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# Targeting tumor-associated hypoxia with bioreductively activatable prodrug conjugates derived from dihydronaphthalene, benzosuberene, and indole-based inhibitors of tubulin polymerization

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A strategy for targeting tumor-associated hypoxia utilizes reductase enzyme-mediated cleavage to convert biologically inert prodrugs to their corresponding biologically active parent therapeutic agents selectively in areas of pronounced hypoxia. Small-molecule inhibitors of tubulin polymerization represent unique therapeutic agents for this approach, with the most promising functioning as both antiproliferative agents (cytotoxins) and as vascular disrupting agents (VDAs). VDAs selectively and effectively disrupt tumor-associated microvessels, which are typically fragile and chaotic in nature. VDA treatment may augment existing tumor-associated hypoxia, thus enhancing the efficacy of hypoxia-selective prodrugs. Structure activity relationship-guided studies in our laboratories led to the discovery of promising lead molecules (OXi6196, KGP05, KGP18, and OXi8006) that bind to the colchicine site on the tubulin heterodimer. A series of bioreductively activatable prodrug conjugates (BAPCs) based on these molecules was synthesized utilizing ether-linked heteroaromatic hypoxia-selective triggers bearing a nitro group. Biological evaluation against the A549 human lung carcinoma cell line (under normoxic *versus* anoxic conditions) revealed several BAPCs with positive hypoxia cytotoxicity ratios. Preliminary *in vivo* evaluation of a representative BAPC (**KGP291**) demonstrated vascular shutdown in nude mice bearing orthotopic 4T1 breast tumors studied by bioluminescence imaging.

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## 1. Introduction

Tumor-associated hypoxia[1] results, in part, from the disproportional distribution of blood vessels throughout tumor tissue, which is further augmented by the fragile and chaotic nature of these microvessels.[2] The resulting regions of low oxygen concentration promote angiogenic signaling and increase metastatic potential. Tumor microcirculation and oxygenation play critical roles in tumor growth, affecting drug delivery, metastatic spread, and responsiveness to cytotoxic chemotherapy and therapeutic irradiation.[3] Studies have shown poor prognosis and increased metastasis for patients with hypoxic tumors with respect to diverse therapies.[4] While tumor-associated hypoxia is associated with an array of challenges in the treatment of cancer, it also presents opportunities for selective drug delivery through appropriate hypoxia-selective prodrug strategies.[5–11] Most solid tumors contain hypoxic regions, and certain reductase enzymes (such as NADPH cytochrome P450 reductase) are capable of irreversibly reducing a variety of substrates under the hypoxic conditions of these tumors.[12–17]

A variety of chemical entities (triggers) are susceptible to one- or two-electron reductase enzymes, which mediate reduction of the trigger and subsequent release of the parent therapeutic agent. Commonly employed triggers include nitro-heterocycles, aromatic *N*-oxides, aliphatic *N*-oxides, quinones, and metal complexes.[26–31] Several bioreductive prodrugs have undergone clinical evaluation (Fig. 1),[18] including evofosfamide (TH-302),[10,19] apaziquone

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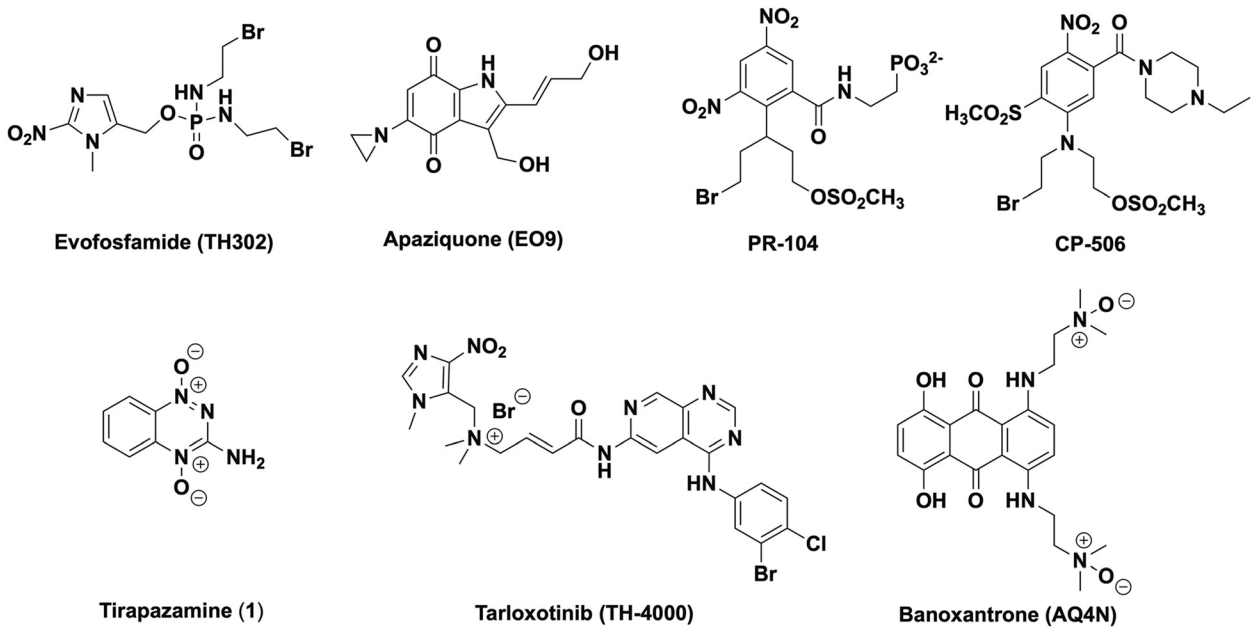



Fig. 1 Representative hypoxia-activated prodrugs evaluated previously in the clinic.[10,18–25]

(EO9),[20] PR-104,[21] tirapazamine,[22,23] tarloxotinib (TH-4000),[18] CP-506,[24] and banoxantrone (AQ4N).[25] Despite significant research efforts in the area of hypoxia targeted cancer therapies, no such agent, to date, has received approval from the United States Food and Drug Administration (FDA). This lack of clinical progress has been attributed in part to the difficulty of selecting patients who have very hypoxic tumors. Efforts are underway to identify tumors in patients that are characterized by molecular features, such as strong hypoxia gene expression signature, indicating potential for treatment benefit.[7,32]

Bioreductively activatable prodrug conjugates (BAPCs) are designed to be biologically inert under aerobic conditions, but under hypoxic conditions enzyme-mediated reduction results in cleavage of the prodrug construct to release the active therapeutic agent. Judicious combination of an appropriate trigger (to facilitate hypoxia-selective release) coupled to a promising therapeutic agent is deemed paramount for success. A foundational study by Davis and co-workers utilized the natural product combretastatin A-4 (CA4) as a tubulin-binding agent in a series of synthesized BAPCs.[43] CA4 is a potent inhibitor of tubulin assembly that demonstrates low nM cytotoxicity against a wide variety of human cancer cell lines.[33] Drawing structural inspiration, in part, from CA4, our previous studies led to the design and synthesis of a variety of small-molecule inhibitors of tubulin polymerization. Representative examples (Fig. 2) include dihydronaphthalene, benzosuberene,

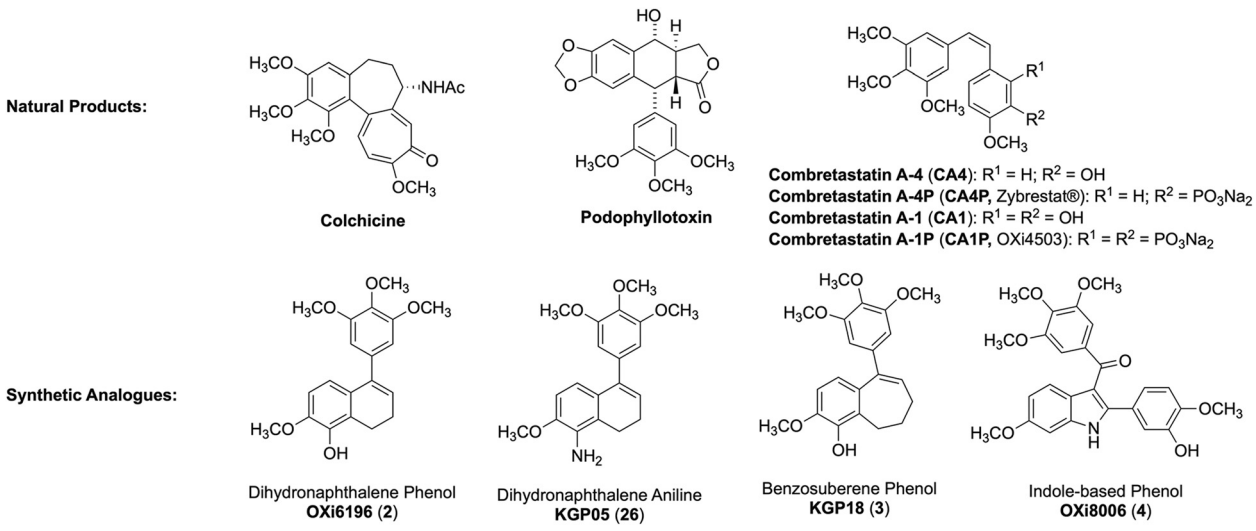



Fig. 2 Representative small-molecule inhibitors of tubulin polymerization.[33–42]

and indole-based analogues.[35–39,44–48] Furthermore, CA4 functions as a vascular disrupting agent (VDA),[49,50] facilitating effective and selective shutdown of blood flow to tumors. While the endothelium of normal blood vessels is remodeled and largely quiescent, the neovasculature of tumors is primitive, more responsive to angiogenic cell signaling, and activated in nature.[51] Consequently, the tumor vasculature offers an excellent, selective target for anticancer therapy. The goal of VDAs is to cause rapid and widespread disruption of existing tumor vasculature leading to blood flow shutdown to the tumor followed by massive tumor necrosis.[51,52] This mechanism is distinct from that of angiogenesis inhibiting agents (AIAs), which prevent the growth of new blood vessels.[51] Davis and co-workers prepared a series of CA4-BAPCs (Fig. 3) that utilized ether-linked nitrothienyl bioreductive triggers.[43] These CA4-based BAPCs demonstrated selective release of CA4 under hypoxic conditions. Guided by these results (Fig. 3), we previously synthesized and evaluated for biological activity a series of phenstatin-BAPCs[53,54] and separately a series of BAPCs based on the natural product combretastatin A-1 (CA1).[55]

