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Bioreductively activatable prodrug conjugates of phenstatin designed to target tumor hypoxia

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ABSTRACT

A variety of solid tumor cancers contain significant regions of hypoxia, which provide unique challenges for targeting by potent anticancer agents. Bioreductively activatable prodrug conjugates (BAPCs) represent a promising strategy for therapeutic intervention. BAPCs are designed to be biologically inert until they come into contact with low oxygen tension, at which point reductase enzyme mediated cleavage releases the parent anticancer agent in a tumor-specific manner. Phenstatin is a potent inhibitor of tubulin polymerization, mimicking the chemical structure and biological activity of the natural product combretastatin A-4. Synthetic approaches have been established for nitrobenzyl, nitroimidazole, nitrofuranyl, and nitrothienyl prodrugs of phenstatin incorporating *nor*-methyl, *mono*-methyl, and *gem*-dimethyl variants of the attached nitro compounds. A series of BAPCs based on phenstatin have been prepared by chemical synthesis and evaluated against the tubulin-microtubule protein system. In a preliminary study using anaerobic conditions, the *gem*-dimethyl nitrothiophene and *gem*-dimethyl nitrofuran analogues were shown to undergo efficient enzymatic cleavage in the presence of NADPH cytochrome P450 oxidoreductase. Each of the eleven BAPCs evaluated in this study demonstrated significantly reduced inhibitory activity against tubulin in comparison to the parent anti-cancer agent phenstatin (IC_{50} = 1.0 μM). In fact, the majority of the BAPCs (seven of the eleven analogues) were not inhibitors of tubulin polymerization (IC_{50} > 20 μM), which represents an anticipated (and desirable) attribute for these prodrugs, since they are intended to be biologically inactive prior to enzyme-mediated cleavage to release phenstatin.

Tumor-associated vasculature has emerged as a promising target for anti-cancer therapies due to its marked differences from vasculature feeding healthy tissue.[1–10] Vasculature associated with healthy tissue forms a well-organized delivery network for oxygen and nutrients to cells.[1–10] In contrast, tumor-associated vasculature is forced to develop rapidly to meet the enhanced demand for significant amounts of nutrients and oxygen required by tumors.[1–10] The rapid growth of tumor-associated vasculature results in compromised structural integrity, which is characterized by weakened vessel walls, increased interstitial pressure, blind ends, and bulges.[1–10] Tumor-associated vasculature is generated rapidly, leading to groups of vessels spaced far apart from each other, and this results in regions of hypoxia that develop when this distance is greater than the diffusion distance of oxygen.[1–10]

One promising therapeutic option for targeting tumor-associated vasculature involves treatment with vascular targeting agents (VTAs), which include angiogenesis-inhibiting agents (AIAs) and vascular disrupting agents (VDAs).[1–10] AIAs, which represent a fairly well investigated therapeutic strategy, act by inhibiting angiogenesis, the formation of new tumor-associated vasculature, while leaving existing vessels intact.[4,11,12] Inhibition of angiogenesis limits tumor growth and also leads to increased blood flow in the remaining vasculature, allowing for increased delivery of

Abbreviations: BAPC, bioreductively activatable prodrug conjugate; CA1, combretastatin A-1; CA4, combretastatin A-4; Et_3N, triethylamine; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; MeOH, methanol; $NaBH_4$, sodium borohydride; TLC, thin layer chromatography; THF, tetrahydrofuran; EtOAc, ethyl acetate; POR, NADPH cytochrome P450 oxidoreductase; DMP, Dess-Martin periodinane; DEAD, diethylazodicarboxylate; DIAD, diisopropyl azodicarboxylate; ADDP, 1,1′-(azodicarbonyl)dipiperidine.

