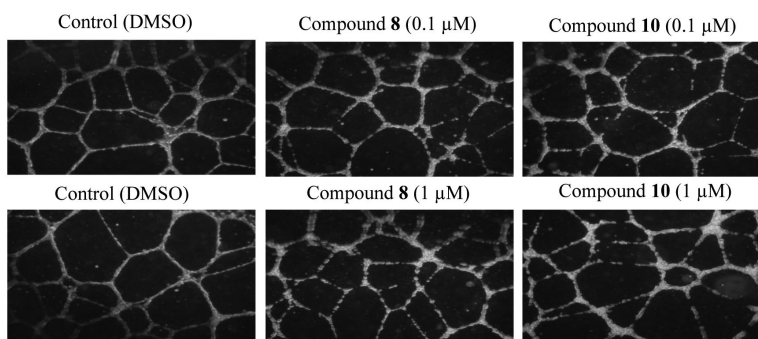


**Figure 2.**  
 Compilation of parent drugs and their amino-acid prodrug conjugates evaluated in this study

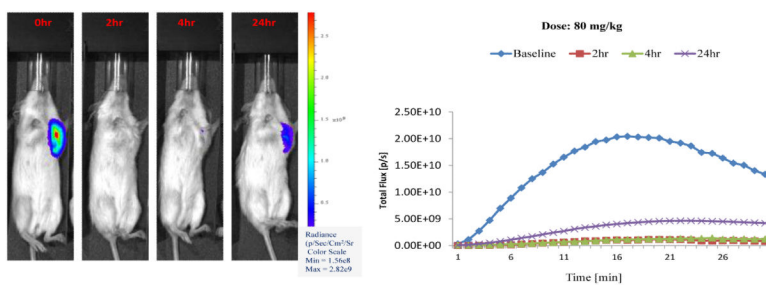


**Figure 3.**

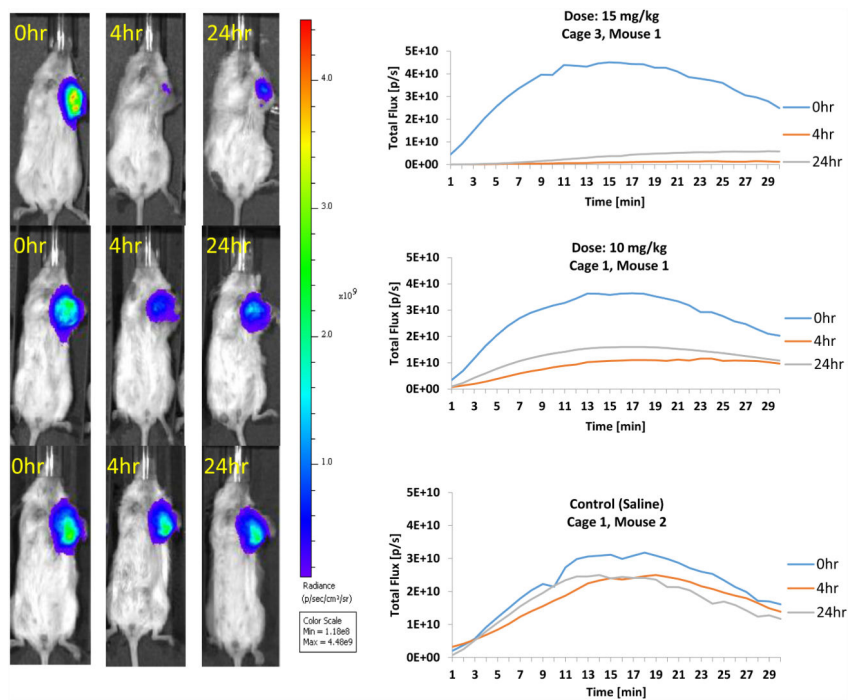
Cleavage of the glycine conjugates of 2' CA4-amine **16** and the amino dihydronaphthalene **44** by LAP. (A) Prodrug **16** was treated with 0.5 units of LAP for 40 h. A single peak corresponding to the product 2' CA4 amine **7** ( $t_R = 31.29$  min) was observed (HPLC chromatogram mobile phase: 26% acetonitrile/74% water containing 0.05% TFA). Inset: Control, compound **16** was incubated for 40 h without LAP. (B) Rate study for the cleavage of prodrug **16** to form 2' CA4 amine **7**. Inset: Calibration curve for 2' CA4 monoamine **7**. (C) Prodrug **44** was treated with 0.05 units of LAP for 2 h. A single peak corresponding to the product amino dihydronaphthalene **32** ( $t_R = 12.10$  min) was observed (HPLC chromatogram mobile phase: 28% acetonitrile/72% water containing 0.05% TFA). Inset: Control, compound **44** was incubated for 2 h without LAP. (D) Rate study for the cleavage of prodrug **44** to form amino dihydronaphthalene **32**. Inset: Calibration curve for amino dihydronaphthalene **32**