A long-standing program in the design, synthesis, and biological evaluation of small-molecule inhibitors of tubulin polymerization resulted in our discovery and development of a wide-variety of molecules, including dihydronaphthalene, benzosuberene and indole analogues inspired, in part, by colchicine and CA4.[35–39,56–58] A subset of these molecules demonstrated dual efficacy by functioning biologically as both potent antiproliferative agents (cytotoxins) and as promising VDAs. There are a variety of potential benefits of these dihydronaphthalene, benzosuberene, and indole analogues in comparison to CA4, including enhanced stability due to replacement of the *Z*-ethylene bridge of CA4 with fused cyclic ring systems. Utilizing the corresponding phenolic-based analogues (OXi6196, KGP18, and OXi8006), along with an aniline-based analogue (KGP05) as the small-molecule parent therapeutic agents (Fig. 2), a series of BAPCs bearing nitrothiophene, nitroimidazole, and nitrofuran bioreductive triggers were synthesized. These BAPCs were designed to undergo reductase-mediated cleavage (Fig. 4) under hypoxic conditions. This type of cleavage is most likely facilitated by the activity of NADPH cytochrome P450 oxidoreductase and may proceed *via* the initially generated radical anion as suggested by pulse radiolysis studies by Davis and co-workers with a CA4-BAPC.[43] Alternatively, further reduction of the radical anion to the corresponding hydroxy amine or amine may then proceed to release the parent therapeutic agent *via* a cascade fragmentation sequence.

## 2. Results and discussion

### 2.1. Synthesis

The synthesis of dihydronaphthalene-BAPCs (**12–18**) and benzosuberene-BAPCs (**19–22**) was accomplished using Mitsunobu conditions (Scheme 1), a strategy previously employed to generate CA4-BAPCs, phenstatin-BAPCs, and CA1-BAPCs, along with other hypoxia-activated prodrugs.[43,54,55,59,60] The potent tubulin binding agents (**OXi6196**, **KGP18**, **OXi8006**) utilized in this study were prepared as previously described.[35–38,45,47,61] The bioreductive triggers (**5–11**) were synthesized utilizing previously reported procedures.[54,59] Coupling of the tubulin binding therapeutic small-molecule with the requisite bioreductive trigger was accomplished using Mitsunobu reaction conditions to generate the ether linkage.[43,54,55,62] Indole-BAPCs (**23–25**) were synthesized in the same manner. **OXi8006** (**4**) and nitrothiophene triggers (**5–7**) were coupled through an ether linkage using Mitsunobu conditions (Scheme 2). Diisopropyl azodicarboxylate (DIAD) and triphenylphosphine were suitable Mitsunobu partners for the *nor*- and *mono*-methyl OXi8006-nitrothiophene BAPCs (**23–24**). However, the use of 1,1′-(azodicarbonyl)dipiperidine (ADDP) and tributylphosphine proved necessary for successful synthesis of the *gem*-dimethyl OXi8006-nitrothiophene BAPC (**25**). While indole-based BAPCs **23** and **24** were obtained with relatively high purity, the indole *gem*-dimethyl analogue **25** only reached a purity level of 74% after purification. Importantly, HPLC indicated that no OXi8006 was present as an impurity. A series of nitrofuran-based and carbamate- or carbonate-based BAPCs were also synthesized (see SI). It should be noted that molecules

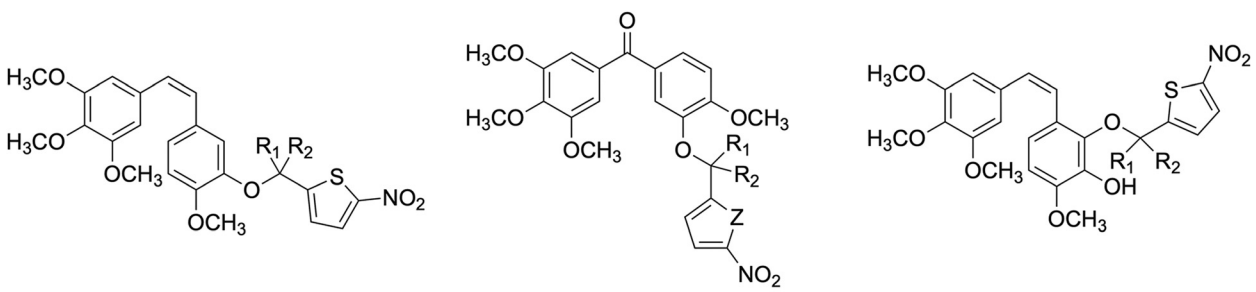



Fig. 3 Representative previously reported BAPCs that incorporate tubulin binding therapeutic agents.[43,54,55]

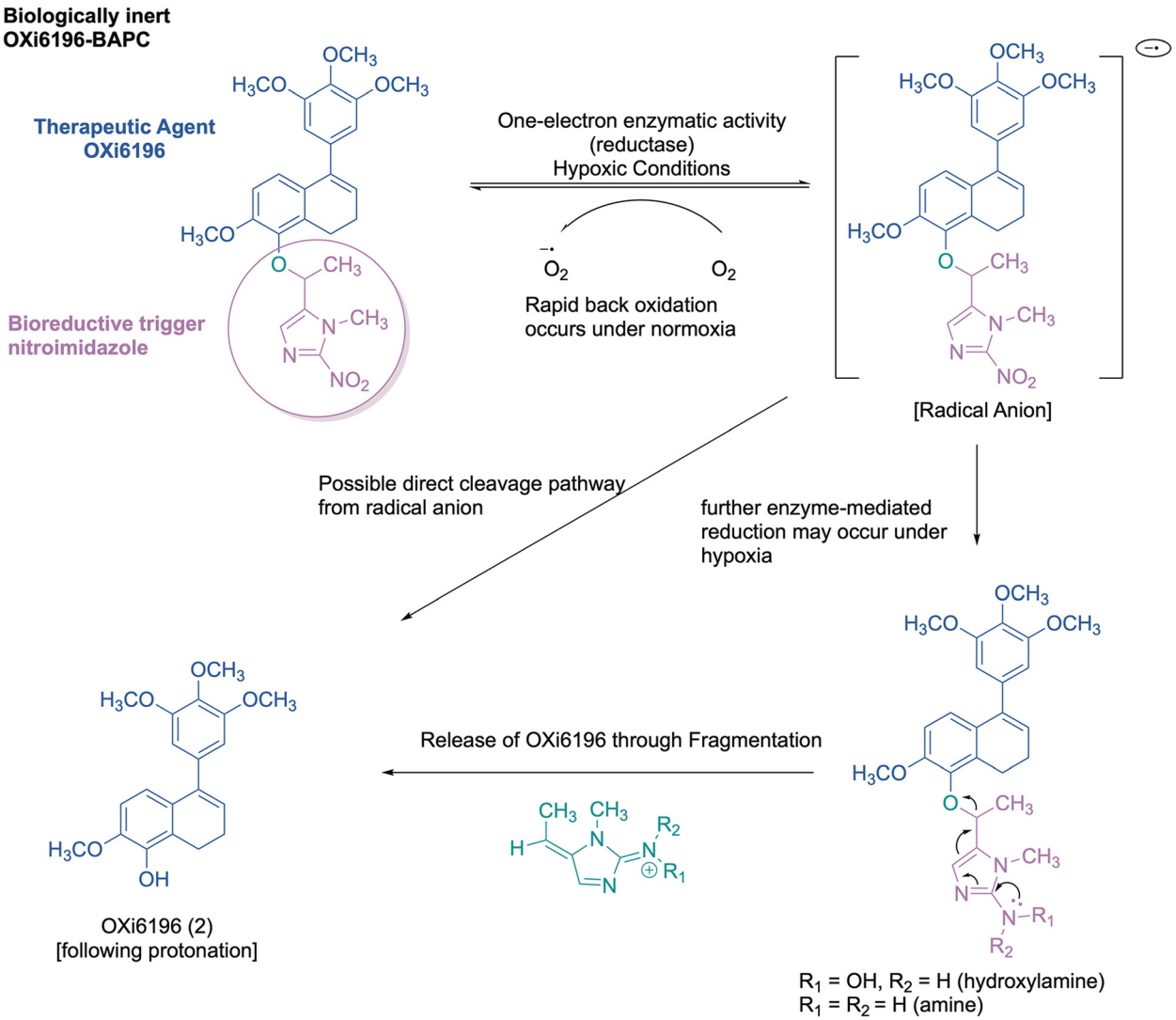
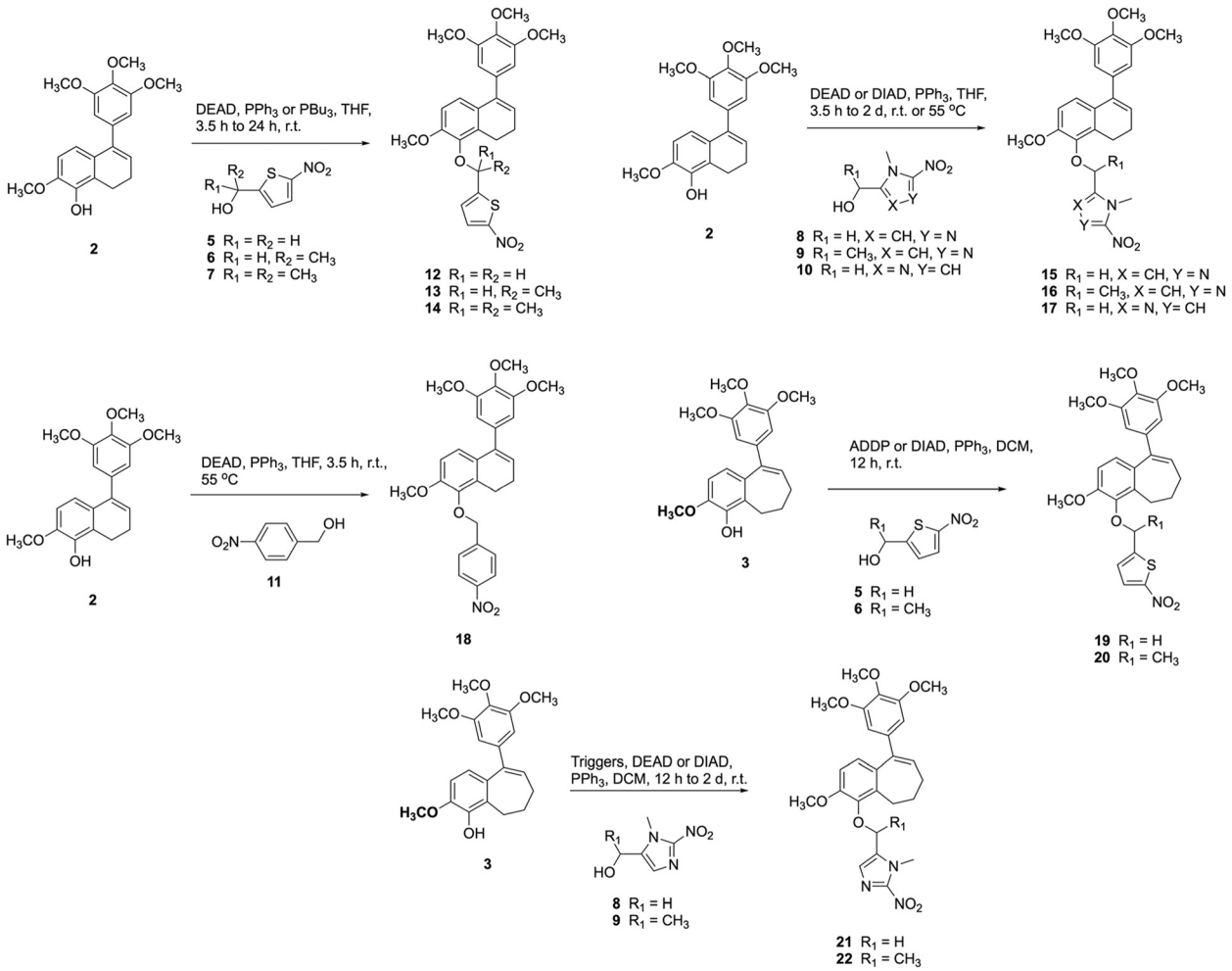