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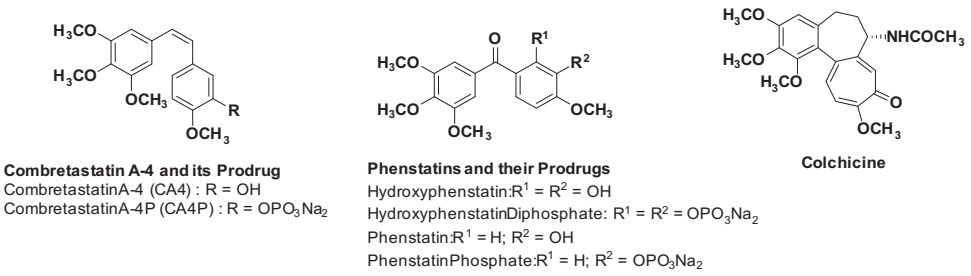


Fig. 1. Colchicine, phenstatin, and combretastatin natural products and their corresponding phosphate salts.

chemotherapy and potentially enhanced tumor damage from radiotherapy to an otherwise difficult therapeutic target due to hypoxia.[4,11,12] VDAs impact tumor vasculature from a mechanistic approach that is distinct from AIAs. One subset of VDAs is comprised of small-molecule inhibitors of tubulin polymerization that target existing tumor-associated vasculature by causing rapid morphology changes (flat to round) of the endothelial cells lining these vessels.[1–10] This leads to irreversible vessel damage and tumor necrosis.[1–10]

A representative clinically relevant small-molecule VDA that disrupts microtubule formation is the natural product combretastatin A-4 (CA4) (Fig. 1). First isolated from the African bush willow tree *Combretum caffrum Kuntze* by the Pettit group, CA4 is a potent inhibitor of tubulin polymerization, functioning through a binding interaction at the colchicine site on tubulin.[13–17] Its corresponding water-soluble phosphate prodrug salt [combretastatin A-4P (CA4P)] has reached advanced clinical trials as a promising VDA.[18–22] However, no small-molecule VDA has yet been approved by the FDA.

Phenstatin, originally synthesized by the Pettit group in 1998, is another potent inhibitor of tubulin polymerization with pronounced anti-cancer activity against human cancer cell lines (Fig. 1).[23,24] Phenstatin was discovered serendipitously by the Pettit group during an attempt to prepare a CA4 analog bearing an epoxide moiety as a replacement for the ethylene bridge.[23] The ketone functionality of phenstatin was the surprising result of a Jacobsen epoxidation reaction intended to form an epoxide from the corresponding olefin.[23] Phenstatin mirrors the biological mechanism of action of CA4, disrupting microtubule assembly through a binding interaction with the colchicine site on tubulin.[24] Phenstatin and its corresponding water-soluble phosphate salt prodrug counterpart demonstrate pronounced cytotoxicity against human cancer cell lines.[24] While hydroxyphenstatin, the diol analog of phenstatin, displays potent inhibition of tubulin polymerization, its diphosphate salt analog is less active against in vitro cancer cell lines and as an inhibitor of tubulin polymerization than its phenstatin phosphate counterpart.[24]

Solid tumors represent inherently challenging therapeutic targets due, in part, to the significant differences between vasculature feeding healthy tissue versus tumor-associated vasculature, which is a contributing factor to the profound regions of hypoxia that often characterize tumors.[3,8,25–36] A combination of blind ends, leaky vessel walls, occlusions, and kinked vessels, along with the increased average distance between capillaries, leads to hypoxic regions where many common radiotherapies and chemotherapeutic options are less effective.[8,25–36] The challenges created by hypoxia and large diffusion distances offer a unique opportunity for targeted therapeutic intervention.[8,25] Bioreductively activatable prodrug conjugates (BAPCs) represent a possible hypoxia-activated treatment method.[8,25] BAPCs are designed to be activated by reductase enzymes, such as NADPH cytochrome P450 oxidoreductase (POR), in regions of hypoxia in the tumor microenvironment, releasing a potent anti-cancer agent in a tumor-specific manner.[8,25]

A series of BAPCs utilizing CA4 as the parent anticancer agent and incorporating *nor*-, *mono*-, and *gem*-dimethyl nitrothiophene triggers was reported in 2006 by Davis and co-workers.[37,38] The CA4-BAPC bearing the *gem*-dimethyl nitrothiophene trigger proved to be the most active of the trio, demonstrating the greatest resistance to cleavage under normoxic conditions *in vitro*, in effect displaying a high selectivity for low-oxygen tumor environments.[37] While the *nor*-methyl nitrothiophene BAPC was only activated at very low oxygen concentrations (<0.01% O_2), the bioreductive triggers for *mono*-methyl and *gem*-dimethyl BAPCs were cleaved over a greater range of oxygen concentrations.[37] The *gem*-dimethyl CA4-BAPC was significantly more effective in hypoxic environments in vitro compared to the *nor*- and *mono*-methyl CA4-BAPCs, releasing approximately 50% CA4 at 0.5% O_2 with the aid of POR.[37]