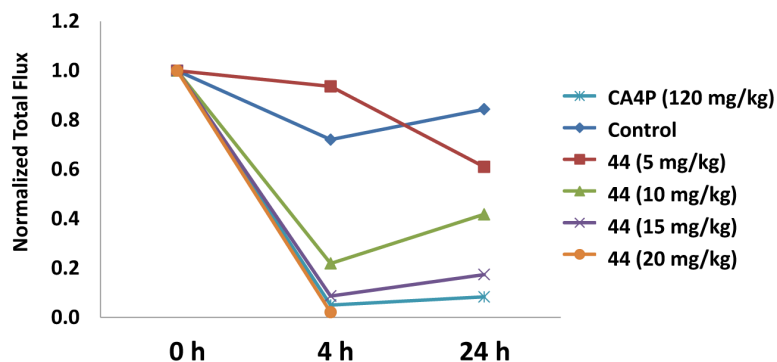
**Figure 4.**  
HUVEC Tubule Disruption at Various Inhibitor Concentrations



**Figure 5.**  
Dynamic BLI of Tumor in Response to Treatment with Compound 10.

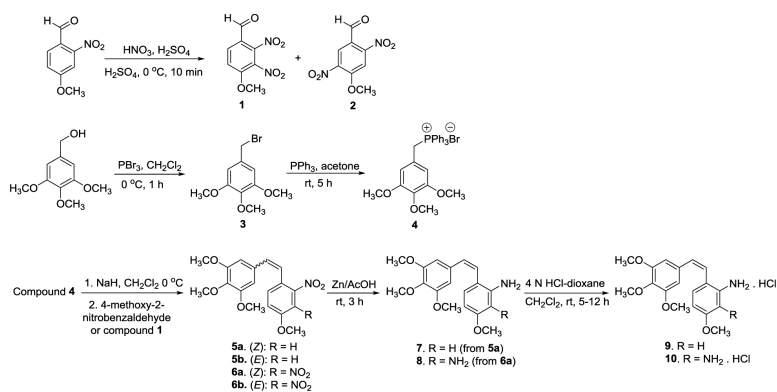


**Figure 6.**  
Dynamic BLI of Tumor in Response to Treatment with Compound 44.

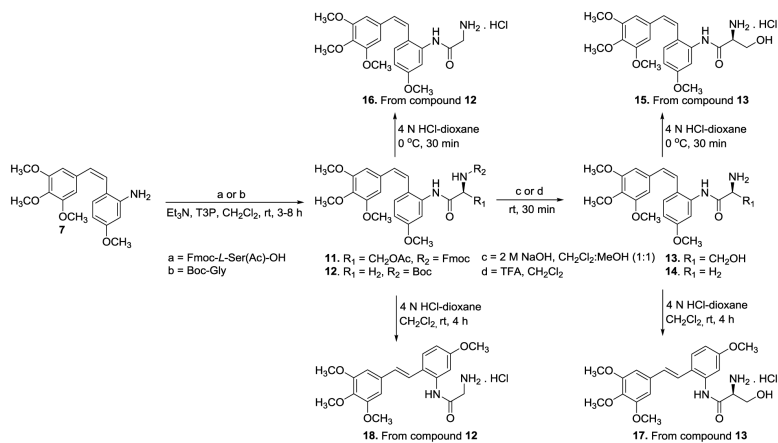


**Figure 7.**

Dose Escalation Data for BLI Evaluation of Compound **44**. CA4P and compound **44** (at 10 or 15 mg/kg) each gave significantly less signal at both 4 h and 24 h compared with baseline. Signal from the saline group was significantly reduced at the 4 h time point. At the 4 h time point compound **44** (5 mg/kg) was not significantly different from saline control, but was significantly different ( $p < 0.05$ ) from compound **44** (10 mg/kg) and compound **44** (15 mg/kg). Similarly compound **44** (15 mg/kg) was significantly different from saline and compound **44** (5 mg/kg), but not compound **44** (10 mg/kg). In general it appeared that a dose of 10, but not 5 mg/kg, generated a significantly greater response, as judged by reduced light emission. CA4P was significantly different from saline, compound **44** (5 mg/kg), and compound **44** (10 mg/kg), but not compound **44** (15 mg/kg) or compound **44** (20 mg/kg)