Fig. 4 Hypoxia-facilitated, enzyme-mediated reduction and cleavage of BAPCs (OXi6196-BAPC as a representative example).

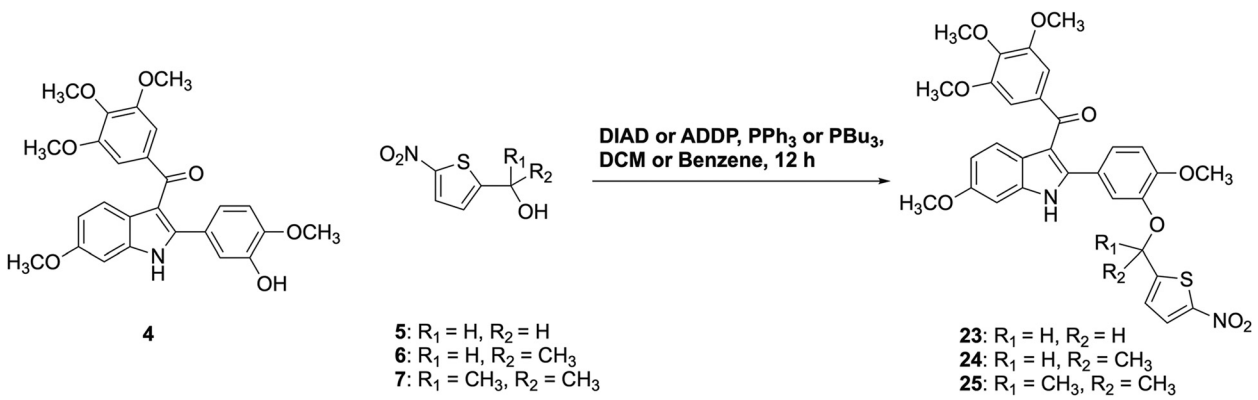
containing a stereogenic center were synthesized and biologically evaluated as racemic mixtures. While there are potential toxicity concerns related to the incorporation of nitroaryl moieties in therapeutic agents,[63] there are also many benefits leading to unique pharmacophore-driven bioactivity against diverse targets.[64] Future studies will be necessary to evaluate the PK, ADME, and toxicology profiles associated with the BAPCs in this study, but a significant number of drugs containing a nitroaryl moiety have received FDA approval.[64]

### 2.2. Biological evaluation

BAPCs and corresponding parent therapeutic agents (**OXi6196**, **KGP18**, and **OXi8006**) were evaluated for their ability to inhibit tubulin assembly and colchicine binding. BAPCs were all found to be inactive ($IC_{50}$ > 20 μM) as inhibitors of tubulin assembly as anticipated (Table S1). This confirmed that attachment of the bioreductive trigger dramatically inhibited binding to the colchicine site by the BAPC. Cytotoxicity under both normoxic and hypoxic conditions was evaluated in the A549 cell line (human lung carcinoma) to observe differential growth inhibition of the A549 cells. Prior studies by us and others demonstrated the feasibility of using A549 cells under hypoxic *versus* normoxic conditions to facilitate activation of hypoxia-selective prodrugs and probes bearing nitroaryl triggers, and separately tirapazamine.[22,43,54,55,65] In addition, lung tumors have been characterized with distinct regions of hypoxia.[66] Two distinct protocols were utilized to evaluate the different BAPCs against the A549 cells under both normoxic and hypoxic conditions. In one protocol (Table 1 data), plates were incubated for 4 h under anaerobic conditions to trigger prodrug cleavage, followed by a 48 h incubation under aerobic conditions without changing the media, in order to assess antimitotic effects. In a second protocol (Table S3), plates were incubated for 4 h under anaerobic conditions followed by 120 h under aerobic conditions after removal of media containing drug (and replacement of media), prior to determination of cytotoxicity by the sulforhodamine B (SRB) assay. Several BAPCs in the




**Scheme 1** Synthetic routes toward **OXi6196** and **KGP18**-BAPCs (**12**–**22**).




**Scheme 2** Synthetic routes toward **OXi8006**-BAPCs (**23**–**25**).

dihydronaphthalene and benzosuberene series (**13**–**16**, **20**–**22**) demonstrated enhanced cytotoxicity (see Table 1) under hypoxic conditions and BAPCs **13**, **16**, and **21** displayed hypoxic cytotoxicity ratios (HCRs) > 7.5, comparable to that of tirapazamine (HCR = 8.9), used as a positive control. The indole series of BAPCs (**23**–**25**) were exemplary with HCR values ranging from 33.8 for BAPC **24** to 79 for BAPC **25** and 79.7 for BAPC **23** (Table 1). HCR values for tirapazamine (TPZ) were consistently lower in this assay compared to the second protocol in which the drug was removed after 4 h of incubation under anaerobic conditions (HCR ≫ 32). In a separate study with BAPC removal after 48 h and longer (120 h) aerobic exposure

Table 1 *In vitro* potency and hypoxia cytotoxicity ratio (HCR) in A549 human cancer cell line

| Compound | $IC_{50}$ [oxic] (μM) ± SEM[a,b] | $IC_{50}$ [anoxic] (μM) ± SEM[a,b] | HCR |
|---|---|---|---|
| **TPZ** (**1**) | 63.5 | 7.1 | 8.9 |
| **OXi6196** (**2**) | 0.0066[c] | ND[d] | ND[d] |
| **KGP18** (**3**) | 0.000027[c] | ND[d] | ND[d] |
| **OXi8006** (**4**) | 0.030[c] | ND[d] | ND[d] |
| **KGP24** (**12**) | 0.46 ± 0.05 | 0.48 ± 0.04 | 1.0 |
| **KGP25** (**13**) | 2.74 ± 1.13 | 0.25 ± 0.08 | 11.0 |
| **KGP66** (**14**) | 0.13 ± 0.06 | 0.05 ± 0.01 | 2.6 |
| **KGP105** (**15**) | 0.25 ± 0.02 | 0.04 ± 0.003 | 6.2 |
| **KGP291** (**16**) | 0.38 ± 0.03 | 0.05 ± 0.004 | 7.6 |
| **KGP30** (**17**)[e] | ND[d] | ND[d] | ND[d] |
| **KGP29** (**18**)[e] | ND[d] | ND[d] | ND[d] |
| **KGP305** (**19**) | 0.05 ± 0.005 | 0.22 ± 0.09 | 0.2 |
| **KGP304** (**20**) | 2.27 ± 1.01 | 0.38 ± 0.12 | 6.0 |
| **KGP293** (**21**) | 0.36 ± 0.02 | 0.04 ± 0.005 | 9.0 |
| **KGP292** (**22**) | 0.67 ± 0.13 | 0.14 ± 0.04 | 4.8 |
| **KGP311** (**23**) | 5.58 ± 3.59 | 0.07 ± 0.04 | 79.7 |
| **KGP343** (**24**) | 4.06 ± 1.89 | 0.12 ± 0.06 | 33.8 |
| **KGP354** (**25**) | 5.53 ± 2.22 | 0.07 ± 0.02 | 79 |

[a] Average of $n \geq 3$ independent determinations. [b] 4 h incubation (oxic or anoxic) followed by 48 h oxic exposure. [c] Values for standard SRB assay for cytotoxicity in A549 cells. [d] ND = not determined. [e] See Table S3 for HCR data under different assay conditions.

time (Table S3), BAPC **17** demonstrated an HCR = 14, while several other BAPCs returned positive HCR values ranging from 4.8 to 0.8 (see Table S3). Positive controls under this assay protocol included TPZ (HCR ≫ 32) and RB6145 (HCR > 3). RB6145 is a nitroimidazole-based bifunctional radiosensitizer prodrug.[67] It should be noted that due, in part, to historical sequencing of assay conditions utilized in our laboratory, not all BAPCs were evaluated for HCR determination under both assay protocols.