The mechanism of cleavage (under hypoxia) for the nitrothiophene trigger begins with a one electron reductase such as POR reducing the nitro group on the trigger (Fig. 2).[37] Once the nitro group on the trigger has been reduced, an electron cascade through the thiophene ring leads to trigger detachment, releasing the active VDA (CA4 in this example).[37] In comparison, under normal oxygen tension, the species obtained after the initial one-electron reduction is simply re-oxidized (by molecular oxygen) and thus does not lead to cleavage.[37]

Although no BAPC has yet been approved by the FDA, two BAPCs that have reached advanced clinical trials are TH-302 and PR-104. TH-302 (from Threshold Pharmaceuticals) is a 2-nitroimidazole based BAPC attached to the DNA alkylating agent bromoisophosphoramide (Fig. 3).[39,40] The prodrug is activated by one electron reductases such as POR, reducing the nitro group on the nitroimidazole trigger in a similar mechanistic pathway to the nitrothienyl trigger cleavage.[8] Highly effective in *in vitro* studies and early *in vivo* studies in mice with hypoxia cytotoxicity ratios (HCR) as high as 600, TH-302 has advanced to Phase III clinical trials after successful Phase I and II studies, although the results of the first Phase III trial were not statistically significant.[41–43] PR-104 (Fig. 3), synthesized and biologically evaluated by Wilson et al. at the University of Auckland, is a nitroaromatic preprodrug of PR-104A, eventually being reduced to the active forms PR104H and PR-104M.[8,44–46] The cytotoxicity of PR-104M derives from its ability to form interstrand DNA crosslinks.[8] PR-104 was taken into Phase I and II clinical trials, yielding promising results in Phase I trials but stalled at Phase II due to dose-limiting toxicity and overall efficacy issues.[8,47,48]

Intrigued by the concept of targeting tumor hypoxia with BAPCs, a series of such prodrugs were prepared by chemical synthesis based on the unique tubulin-active anticancer agent, phenstatin. Utilizing a combination of synthetic pathways previously described in the literature along with our modifications designed to improve yield and reaction efficiency, a selected subset of *nor*-, *mono*-, and *gem*-dimethyl nitrothiophene, nitrobenzyl,

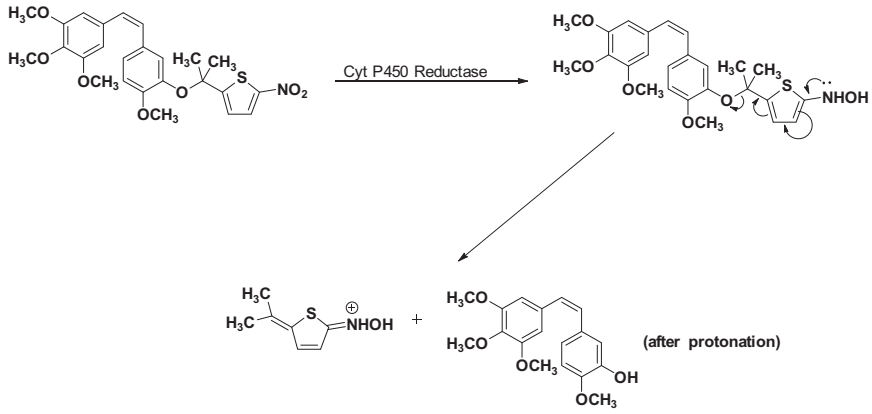


Fig. 2. *gem*-Dimethyl nitrothienyl trigger release from CA4.[37]