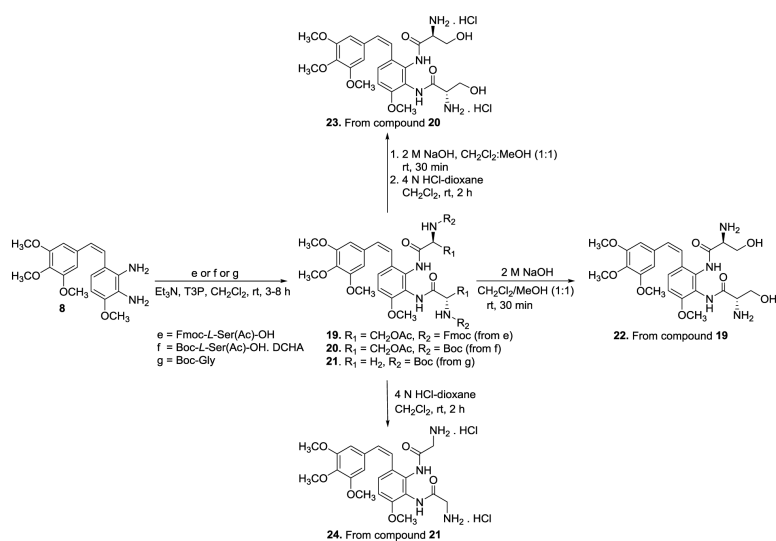


**Scheme 1.**  
 Synthesis of Combretastatin Amines<sup>15,16</sup>

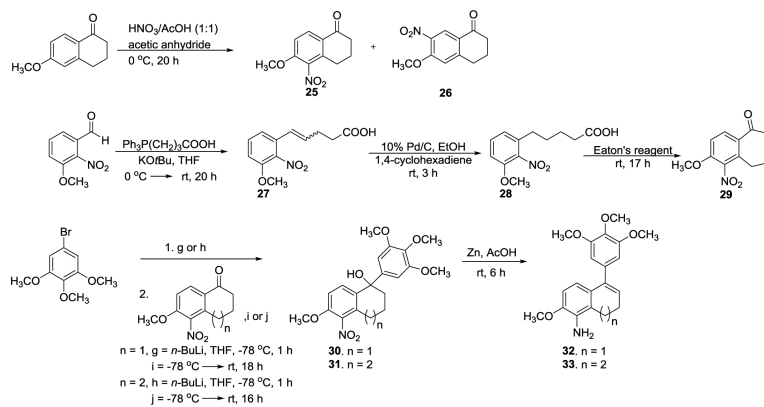


**Scheme 2.**  
 Synthesis of Amino Acid Prodrug Conjugates of 2'-Combretastatin Amines<sup>15,53</sup>

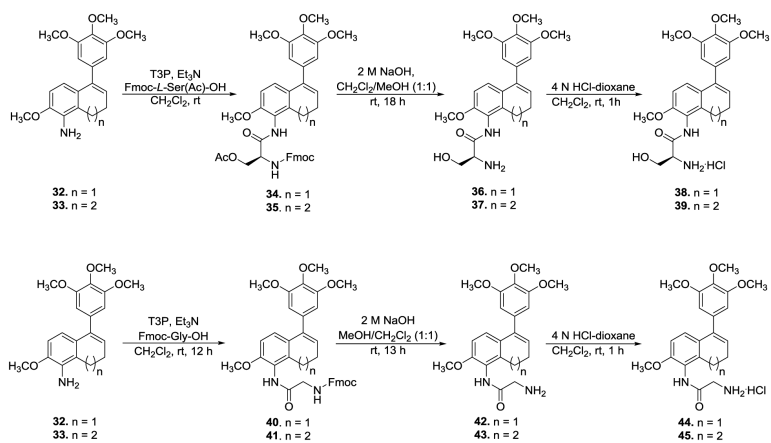




**Scheme 3.**  
 Synthesis of Amino Acid Prodrug Conjugates of Combretastatin Diamine<sup>15,16,53</sup>



**Scheme 4.** Synthesis of Dihydronaphthalene<sup>42,44</sup> and Benzosuberene Amines<sup>42,45–47</sup>

**Scheme 5.**

Synthesis of Amino Acid Prodrug Conjugates of Amino Dihydronaphthalene and Benzosuberene<sup>45,47</sup> [Note: A portion of Scheme 5 was reproduced from reference 47 with permission from Elsevier.]

**Table 1**

Cytotoxicity data for the amino-based combretastatin, dihydronaphthalene, and benzosuberene analogues against human cancer cell lines [SK-OV-3 (ovarian), NCI-H460 (lung), and DU-145 (prostate)]

Compound	GI <sub>50</sub> (nM) SRB Assay <sup>a</sup>		
	SK-OV-3	NCI-H460	DU-145
CA1 <sup>b</sup>	38.4±24.2	15.3±15.8	32.6±17.3
CA4 <sup>c</sup>	5.06±0.145	5.00±0.359	6.02±0.661
3' CA4-amine <sup>d</sup>	4.45±0.870	4.49±0.894	3.30±0.281