Representative BAPCs were evaluated for their stability (phosphate buffer, pH 7.4) and their ability to undergo enzymatic cleavage by NADPH cytochrome P450 oxidoreductase (POR), an enzyme implicated in the bioreductive activation of a number of prodrugs,[68,69] in a cell-free assay (Table S4). While positive HCR values correlated, in general, with observed enzyme-mediated cleavage (to release the parent therapeutic agent), the observed cleavage was often fairly low, except in the case of BAPC **14** (98% cleavage) and BAPC **25** (100% cleavage). In general, the BAPCs evaluated showed excellent stability in phosphate buffer.

**Preliminary *in vivo* evaluation of BAPC KGP291 (16) as a hypoxia activated VDA.** The dynamic and longitudinal effects of a BAPC being activated to release its linked therapeutic agent, which functions as both a cytotoxin and VDA, can be assessed with various non-invasive imaging modalities.[70] Bioluminescence imaging (BLI) is a widely used optical technique for preclinical research.[70–72] The light emission of BLI is based on the expression of the luciferase enzyme and the presence of the substrate luciferin. The effect of VDAs can be observed through reduced delivery of the substrate luciferin, due to VDA-induced damage to tumor-associated microvessels, and consequent diminished light emission, as has been demonstrated with several VDAs.[70,73–75] It is noteworthy that our prior research with various mouse models of cancer involving the parent therapeutic agents utilized in this study, including OXi6196 (parent agent of BAPC KGP291), provided pertinent guidance on initial dose selection for the *in vivo* study.[73–75] We performed a preliminary dose escalation study on three nude mice with 4T1-luc tumors[76] with respective doses of 29, 53, or 61 mg $kg^{-1}$ **KGP291** administered intraperitoneally. Two additional mice served as controls, with one treated with vehicle (10% DMSO : 90% sesame oil) and the other treated with single dose **OXi6197** (30 mg $kg^{-1}$), the phosphate salt prodrug of compound **2**. Tumors treated with single dose **KGP291** (61 mg $kg^{-1}$) and **OXi6197** showed dramatic vascular shutdown within 4 h, evidenced by substantial reduction in the BLI signal at 4 and 24 h after administration of each compound (Fig. 5 and 6). These results provide preliminary evidence that KGP291 underwent *in vivo* reductase enzyme-mediated cleavage to release parent agent OXi6196 in the tumor microenvironment, leading to tumor-associated microvessel damage. This damage was initially observed at 4 h and was sustained at 24 h for mice receiving the higher doses (53 and 61 mg $kg^{-1}$) as fresh injections of luciferin at each of those time points resulted in diminished BLI signal compared to control. The effect was most evident in the mouse receiving 61 mg $kg^{-1}$ of KGP291. All animal experiments and procedures were carried out in accordance with the State of Texas and United States (US) Federal guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center under APN101222 and APN102169.

## 3. Conclusion

In this study, potent colchicine binding site inhibitors and VDAs (**OXi6196**, **KGP18**, and **OXi8006**) were coupled to a series

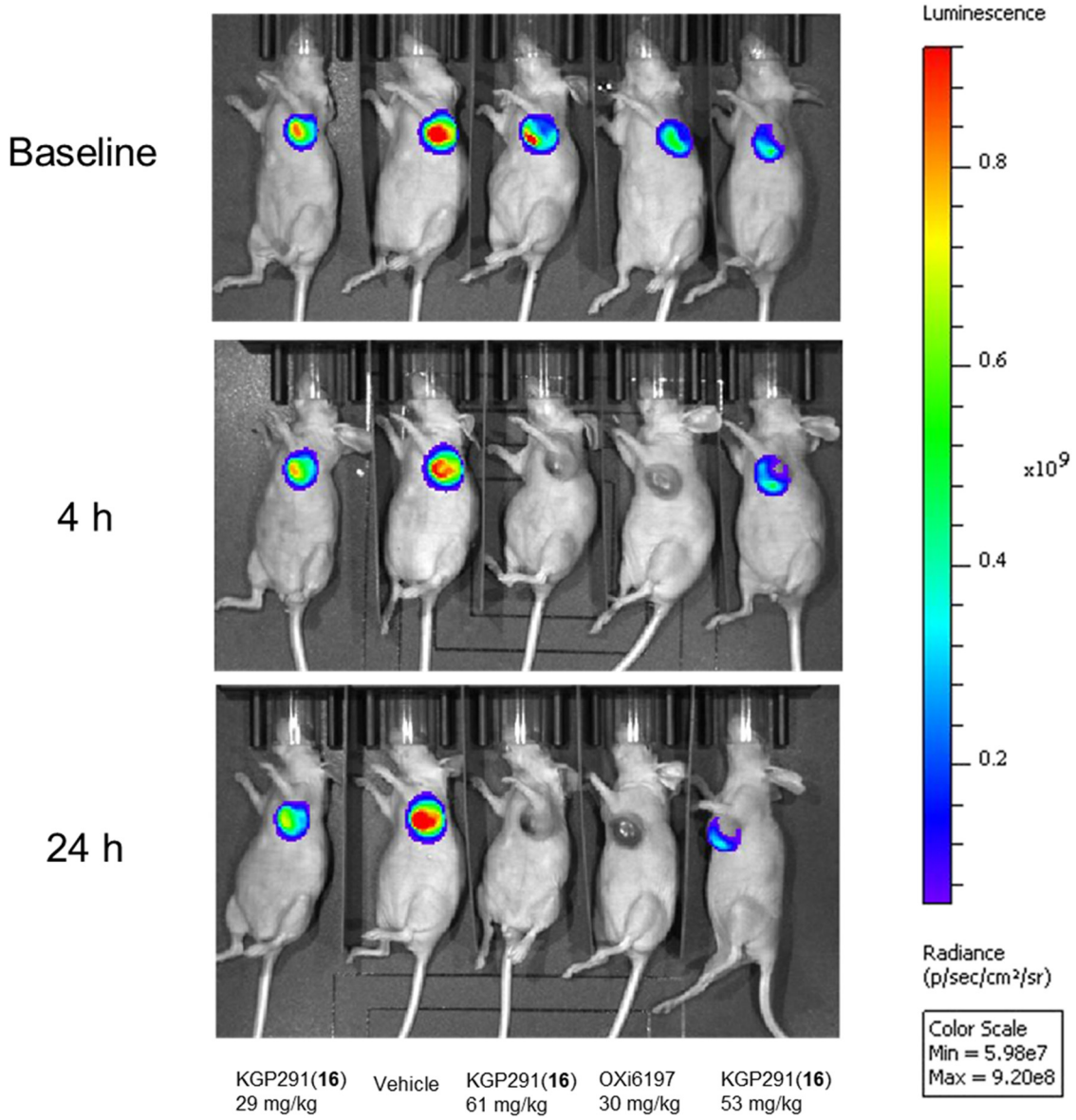



**Fig. 5** BLI monitoring of tumor response to **KGP291**. Baseline shows mice at 20 min time point following administration of luciferin (120 mg $kg^{-1}$) subcutaneously to five athymic nude mice bearing orthotopic 4T1-luc tumors growing in frontal mammary fat pad. Immediately following baseline BLI, mice were treated by intraperitoneal injection as follows: (left to right) **KGP291** (29 mg $kg^{-1}$); vehicle (10% DMSO : 90% sesame oil); **KGP291** (61 mg $kg^{-1}$); **OXi6197** (30 mg $kg^{-1}$) in saline vehicle; **KGP291** (53 mg $kg^{-1}$). BLI was repeated at 4 h and 24 h after treatment.

of bioreductive triggers (nitrothiophene, nitroimidazole, and nitrophenyl) to generate corresponding BAPCs. Prodrug constructs were screened for inhibition of tubulin polymerization and percent inhibition of colchicine binding; all BAPCs demonstrated significant reduction in activity compared to the corresponding parent drug in these assays. BAPCs were evaluated for inhibition of growth ($GI_{50}$) of A549 human cancer cells under normoxic and hypoxic conditions (COY chamber) using an SRB assay. Selectivity for drug release under hypoxic conditions was determined by hypoxic cytotoxicity ratio (HCR), and compounds **13**, **15**, **16**, **20**, and **21** produced positive HCR values (10.9, 6.2, 7.8, 6.0, and 7.8 respectively). The indole BAPC series (**23**, **24** and **25**) showed exemplary HCR values (76.6, 34.9, and 82.2). Preliminary *in vivo* BLI evaluation of compound **16** (61 mg $kg^{-1}$) against orthotopic 4T1-luc tumors (nude mouse model) showed a dramatic decrease in signal after 4 h and continued signal reduction after 24 h. Evidence of vascular shutdown (imaged by BLI), suggests compound **16** and related BAPCs are promising candidates for further development as antiproliferative agents and VDAs.

# 4. Experimental section

## 4.1. Chemistry

**4.1.1. General materials and methods.** Dichloromethane ($CH_2Cl_2$), acetonitrile, dimethylformamide (DMF), methanol, ethanol (EtOH), and tetrahydrofuran (THF) were used in their anhydrous forms, as obtained from the chemical suppliers.