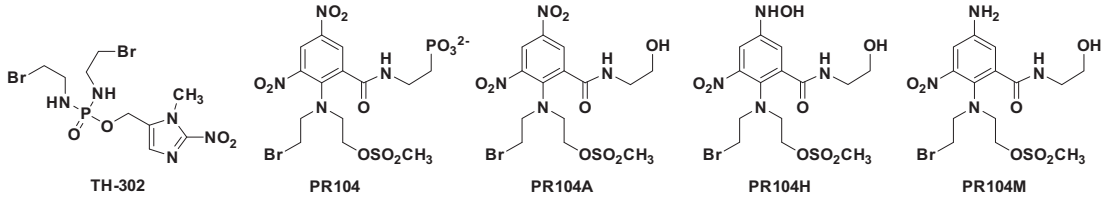


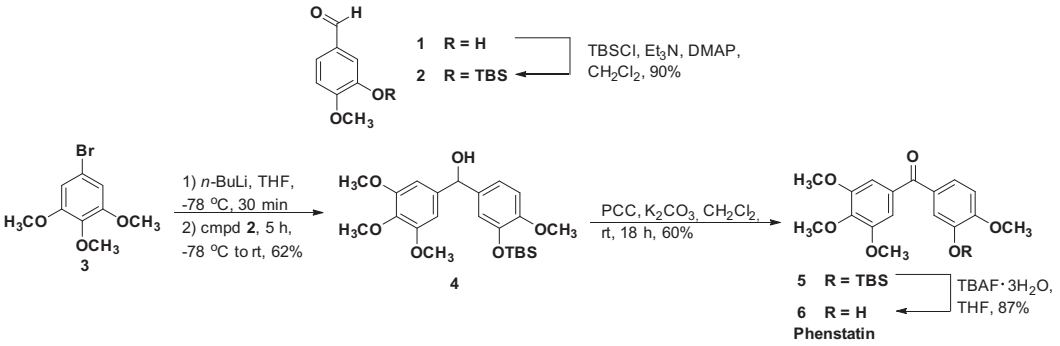
Fig. 3. TH-302, PR104, PR104A, PR104H, and PR104M.[8]

nitroimidazole, and nitrofuran triggers were synthesized and linked to phenstatin.[37,38] Preliminary biological assessment of these phenstatin BAPCs evaluated their ability to inhibit tubulin polymerization, and a subset were evaluated for their suitability as substrates for enzymatic-mediated cleavage to release the parent anticancer agent, phenstatin.

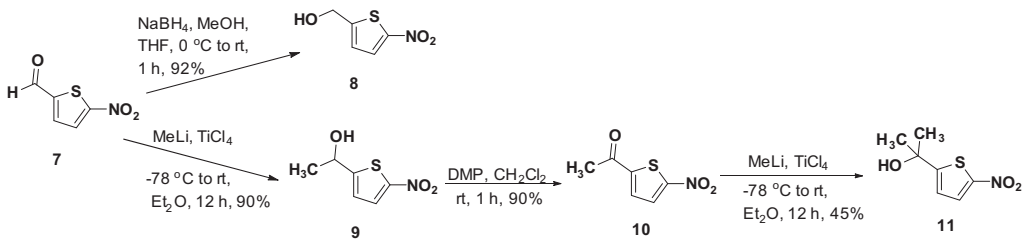
While each of the phenstatin-based BAPCs represents a new chemical entity, phenstatin (Scheme 1) and the prodrug triggers were synthesized utilizing methodology previously described.[23,49] Isovanillin was protected as its corresponding *tert*-butyldimethylsilyl ether, aldehyde **2**.[23,49] Halogen-metal exchange of aryl bromide **3**, followed by the introduction of aldehyde **2**, afforded the secondary alcohol **4**, which, upon oxidation, generated phenstatin precursor **5**.[23,49] Removal of the TBS protecting group yielded phenstatin **6**.[23,49]

The *nor*-methyl nitrothiophene trigger **8** was generated in excellent yield through reduction of aldehyde **7**, as reported by Davis et al. (Scheme 2).[37] The Davis route to the *gem*-dimethyl nitrothiophene trigger **11** proved less effective in our hands, generating the product but only in low yield. This motivated us to consider a modified synthetic methodology toward the *gem*-dimethyl trigger **11**. Methylation conditions described by Reetz et al. provided an improved synthetic route that generated both *mono*- and *gem*-dimethyl nitrothiophene triggers from aldehyde **7** in good yield.[50] *Mono*-methyl trigger **9** was synthesized through methylation of aldehyde **7** with methyllithium and titanium tetrachloride.[50] Oxidation with Dess-Martin periodinane (DMP) generated ketone **10** in high yield, and further methylation of ketone **10** furnished the *gem*-dimethyl trigger **11** (Scheme 2).[50,51] Further investigation determined that trimethyl aluminum was a more effective methylation agent for the conversion of aldehyde **7** and ketone **10** to their corresponding *mono*- and *gem*- triggers **9** and **11**, respectively, in comparison to the methyllithium/titanium tetrachloride method (see Supplementary data).