3' CA4-L-serinamide <sup>d</sup>	9.02±4.81	24.1±2.00	16.4±7.29
AVE8062 <sup>d</sup>	3.96±0.912	3.87±0.297	3.34±0.261
7 <sup>e</sup>	26.6±22.6	40.1±17.1	33.1±21.4
8 <sup>f</sup>	23.5±23.4	35.9±21.8	39.1±26.5
9	7.35±3.50	26.3±16.1	28.5±23.2
10 <sup>f</sup>	22.8±15.2	124±116	205±241
13 <sup>e</sup>	39.5±21.1	33.3±19.0	34.7±8.27
14	32.3±6.38	53.2±5.23	43.6±2.43
15 <sup>e</sup>	51.6±6.88	31.7±4.87	48.4±3.36
16	48.5±8.53	29.2±5.67	44.7±3.53
17	2290±1170	2240±1450	4550±2700
18	427±111 (D)	418±137 (D)	630±306 (D)
	426±94.1 (W)	380±60.2 (W)	674±255 (W)
22	25900±2550	24400±4420	33600±2600
23	>86600	>86600	>86600
24	39300±13500 (D)	>68500 (D)	>64700 (D)
	>56400 (W)	>96600 (W)	>90400 (W)
32 <sup>g</sup>	0.308±0.402	0.290±0.126	0.111±0.114
33 <sup>h</sup>	0.137±0.0596	1.85±1.87	2.26±1.58
36	13700±9700	31100±9940	40600±32300
37	44900±4100	45900±1790	>81300
38	11600±10200	18600±7670	26000±15700
39	47000±21400	53800±864	69400±31300
42 <sup>i</sup>	13.8±7.54	36.5±0.899	50.1±4.80
43	195±31.9	566±38.2	507±188
44	48.5±32.5	42.0±3.72	58.5±12.0
45	203±29.4	567±56.6	773±302

D = compound dissolved in DMSO. W = compound dissolved in water

<sup>a</sup> Average of n = 3 independent determinations.