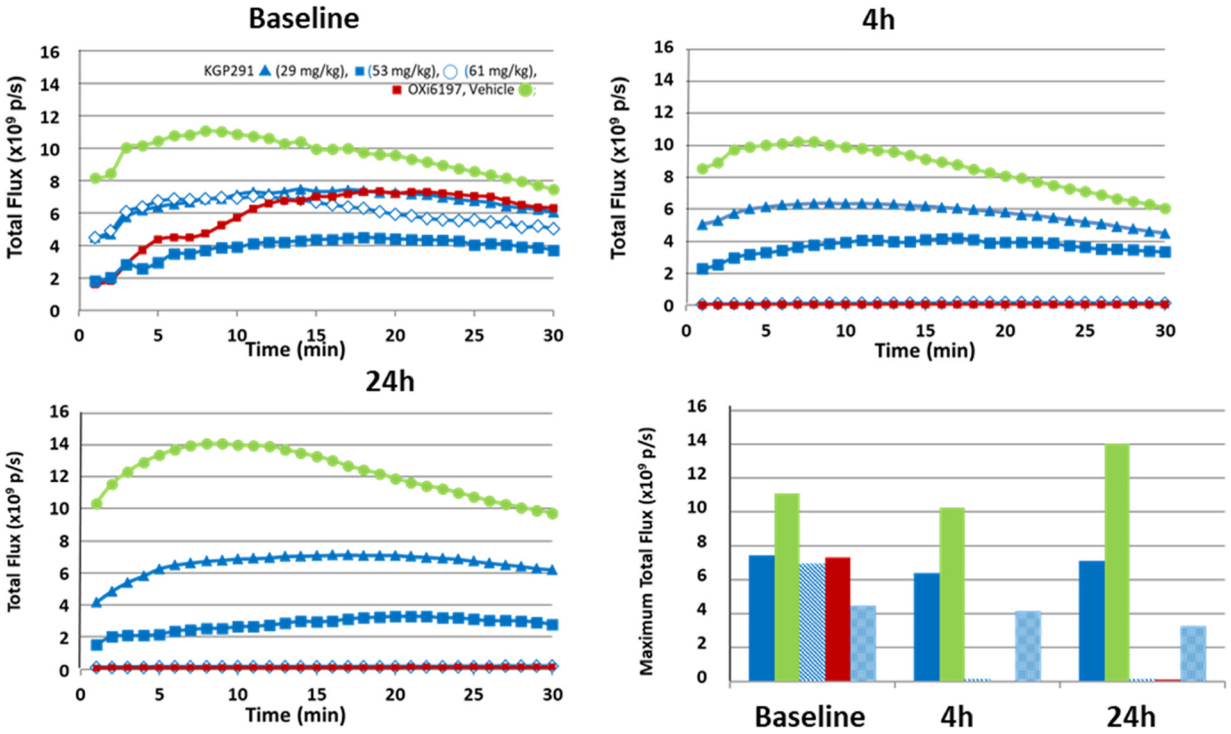



Fig. 6 Dynamic light emission time courses with respect to vascular disruption. Variation of signal intensity is shown at baseline, 4 h and 24 h. Mouse represented by lines with red squares received **OXi6197**; mouse represented by green circles received vehicle; mouse represented by solid blue triangles were treated with **KGP291** (29 mg $kg^{-1}$); mouse represented by solid blue squares received **KGP291** (53 mg $kg^{-1}$); and mouse represented by open blue diamonds received **KGP291** (61 mg $kg^{-1}$). At baseline, all tumors showed similar light emission kinetics (upper left). 4 h and 24 h later (upper right and lower left, respectively), the tumors receiving **OXi6197** and the highest dose of **KGP291** showed substantially reduced signal, while the tumors receiving vehicle alone and two lower doses of **KGP291** showed little change. The histogram (lower right) shows the maximum light emission for each dynamic curve. Following 61 mg $kg^{-1}$ **KGP291**, BLI intensity was significantly reduced compared with lower doses or vehicle ($p < 0.05$).

Reactions were performed under an inert atmosphere using nitrogen gas, unless specified. Thin-layer chromatography (TLC) plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used to monitor reactions. Purification of intermediates and products was carried out with a Biotage Isolera flash purification system using silica gel (200–400 mesh, 60 Å). Intermediates and products synthesized were characterized on the basis of their $^1$H NMR (600 or 500 MHz) and $^{13}$C NMR (151 or 126 MHz) spectroscopic data using a Bruker Avance III 600 MHz or a Varian VNMRs 500 MHz instrument. Spectra were recorded in $CDCl_3$, $(CD_3)_2SO$, or $(CD_3)_2CO$. All chemical shifts are expressed in ppm ($\delta$), coupling constants ($J$) are presented in Hz, and peak patterns are reported as broad (br), singlet (s), doublet (d), triplet (t), quartet (q), septet (sept), double doublet (dd), 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 ($\lambda$ = 190–400 nm), a Zorbax XDB-C18 HPLC column (4.6–150 mm, 5 μm), and a Zorbax reliance cartridge guard-column; solvent A, acetonitrile, solvent B, $H_2O$; gradient, 50% A/50% B to 100% A/0% B over 0 to 30 min; post-time 10 min; flow rate 1.0 mL $min^{-1}$; injection volume 20 μL; monitored at wavelengths of 210, 254, 280, 300, and 320 nm. Mass spectrometry was carried out under positive ESI (electrospray ionization) using a Thermo Scientific LTQ Orbitrap Discovery instrument.

**Experimental procedures for OXi6196, KGP18, OXi8006 and bioreductive triggers.** Procedures for the synthesis of **OXi6196** (**2**), **KGP18** (**3**), **OXi8006** (**4**) and bioreductive triggers (**5–11**) can be found in our previously published work.[35–39,48,58]

**Experimental procedures for the synthesis of BAPCs**

*4.1.1.1. 2-(((2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)oxy)methyl)-5-nitrothiophene (**12**).* **OXi6196** (**2**) (0.340 g, 1.00 mmol), (5-nitrothiophen-2-yl)methanol **5** (0.080 g, 0.500 mmol), and $PPh_3$ (0.267 g, 1.00 mmol) were dissolved in THF (2.0 mL) at room temperature. DEAD (0.158 mL, 1.00 mmol) was added dropwise, and the reaction mixture was stirred at 55 °C for 3.5 h. The reaction was cooled to room temperature, and the solvent was removed under reduced pressure. Purification by flash chromatography using a pre-packed (25 g) silica column [solvent A: EtOAc; solvent B: hexanes; gradient 0% A/100% B (1 CV), 0% A/100% B → 30% A/70% B (13 CV), 30% A/70% B (2 CV); flow rate: 75 mL $min^{-1}$; monitored at 254 and 280 nm] afforded compound **12** (0.040 g, 0.083 mmol, 17% yield) as a yellow oil. $^1$H NMR ($(CD_3)_2CO$, 500 MHz): $\delta$ 7.98 (1H, d, $J$ = 4.2 Hz), 7.24 (1H, dt, $J$ = 4.2, 0.9 Hz), 6.87 (1H, d, $J$ = 8.6 Hz), 6.84 (1H, d, $J$ = 8.6 Hz), 6.58 (2H, s), 5.99 (1H, t, $J$ = 4.7 Hz), 5.31 (2H, br s), 3.91 (3H, s), 3.81 (6H, s), 3.76

(3H, s), 2.85 (2H, t, $J$ = 8.1 Hz), 2.29 (2H, td, $J$ = 8.0, 4.7 Hz). $^{13}$C NMR ($(CD_3)_2CO$, 151 MHz): $\delta$ 153.3, 151.8, 151.4, 149.8, 143.6, 139.5, 137.7, 136.3, 130.5, 128.8, 128.6, 125.9, 124.9, 122.1, 109.3, 106.1, 68.6, 59.7, 55.5, 55.2, 22.6, 21.1. HRMS: obsd 506.1244 [M + $Na^+$], calcd for $C_{25}H_{25}NNaO_7S^+$: 506.1244. HPLC purity 100%, retention time – 16.4 min.

*4.1.1.2. 2-(1-((2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)oxy)ethyl)-5-nitrothiophene (**13**).* The procedure was similar to compound **12**, except that bioreductive trigger **6** was used instead of **5** to afford compound **13** (0.115 g, 0.231 mmol, 46% yield) as a yellow solid. $^1$H NMR ($(CD_3)_2CO$, 500 MHz): $\delta$ 7.95 (1H, d, $J$ = 4.2 Hz), 7.16 (1H, dd, $J$ = 4.2, 0.9 Hz), 6.85 (1H, d, $J$ = 8.6 Hz), 6.81 (1H, d, $J$ = 8.6 Hz), 6.58 (2H, s), 5.98 (1H, t, $J$ = 4.7 Hz), 5.68 (1H, qd, $J$ = 6.4, 0.9 Hz), 3.87 (3H, s), 3.81 (6H, s), 3.75 (3H, s), 2.78 (2H, ddd, $J$ = 10.8, 8.9, 6.7 Hz), 2.34–2.14 (2H, m), 1.71 (3H, d, $J$ = 6.5 Hz). $^{13}$C NMR ($(CD_3)_2CO$, 126 MHz): $\delta$ 155.6, 153.3, 151.9, 150.7, 142.2, 139.6, 136.4, 130.9, 128.7, 128.6, 125.0, 124.2, 121.9, 109.3, 106.1, 106.1, 74.8, 59.7, 55.5, 55.1, 27.7, 21.7, 21.3. HRMS: obsd 520.1400 [M + $Na^+$], calcd for $C_{26}H_{27}NNaO_7S^+$: 520.1400. HPLC purity 98.6%, retention time – 18.7 min. This compound was synthesized as a racemic mixture.