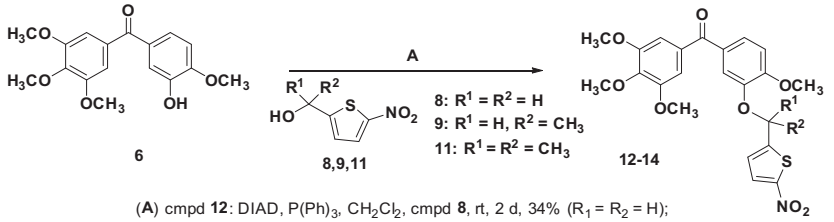
A Mitsunobu reaction was utilized to conjugate the bioreductive triggers to phenstatin to generate the requisite BAPCs (Scheme 3).[37,49,52,53] Depending on the reactivity of the bioreductive triggers involved in each reaction, a combination of either diethyl azodicarboxylate (DEAD), diisopropyl azodicarboxylate (DIAD), or 1,1′-(azodicarbonyl)dipiperidine (ADDP) and triphenylphosphine or tributylphosphine were employed to generate the ether linkage.[37,52,53] Synthesis of the phenstatin *nor*-methyl



Scheme 1. Synthesis of Phenstatin **6**.[23,49]

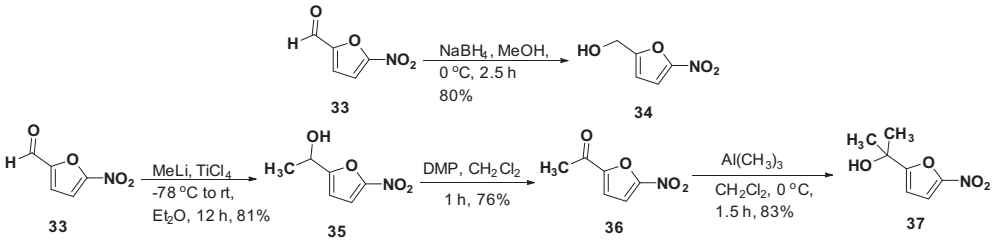


Scheme 2. Synthesis of nitrothiophene triggers.[37,50,51]



(**A**) cmpd **12**: DIAD, $P(Ph)_3$, CH_2Cl_2, cmpd **8**, rt, 2 d, 34% ($R_1 = R_2 = H$);
cmpd **13**: DIAD, $P(Ph)_3$, CH_2Cl_2, cmpd **9**, rt, 2 d, 30% ($R_1 = H$, $R_2 = CH_3$);
cmpd **14**: ADDP, $P(Bu)_3$, CH_2Cl_2, cmpd **11**, rt, 2 d, 7% ($R_1 = R_2 = CH_3$);

Scheme 3. Synthesis of the phenstatin nitrothiophene BAPCs.[37,49,52,53]



Scheme 4. Synthesis of the nitrofuran triggers.[37,50,51,64]

nitrothiophene BAPC **12** and its corresponding *mono*-methyl BAPC **13** involved the reaction of phenstatin with nitrothiophene **8** (or **9**), DIAD, and triphenylphosphine.[37,49] The phenstatin *gem*-dimethyl BAPC **14** was synthesized in a Mitsunobu reaction utilizing ADDP, tributylphosphine, phenstatin, and nitrothiophene trigger **11**.[37,49]

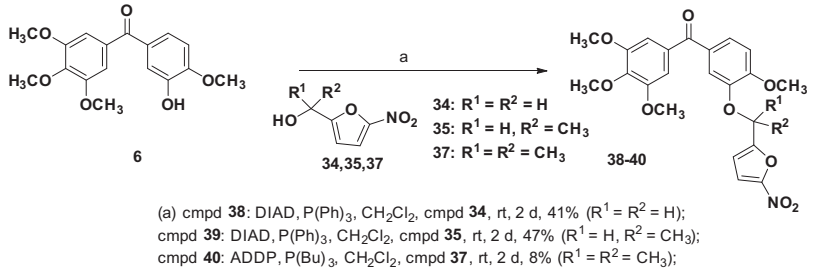
The phenstatin nitrobenzyl BAPCs were synthesized in a similar fashion to the phenstatin nitrothiophene BAPCs, utilizing a Mitsunobu reaction to generate the critical ether linkage (Scheme S1, Supplementary data).[37,49,52,53] Synthesis of the *nor*-methyl nitrobenzyl BAPC **20** and the *mono*-methyl nitrobenzyl BAPC **21** was achieved through the reaction of phenstatin, DIAD, and triphenylphosphine, with the appropriate trigger (4-nitrobenzyl alcohol **19** or *mono*-methyl trigger **16**, respectively).[37,49] A reaction of tributylphosphine, ADDP, phenstatin, and the *gem*-dimethyl nitrobenzyl trigger **18** furnished the *gem*-dimethyl nitrobenzyl BAPC **22**.[37,49] The synthetic route reported by Reetz and co-workers was utilized for the synthesis of the nitrobenzyl triggers **16** and **18** (Scheme S2, Supplementary data).[50]