<sup>b</sup> Data from ref.<sup>74</sup>, see ref. 14 for additional data.

<sup>c</sup> For additional data, see ref. 14

<sup>d</sup> For additional data, see ref. 15,36,37,53

<sup>e</sup> For additional data, see ref. 15

<sup>f</sup> For additional data, see ref. 16

<sup>g</sup> For additional data, see ref. 42,43

<sup>h</sup> For additional data, see ref. 46,47

<sup>i</sup> For additional data, see ref. 47

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**Table 2**

Inhibition of tubulin polymerization and percent inhibition of colchicine binding

Compound	Inhibition of Tubulin Polymerization IC <sub>50</sub> (±M) ±SD	Inhibition of Colchicine Binding (% Inhibition±SD)	
		1 μM	5 μM
CA1	1.9 <sup>a</sup>	ND	99.6±0.7 <sup>b</sup>
CA4	1.2±0.049	84±1.1	98±0.10
3' CA4-amine <sup>c</sup>	1.2±0.056 <sup>c</sup>	84±1.8	97±1.9
3' CA4-L-serinamide <sup>c</sup>	14±1.1 <sup>d</sup>	ND	ND
AVE8062 <sup>c</sup>	13 ± 0.28 <sup>d</sup>	ND	ND
7	1.1±0.057 <sup>d</sup>	55±3.8	88±0.93
8	1.5±0.13 <sup>e</sup>	55±0.14	87±0.19
9	2.5±0.16	ND	75±0.90
10	2.3±0.21 <sup>e</sup>	46±2.3	86±3.8
32 <sup>f</sup>	0.62±0.019	65±3.6	92±0.16
33	1.3±0.11 <sup>g</sup>	ND	82±2.4

ND = Not determined for this study

<sup>a</sup>Data from ref. 16, see ref. 14 for additional data.<sup>b</sup>Data from ref. 14<sup>c</sup>For additional data, see ref. 15,37<sup>d</sup>For additional data, see ref. 15<sup>e</sup>For additional data, see ref. 16<sup>f</sup>For additional data, see refs. 42,43<sup>g</sup>For additional data, see refs. 46,47

**Table 3**

Activity of LAP toward amino acid prodrug conjugates

Compound	Prodrug Type	% Cleavage <sup>a</sup>	Specific Activity of LAP (μM/min/enzyme unit)
15	Serinamide	62%	1.2
16	Glycinamide	100%	180
17	Serinamide	90%	C <sup>e</sup>
18	Glycinamide	100%	C <sup>e</sup>
22	Serinamide	NC	ND
23	Serinamide	NC	ND
24	Glycinamide	C <sup>b</sup>	ND
38	Serinamide	C <sup>c</sup> , <10%	ND
39	Serinamide	C <sup>c</sup> , <10%	ND
44	Glycinamide	100%	15.5
45	Glycinamide	100%	0.12
3' CA4-L-serinamide	Serinamide	C <sup>d</sup> , 95%	ND
AVE8062	Serinamide	C <sup>d</sup> , 95%	ND

ND: Not determined

<sup>a</sup>Percentage of cleavage is calculated based on the area ratio of the parent drug and prodrug at a specific wavelength. NC: No cleavage observed using 1.5 units LAP. C: Cleaved (a rate study was not carried out).

<sup>b</sup>, disappearance of the bis-glycinamide prodrug **24** was observed, but less than 50% of the expected final product **8** was detected.

<sup>c</sup>, the serinamide prodrug was cleaved, but less than 10% of the expected product was obtained.

<sup>d</sup>, used as positive controls.<sup>52</sup>

<sup>e</sup>, the prodrug was readily cleaved, but the absolute rate could not be determined because the parent compound (*E*-isomer of 2' CA4-amine) was not available since isomerization (*Z* to *E*) took place as a byproduct during salt formation after installation of the amino acid prodrug).