*4.1.1.3. 2-(2-((2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)oxy)propan-2-yl)-5-nitrothiophene (**14**).* The procedure was similar to compound **12**, except that bioreductive trigger **7** was used instead of **5** to afford compound **14** (0.035 g, 0.068 mmol, 16%) as an orange solid. $^1$H NMR ($(CD_3)_2CO$, 600 MHz): $\delta$ 7.87 (1H, d, $J$ = 4.3 Hz), 7.03 (1H, d, $J$ = 4.3 Hz), 6.70 (1H, d, $J$ = 8.4 Hz), 6.58 (2H, s), 6.54 (1H, d, $J$ = 8.4 Hz), 5.95 (1H, t, $J$ = 4.7 Hz), 3.82 (3H, s), 3.80 (6H, s), 3.75 (3H, s), 2.81 (2H, t, $J$ = 7.9 Hz), 2.30 (2H, td, $J$ = 7.9, 4.7 Hz), 1.63 (6H, s). $^{13}$C NMR ($(CD_3)_2CO$, 126 MHz): $\delta$ 166.2, 153.1, 146.6, 142.5, 139.9, 137.5, 136.8, 129.3, 128.6, 124.5, 122.4, 121.4, 117.1, 107.8, 106.1, 70.9, 59.7, 55.5, 55.3, 31.4, 31.3, 22.7, 20.2. HPLC purity 91.2%, retention time – 21.6 min. While purification beyond 91.2% by flash chromatography proved challenging, there was no parent agent (to the best of our knowledge) as part of the impurities, thus this did not influence the biological evaluation.

*4.1.1.4. 5-(((2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)oxy)methyl)-1-methyl-2-nitro-1H-imidazole (**15**).* **OXi6196** (**2**) (0.363 g, 1.06 mmol), (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol **8** (0.200 g, 1.27 mmol), and DIAD (0.280 mL, 1.43 mmol) were dissolved in THF (70 mL) at room temperature. $PPh_3$ (0.557 g, 2.12 mmol) was added, and the reaction mixture was stirred for 48 h. The solvent was removed under reduced pressure. Purification by flash chromatography using a pre-packed (50 g) silica column [solvent A: EtOAc; solvent B: hexanes; gradient 10% A/90% B (1 CV), 10% A/90% B → 80% A/20% B (13 CV), 80% A/20% B (2 CV); flow rate: 50 mL $min^{-1}$; monitored at 254 and 280 nm] afforded compound **15** (0.179 g, 0.371 mmol, 35% yield) as a yellow solid. $^1$H NMR ($CDCl_3$, 600 MHz): $\delta$ 7.14 (1H, s), 6.85 (1H, d, $J$ = 8.5 Hz), 6.69 (1H, d, $J$ = 8.5 Hz), 6.54 (2H, s), 5.97 (1H, t, $J$ = 4.6 Hz), 5.01 (2H, s), 4.24 (3H, s), 3.89 (3H, s), 3.85 (3H, s), 3.85 (6H, s), 2.76 (2H, t, $J$ = 7.9 Hz), 2.31 (2H, td, $J$ = 7.8, 4.6 Hz). $^{13}$C NMR ($CDCl_3$, 151 MHz): $\delta$ 153.0, 151.6, 143.0, 139.4, 137.2, 136.4, 133.9, 130.7, 129.1, 129.0, 125.4, 122.6, 109.0, 107.2, 105.8, 63.0, 61.0, 56.2, 55.6, 34.6, 22.8, 21.1. HRMS: obsd 504.1740 [M + $Na^+$], calcd for $C_{25}H_{27}N_3NaO_7^+$: 504.1741. HPLC purity 96.2%, retention time – 7.3 min.

*4.1.1.5. 5-(1-((2-Methoxy-5-(3,4,5-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl)oxy)ethyl)-1-methyl-2-nitro-1H-imidazole (**16**).* The procedure was similar to compound **15**, except that bioreductive trigger **9** was used instead of **8** to afford compound **16** (0.0694 g, 0.140 mmol, 24%) as a yellow solid. $^1$H NMR ($CDCl_3$, 600 MHz): $\delta$ 7.14 (1H, s), 6.77 (1H, d, $J$ = 8.5 Hz), 6.62 (1H, d, $J$ = 8.6 Hz), 6.47 (2H, s), 5.91 (1H, t, $J$ = 4.6 Hz), 5.53 (1H, q, $J$ = 6.6 Hz), 4.11 (3H, s), 3.82 (3H, s), 3.79 (3H, s), 3.78 (6H, s), 2.69 (2H, td, $J$ = 9.3, 6.8 Hz), 2.27–2.14 (2H, m), 1.61 (3H, d, $J$ = 6.6 Hz). $^{13}$C NMR ($(CD_3)_2CO$, 126 MHz): $\delta$ 153.3, 152.0, 141.6, 139.6, 138.8, 137.6, 136.3, 131.6, 128.7, 126.3, 125.0, 122.0, 109.2, 106.0, 68.9, 59.7, 55.5, 55.1, 34.1, 22.6, 21.7, 17.6. HRMS: obsd 496.2077 [M + $H^+$], calcd for $C_{26}H_{30}N_3O_7^+$: 496.2078. HPLC purity 94.9%, retention time – 10.0 min. This compound was synthesized as a racemic mixture.

*4.1.1.6. 2-[2-Methoxy-5-(3′,4′,5′-trimethoxyphenyl)-7,8-dihydronaphthalen-1-yl-oxymethyl]-1-methyl-5-nitro-1H-imidazole (**17**).* The procedure was similar to compound **12**, except that bioreductive trigger **10** was used instead of **5** to afford compound **17** (0.090 g, 0.187 mmol, 37%); $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 7.98 (1H, s), 6.85 (1H, d, $J$ = 8.6 Hz), 6.69 (1H, d, $J$ = 8.6 Hz), 6.54 (2H, br s, Ar*H*), 5.98 (1H, t, $J$ = 4.7 Hz), 5.10 (2H, s), 4.24 (3H, s), 3.89 (3H, s), 3.85 (9H, s), 2.80 (2H, t, $J$ = 7.8 Hz), 2.33 (2H, m); $^{13}$C NMR ($CDCl_3$, 125 MHz) $\delta$ 153.0, 151.6, 148.4, 143.1, 139.4, 137.2, 136.4, 131.8, 130.9, 129.2, 125.5, 122.7, 108.9, 105.8, 66.2, 60.9, 56.1, 55.6, 33.9, 22.8, 21.0; HRMS (EI): obsd 481.1851 [$M^+$], calcd for $C_{25}H_{27}N_3O_7^+$: 481.1849. HPLC purity 93.0%, retention time – 16.5 min. While purification beyond 93.0% by flash chromatography proved challenging, there was no parent agent (to the best of our knowledge) as part of the impurities, thus this did not influence the biological evaluation.

*4.1.1.7. 7-Methoxy-8-(4-nitrobenzyloxy)-4-(3′,4′,5′-trimethoxyphenyl)-1,2-dihydronaphthalene (**18**).* The procedure was similar to compound **12**, except that bioreductive trigger **11** was used instead of **5** to afford compound **18** (0.100 g, 0.210 mmol, 42%); $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 8.26 (2H, d, $J$ = 8.8 Hz), 7.68 (2H, d, $J$ = 8.6 Hz), 6.84 (1H, d, $J$ = 8.6 Hz), 6.71 (1H, d, $J$ = 8.6 Hz), 6.55 (2H, br s), 5.97 (1H, t, $J$ = 4.7 Hz), 5.11 (2H, s), 3.89 (3H, s), 3.87 (3H, s), 3.85 (6H, s), 2.85 (2H, t, $J$ = 8.0 Hz), 2.31 (2H, m); $^{13}$C NMR ($CDCl_3$, 125 MHz) $\delta$ 153.0, 151.9, 147.6, 145.4, 144.0, 139.5, 137.2, 136.6, 130.7, 129.0, 128.2, 125.4, 123.6, 122.1, 109.1, 105.9, 73.2, 61.0, 56.2, 55.7, 22.9, 21.3; HRMS (EI): obsd 477.1794 [$M^+$], calcd for $C_{27}H_{27}NO_7^+$: 477.1788. HPLC purity 100%, retention time – 16.50 min.

*4.1.1.8. 2-(((3-Methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)oxy)methyl)-5-nitrothiophene (**19**).* **KGP18** (**3**) (0.301 g, 0.844 mmol), (5-nitrothiophen-2-yl)methanol **5**

(0.129 g, 0.810 mmol), and $PPh_3$ (0.435 g, 1.66 mmol) were dissolved in $CH_2Cl_2$ (7 mL). ADDP (0.333 g, 1.32 mmol) was added, and the solution was stirred at room temperature for 12 h. The solvent was removed under reduced pressure. Purification on silica gel using isocratic 7.5% EtOAc: 92.5% hexanes as eluent, followed by recrystallization using $Et_2O$, afforded compound **19** (0.050 g, 0.010 mmol, 12% yield) as a yellow solid. $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 7.85 (1H, d, $J$ = 4.2 Hz), 7.03 (1H, d, $J$ = 4.2 Hz), 6.83 (1H, d, $J$ = 8.6 Hz), 6.80 (1H, d, $J$ = 8.6 Hz), 6.48 (2H, s), 6.34 (1H, t, $J$ = 7.3 Hz), 5.22 (2H, d, $J$ = 0.5 Hz), 3.92 (3H, s), 3.86 (3H, s), 3.81 (6H, s), 2.73 (2H, t, $J$ = 6.8 Hz), 2.07 (2H, p, $J$ = 7.0 Hz), 1.93 (2H, q, $J$ = 7.0 Hz). $^{13}$C NMR ($CDCl_3$, 126 MHz): $\delta$ 152.9, 151.8, 151.1, 149.2, 143.8, 142.6, 138.2, 137.5, 135.9, 134.0, 128.2, 127.3, 126.1, 125.0, 109.4, 105.3, 69.4, 60.9, 56.2, 55.7, 34.4, 25.5, 24.4. HRMS: obsd 520.1394 [M + $Na^+$], calcd for $C_{26}H_{27}NNaO_7S^+$: 520.1400. HPLC purity 98.8%, retention time – 16.5 min.