The nitroimidazole phenstatin BAPCs (Scheme S3, Supplementary data) were generated through a Mitsunobu reaction analogous to the chemistry described previously for the nitrobenzyl and nitrothiophene BAPCs.[37,49,51–54] The *nor*-methyl nitroimidazole BAPC **31** was synthesized utilizing a Mitsunobu reaction with phenstatin, DIAD, triphenylphosphine, and nitroimidazole **28**.[37,49] Triphenylphosphine, DIAD, phenstatin, and *mono*-methyl nitroimidazole **30** reacted to yield the *mono*-methyl nitroimidazole BAPC **32**.[37,49] The synthesis of the nitroimidazole triggers followed a route developed by Conway et al. (Scheme S4, Supplementary data).[51,54,64] Despite several attempts directed towards methylation of the nitroimidazole ketone, the *gem*-dimethyl nitroimidazole trigger was not successfully synthesized in our hands (Scheme S5, Supplementary data).[50,51,55–63]

The synthetic route for the nitrofuran bioreductive triggers (Scheme 4) was based on the new route to the nitrothiophene triggers shown in Scheme 2.[37,50,51,64] The *nor*-methyl nitrofuran trigger **34** was generated through the reduction of aldehyde **33**.[37] Aldehyde **33** was methylated to yield *mono*-methyl nitrofuran trigger **35**.[50,51] The synthesis of the *gem*-dimethyl nitrofuran trigger **37** was achieved in high yield by oxidation of *mono*-methyl trigger **35** to its corresponding ketone **36**, followed by methylation to generate the *gem*-dimethyl nitrofuran trigger **37**.[50,51]

Mitsunobu chemistry was once again employed to form the requisite ether linkage between the nitrofuran triggers and phenstatin, generating the nitrofuran BAPCs (Scheme 5).[37,49,54,64] The *nor*-methyl nitrofuran BAPC **38** and the *mono*-methyl nitrofuran BAPC **39** were generated through the reaction of phenstatin, DIAD, and triphenylphosphine with the appropriate trigger (*nor*-methyl nitrofuran **34** or *mono*-methyl nitrofuran **35**, respectively).[37,49] The *gem*-dimethyl nitrofuran BAPC **40** was likewise synthesized via a Mitsunobu reaction with nitrofuran **37**.[37,49]

The phenstatin BAPCs (Fig. 4), as well as phenstatin, were evaluated for their ability to inhibit tubulin polymerization and compete for the colchicine binding site (Table 1). In addition to the *mono*-methyl nitrothiophene BAPC **13** and the *gem*-dimethyl nitrofuran BAPC **40**, the entire nitrobenzyl series (**20**, **21**, **22**) and the nitroimidazole series (**31**, **32**) all proved to be inactive



Scheme 5. Synthesis of the phenstatin nitrofuran BAPCs.[37,49,54,64]

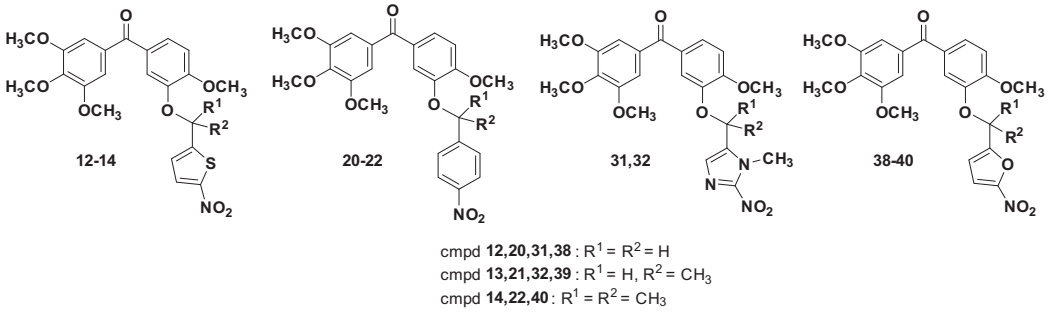


Fig. 4. Phenstatin BAPCs prepared by chemical synthesis.