*4.1.1.9. 2-(1-((3-Methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)oxy)ethyl)-5-nitrothiophene (20).* **KGP18** (**3**) (1.50 g, 4.20 mmol), 1-(5-nitrothiophen-2-yl)ethan-1-ol **6** (0.650 g, 3.75 mmol), and $PPh_3$ (1.37 g, 7.29 mmol) were dissolved in $CH_2Cl_2$ (40 mL). DIAD (1.15 mL, 5.08 mmol) was added, and the solution was stirred at room temperature for 12 h. The solvent was removed under reduced pressure, and the crude mixture was purified on silica gel using isocratic 5% EtOAc : 95% hexanes as eluent, followed by recrystallization using $Et_2O$ to afford compound **20** (0.869 g, 1.70 mmol, 45% yield) as a yellow solid. $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 7.81 (1H, d, $J$ = 4.4 Hz), 6.94 (1H, dd, $J$ = 4.2, 0.7 Hz), 6.80 (1H, d, $J$ = 8.6 Hz), 6.77 (1H, d, $J$ = 8.6 Hz), 6.45 (2H, s), 6.32 (1H, t, $J$ = 7.1 Hz), 5.62 (1H, qd, $J$ = 6.4, 0.7 Hz), 3.87 (3H, s), 3.86 (3H, s), 3.80 (6H, s), 2.74 (1H, m), 2.64 (1H, m), 2.06 (1H, m), 1.91 (3H, m), 1.73 (3H, d, $J$ = 6.6 Hz). $^{13}$C NMR ($CDCl_3$, 126 MHz): $\delta$ 155.3, 152.9, 151.1, 142.6, 142.4, 138.3, 137.4, 136.6, 134.0, 128.1, 127.3, 125.8, 123.3, 109.4, 105.2, 74.6, 60.9, 56.1, 55.6, 34.1, 25.6, 24.4, 21.8. HRMS: obsd 534.1556 [M + $H^+$], calcd for $C_{27}H_{29}NNaO_7S^+$: 534.1557. HPLC purity 99.0%, retention time – 20.1 min. This compound was synthesized as a racemic mixture.

*4.1.1.10. 5-(((3-Methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)oxy)methyl)-1-methyl-2-nitro-1H-imidazole (21).* **KGP18** (**3**) (0.250 g, 0.702 mmol), (1-methyl-2-nitro-1*H*-imidazol-5-yl)methanol **8** (0.123 g, 0.842 mmol), and DEAD (0.144 mL, 0.913 mmol) were dissolved in $CH_2Cl_2$ (60 mL). $PPh_3$ (0.368 g, 1.40 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure. Purification by flash chromatography using a pre-packed (50 g) silica column [solvent A: EtOAc; solvent B: hexanes; gradient 10% A/90% B (1 CV), 10% A/90% B → 80% A/20% B (13 CV), 80% A/20% B (2 CV); flow rate: 50 mL $min^{-1}$; monitored at 254 and 280 nm] afforded compound **21** (0.143 g, 0.288 mmol, 41% yield) as an orange crystal. $^1$H NMR ($CDCl_3$, 600 MHz): $\delta$ 7.15 (1H, s), 6.84 (1H, d, $J$ = 8.5 Hz), 6.79 (1H, d, $J$ = 8.5 Hz), 6.46 (2H, s), 6.34 (1H, t, $J$ = 7.3 Hz), 5.05 (2H, s), 4.25 (3H, s), 3.89 (3H, s), 3.86 (3H, s), 3.80 (6H, s), 2.68 (2H, t, $J$ = 6.9 Hz), 2.07–1.99 (2H, m), 1.93 (2H, q, $J$ = 7.1 Hz). $^{13}$C NMR (CDCl3, 151 MHz): $\delta$ 152.9, 151.2, 143.4, 142.6, 138.1, 137.6, 135.9, 134.2, 134.0, 128.8, 127.3, 126.4, 109.3, 105.3, 63.6, 60.9, 56.2, 55.6, 34.6, 34.4, 25.5, 24.4. HRMS: obsd 518.1898 [M + $Na^+$], calcd for $C_{26}H_{29}N_3NaO_7^+$: 518.1898. HPLC purity 98.1%, retention time – 10.7 min.

*4.1.1.11. 5-(1-((3-Methoxy-9-(3,4,5-trimethoxyphenyl)-6,7-dihydro-5H-benzo[7]annulen-4-yl)oxy)ethyl)-1-methyl-2-nitro-1H-imidazole (22).* The procedure was similar to compound **20**, except that bioreductive trigger **9** was used instead of **6** to afford compound **22** (0.122 g, 0.239 mmol, 34% yield) as an orange solid. $^1$H NMR ($CDCl_3$, 600 MHz): $\delta$ 7.14 (1H, s), 6.75 (1H, d, $J$ = 8.5 Hz), 6.71 (1H, d, $J$ = 8.5 Hz), 6.38 (2H, s), 6.26 (1H, t, $J$ = 7.0 Hz), 5.57 (1H, q, $J$ = 6.6 Hz), 4.09 (3H, s), 3.81 (3H, s), 3.79 (3H, s), 3.73 (6H, s), 2.63 (1H, dt, $J$ = 12.6, 6.3 Hz), 2.54 (1H, dt, $J$ = 13.7, 6.8 Hz), 2.05–1.97 (1H, m), 1.85 (3H, m), 1.64 (3H, d, $J$ = 6.6 Hz). $^{13}$C NMR ($CDCl_3$, 151 MHz): $\delta$ 152.9, 151.1, 146.3, 142.5, 141.7, 138.6, 138.2, 137.5, 136.8, 134.3, 127.4, 126.7, 126.1, 109.2, 105.3, 68.7, 60.9, 56.2, 55.5, 34.7, 34.1, 25.6, 24.3, 18.4. HRMS: obsd 532.2054 [M + $Na^+$], calcd for $C_{27}H_{31}N_3NaO_7^+$: 532.2053. HPLC purity 97.5%, retention time – 11.5 min. This compound was synthesized as a racemic mixture.

*4.1.1.12. (6-Methoxy-2-(4-methoxy-3-((5-nitrothiophen-2-yl)methoxy)phenyl)-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (23).* The procedure was similar to compound **20**, except that **OXi8006** (**4**) was used instead of **KGP18** (**3**) and bioreductive trigger **5** was used instead of **6** to afford compound **23** (0.080 g, 0.11 mmol, 13% yield) as a yellow solid. $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 8.62 (1H, s), 7.81 (1H, d, $J$ = 9.5 Hz), 7.78 (1H, d, $J$ = 4.0 Hz), 7.10 (1H, dd, $J$ = 8.0, 2.0 Hz), 6.99 (2H, s), 6.95 (1H, d, $J$ = 4.0 Hz), 6.89 (2H, m), 6.85 (1H, d, $J$ = 2.0 Hz), 6.81 (1H, d, $J$ = 8.0 Hz), 4.91 (2H, s), 3.85 (3H, s), 3.83 (3H, s), 3.82 (3H, s), 3.67 (6H, s). $^{13}$C NMR ($CDCl_3$, 126 MHz): $\delta$ 192.0, 157.5, 152.8, 151.8, 150.6, 148.1, 147.0, 141.8, 141.6, 136.5, 134.9, 128.5, 125.1, 124.9, 123.1, 122.9, 122.5, 117.3, 113.0, 111.94, 111.87, 107.5, 94.7, 66.9, 61.1, 56.2, 56.1, 55.8. HRMS: obsd 605.1587 [M + $H^+$], calcd for $C_{31}H_{29}N_2O_9S^+$: 605.1588. HPLC purity 94.7%, retention time – 7.74 min.

*4.1.1.13. (6-Methoxy-2-(4-methoxy-3-(1-(5-nitrothiophen-2-yl)ethoxy)phenyl)-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (24).* The procedure was similar to compound **20**, except that **OXi8006** (**4**) was used instead of **KGP18** (**3**) to afford compound **24** (0.19 g, 0.31 mmol, 32% yield) as a yellow solid. $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ 8.97 (1H, s), 7.75 (1H, d, $J$ = 9.5 Hz), 7.68 (1H, d, $J$ = 4.0 Hz), 7.05 (1H, dd, $J$ = 8.0, 2.0 Hz), 6.99 (2H, s), 6.86 (1H, d, $J$ = 2.0 Hz), 6.84 (2H, m), 6.79 (1H, dd, $J$ = 4.0, 1.0 Hz), 6.71 (1H, d, $J$ = 8.0 Hz), 5.13 (1H, q, $J$ = 6.0 Hz), 3.82 (3H, s), 3.80 (3H, s), 3.78 (3H, s), 3.66 (6H, s), 1.53 (3H, d, $J$ = 6.0 Hz). $^{13}$C NMR ($CDCl_3$, 126 MHz): $\delta$ 191.9, 157.4, 155.3, 152.7, 151.4, 150.9, 146.1, 141.7, 141.5, 136.5, 134.7, 128.6, 124.8, 123.4, 123.1, 123.1, 122.4, 119.7, 112.9, 112.1, 111.8, 107.4, 94.6, 74.5, 61.0, 56.2, 56.0, 55.7, 22.9. HRMS: obsd 619.1742 [M + $H^+$], calcd for $C_{32}H_{31}N_2O_9S^+$: 619.1745. HPLC purity 85.8%, retention time – 15.73 min. While purification beyond 85.8% by flash chromatography proved challenging, there was no parent agent (to the best of our knowledge) as part of the impurities, thus this did not

influence the biological evaluation. This compound was synthesized as a racemic mixture.

*4.1.1.14. (6-Methoxy-2-(4-methoxy-3-((2-(5-nitrothiophen-2-yl) propan-2-yl)oxy)phenyl)-1H-indol-3-yl)(3,4,5-trimethoxyphenyl) methanone (25).* **OXi8006** (**4**) (0.50 g, 1.08 mmol), 2-(5-nitrothiophen-2-yl)propan-2-ol **7** (0.22 g, 1.15 mmol) and ADDP (0.27 g, 1.08 mmol) were dissolved in benzene (10 mL). $PBu_3$ (0.27 mL, 1.08 mmol) was added dropwise to the solution and the reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. Purification by flash chromatography using a pre-packed (50 g) silica gel column [solvent A, EtOAc, solvent B, hexanes; gradient 5% A/95% B (1 CV), 5% A/95% B → 40% A/60% B (12 CV), 40% A/60% B (1 CV); flow rate, 25 mL $min^{-1}$; monitored at 254 and 280 nm] afforded compound **25** (0.03 g, 0.05 mmol, 5% yield) as a yellow solid. $^1H$ NMR ($CDCl_3$, 500 MHz): $\delta$ 8.68 (1H, s), 7.67 (1H, d, $J$ = 9.0 Hz), 7.65 (1H, d, $J$ = 4.0 Hz), 7.16 (1H, dd, $J$ = 8.5, 2.0 Hz), 7.03 (2H, s), 6.87 (1H, d, $J$ = 2.0 Hz), 6.84 (2H, m), 6.78 (1H, d, $J$ = 8.5 Hz), 6.76 (1H, d, $J$ = 4.5 Hz), 3.85 (3H, s), 3.83 (3H, s), 3.74 (3H, s), 3.71 (6H, s), 1.51 (6H, s). $^{13}C$ NMR ($CDCl_3$, 126 MHz): $\delta$ 191.5, 161.1, 157.4, 154.1, 152.8, 150.7, 143.4, 141.6, 141.3, 136.4, 134.5, 128.4, 125.4, 124.8, 124.8, 123.1, 122.4, 122.2, 112.9, 112.2, 111.8, 107.6, 94.6, 80.7, 61.0, 55.3, 55.8, 55.8, 28.7. HRMS: obsd 633.1899 [M + $H^+$], calcd for $C_{33}H_{33}N_2O_9S^+$: 633.1901. HPLC purity 74.5%, retention time – 16.37 min. While purification beyond 74.5% by flash chromatography proved challenging, there was no parent agent (to the best of our knowledge) as part of the impurities, thus this did not influence the biological evaluation.

### 4.2. Biological evaluation

**4.2.1. Cell culture.** A549 cells (human non-small-cell lung carcinoma, ATCC) were cultured in MEM-alpha medium containing 10% fetal bovine serum (FBS), 17 mM D-glucose, 1% glutamine, and 1% gentamycin sulfate. For the bioreductive assay, the same medium was used with the addition of 0.02 mM 2′-deoxycytidine hydrochloride. Cells were maintained in a log growth phase in a humidified 37 °C incubator under 95% air plus 5% $CO_2$.

**4.2.2. Differential cytotoxicity.** The bioreductive assay was modified from Jaffar *et al.*[55,77] For the anoxic assay, A549 cells were cultured in a Coy chamber in medium that had been preconditioned in the anaerobic chamber. Cells were plated at 6000 cells/100 μL per well into 96-well plates that had been degassed in the anaerobic chamber and allowed to attach for 2 h. Compounds to be tested were serially diluted in preconditioned medium from 10 mg $mL^{-1}$ stock solutions in DMSO to 2× the final concentrations; 100 μL was added per well in duplicate for each experiment. The final concentrations of control and test compounds ranged from 50 μg $mL^{-1}$ to 5 pg $mL^{-1}$. Plates were incubated for 4 h under anaerobic conditions and then either for 48 h under aerobic conditions without media change, or for 120 h under aerobic conditions after removal and replacement of media containing drug, prior to sulforhodamine B (SRB)[78–81] determination of cytotoxicity as previously reported.[22,23,55,78–83] Briefly, treated and control cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB dye for 30 min, and subsequently washed 4 times with 1% acetic acid in water. The plates were air dried, and the protein-bound SRB dye was solubilized with 10 mM Tris base. The plates were read at 540 nm and 630 nm (to adjust for background) with an automated Biotek Elx800 plate reader. $IC_{50}$ values were determined with Excel software.[80] An identical procedure was used for the normoxic arm of the assay with the exception that all incubations were carried out under 95% air plus 5% $CO_2$. Tirapazamine was used as a positive control, as previously described.[22,23,82]

**4.2.3. NADPH-cytochrome P450 oxidoreductase cleavage Assay.**[55,84]. The enzymatic activity of rat NADPH-cytochrome P450 oxidoreductase supersome (Corning) was assessed with cytochrome c as substrate and protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid). For the bioreductive enzyme assay,[54,55] BAPC prodrugs were diluted from 10 mM stock solutions in DMSO and added to 200 mM pH 7.4 potassium phosphate buffer containing 0.1% Triton X-100 (to facilitate BAPC solubility) and 400 μM freshly dissolved protocatechuic acid. The resulting solution was evacuated and flushed with nitrogen (3 times) followed by the addition of protocatechuate 3,4-dioxygenase (PCD, Sigma-Aldrich) to react with substrate PCA to remove remaining traces of $O_2$. POR and NADPH were then introduced sequentially. The anaerobic reaction mixture was incubated for designated times at 37 °C, cooled on ice, and treated with a 2× volume of acetonitrile to precipitate proteins. After centrifugation and syringe (0.2 μm) filtration, the samples were analyzed by HPLC using various gradients of acetonitrile/water for elution. Solutions without the POR enzyme were used as controls for stability studies. Standard curves were determined for each substrate and product for quantitation.

**4.2.4. Colchicine binding assay.** Inhibition of [$^3H$]colchicine binding to tubulin was measured using 100 μL reaction mixtures containing 1.0 μM tubulin, 5.0 μM [$^3H$]colchicine (from Perkin-Elmer), 5% (v/v) dimethyl sulfoxide, potential inhibitors at 1.0 or 5.0 μM, as indicated, and components that stabilize the colchicine binding activity of tubulin (1.0 M monosodium glutamate [adjusted to pH 6.6 with HCl in a 2.0 M stock solution], 0.5 mg $mL^{-1}$ bovine serum albumin, 0.1 M glucose-1-phosphate, 1.0 mM $MgCl_2$, and 1.0 mM GTP). Incubation was for 10 min at 37 °C, when the binding reaction in control reaction mixtures is 40–60% complete. Reactions were stopped with 2.0 mL of ice-cold water, and the reaction mixtures were placed on ice. Each sample was poured onto a stack of two DEAE-cellulose filters (Whatman), followed by 6 mL of ice-cold water. The samples were aspirated under reduced vacuum. The filters were washed three times with 2 mL water and placed into vials containing 5 mL of Biosafe II scintillation cocktail. Samples were counted 18 h later in a Beckman scintillation counter. Samples with inhibitors were compared to samples with no inhibitor, and percent inhibition was determined. All samples were corrected for radiolabel bound to the filters in the absence of tubulin.

**4.2.5. Inhibition of tubulin polymerization.** Tubulin polymerization experiments were performed in 0.25 mL reaction mixtures (final volume) that contained 1 mg $mL^{-1}$ (10 μM) purified bovine brain tubulin, 0.8 M monosodium glutamate (pH 6.6), 4% (v/v) dimethyl sulfoxide, 0.4 mM GTP, and different compound concentrations. All reaction components except GTP were preincubated for 15 min at 30 °C in 0.24 mL. The mixtures were cooled to 0 °C, and 10 μL of 10 mM GTP was added. Reaction mixtures were transferred to cuvettes held at 0 °C in Beckman DU-7400 or DU-7500 spectrophotometers equipped with electronic temperature controllers. The temperature was jumped to 30 °C, taking about 30 s, and polymerization was followed at 350 nm for 20 min. The $IC_{50}$ was defined as the compound concentration that inhibited extent of polymerization by 50% after 20 min.

**4.2.6. *In vivo* bioluminescence imaging (BLI) with KGP291 (16).** 4T1-luc cells [$1 \times 10^6$ in 100 μL PBS with 50% Matrigel® (original cell line from ATCC, with transfected cell line provided by Dr. Edward Graves, Stanford University)] were injected directly into the left upper mammary fat pad of five female athymic nude mice. Tumors were allowed to grow over about 10 days, and then BLI was performed using an IVIS® Spectrum system (Perkin-Elmer (Xenogen), Alameda, CA), as described in detail previously.[55] Briefly, D-luciferin (128 mg $kg^{-1}$ sodium salt in PBS in a total volume of 80 μL; Gold Biotechnology Inc., St. Louis, MO) was administered subcutaneously (SC) in the foreback neck region. Immediately after luciferin injection, a series of BLI images was acquired over a period of 30 min using auto exposure time. Following baseline BLI, mice were injected intraperitoneally (IP) respectively with either 29, 53, or 61 mg $kg^{-1}$ of **KGP291** in vehicle (10% DMSO : 90% sesame oil). In addition, the vehicle and 30 mg $kg^{-1}$ OXi6197 (also called **KGP04**)[45] in vehicle were used as controls. Dynamic BLI was repeated 4 and 24 h post-treatment with administration of fresh luciferin on each occasion. All animal experiments and procedures were carried out in accordance with the State of Texas and United States (US) Federal guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center under APN101222 and APN102169.

## Conflicts of interest

A portion of the research presented in this manuscript was funded by Mateon Therapeutics, Inc. (formerly OXiGENE Inc.), and this previous relationship is properly indicated in the Acknowledgements section. In addition, one of the authors (KGP) was formerly a paid consultant with Mateon Therapeutics, Inc. and is a current shareholder. While Mateon Therapeutics (OXiGENE Inc.) no longer exists as a corporate entity, we appreciated the long-term scientific collaboration and note that there is no actual conflict of interest associated with the scientific content and data presented in this manuscript.

## Data availability

Supplementary information: Details regarding the synthesis of additional analogues, further biological evaluation, and compound characterization are provided in the SI. See DOI: https://doi.org/10.1039/D5MD00564G.

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