Table 1
Inhibition of tubulin polymerization and percent inhibition of colchicine binding.

Compd	Inhibition of tubulin assembly IC_{50} (μM)±SD	Inhibition of colchicine binding % inhibition μM ± SD 5 μM
42 CA4	0.73 ± 0.04	98 ± 0.1
6 Phenstatin	1.0 ± 0.2[a]	85 ± 2
12	16 ± 0.6	19 ± 2
13	>20	15 ± 0.09
14	9.0 ± 1	16 ± 3
20	>20	11 ± 4
21	>20	5.8 ± 5
22	>20	8.8 ± 5
31	>20	7.1 ± 0.01
32	>20	11 ± 5
38	15 ± 1	17 ± 0.01
39	12 ± 0.5	16 ± 3
40	>20	13 ± 5

[a] Data from reference **23**.

Table 2
Bioreductive trigger hydrolysis (untreated) and cleavage of POR-Treated BAPCs.

Compd	Hydrolysis percentage in pH 7.4 phosphate buffer for 24 h	Cleavage percentage of POR-treated for 24 h[a]
14	0	100
22	0	0
40	0	100

[a] Anoxic conditions.

(IC_{50} > 20 μM) as inhibitors of tubulin polymerization. This is an important and desirable attribute for these phenstatin-based BAPCs, since they are designed to be biologically inactive until enzyme-mediated prodrug cleavage releases the active anticancer agent (phenstatin), which itself is a potent inhibitor of tubulin polymerization (IC_{50} = 1.0 μM). The other four BAPCs (**12**, **14**, **38**, **39**) evaluated in this study demonstrated significantly reduced (IC_{50} range of 9–16 μM) inhibition of tubulin polymerization, in comparison to phenstatin. The limited activity of these four BAPCs might be attributed to partial trigger cleavage under the assay conditions, leading to generation of the tubulin-active parent anticancer agent phenstatin. This hypothesis of partial cleavage has not been confirmed or further investigated to date.

Based on the pioneering work by Davis and co-workers with CA4-BAPCs, which demonstrated the potency of the *gem*-dimethyl variant (in comparison to the corresponding *nor*- and *mono*-methyl analogues), the three phenstatin-based *gem*-dimethyl BAPCs prepared in this study were subjected to further initial evaluation. A stability study carried out in pH 7.4 potassium phosphate buffer solution on the three *gem*-dimethyl trigger BAPCs (**14**, **22**, **40**) demonstrated promising results, with each BAPC remaining structurally intact over a 24 h period with no observable (by HPLC analysis) cleavage or degradation. These same three BAPCs (**14**, **22**, **40**) were treated (in separate experiments) with NADPH cytochrome P450 oxidoreductase (POR) to evaluate them as substrates for this enzyme under anoxic conditions. The *gem*-dimethyl furan and thiophene compounds (**14** and **40**, respectively) were fully cleaved over the course of 24 h, while, interestingly, the *gem*-dimethyl benzyl compound **22** did not undergo cleavage in the 24 h assay (Table 2). The reduction potential for the nitrobenzyl trigger is less than the reduction potential (less electron-philic) than that of the nitrofuran, nitroimidazole, and nitrothiophene triggers, possibly explaining its resistance to cleavage by POR under these assay conditions.[64]

In conclusion, a series of eleven promising phenstatin-based BAPCs were prepared by chemical synthesis and had little or no activity as inhibitors of tubulin assembly or binding of colchicine to tubulin, in comparison to the parent anticancer agent phenstatin, a potent tubulin inhibitor. In preliminary studies, the three phenstatin-based *gem*-dimethyl BAPCs (**14**, **22**, **40**) demonstrated aqueous solution stability (over 24 h), and two of the BAPCs (**14**, **40**) were suitable substrates for POR. These BAPCs have the potential to be therapeutic agents that target hypoxic tumor cells.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.11.093.

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