Synthesis and β-lactamase-mediated activation of a cephalosporin–taxol prodrug

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Background: Enzyme-activatable prodrugs in conjunction with antibody–enzyme fusion proteins may enhance the anti-tumor efficacy of antibodies and reduce the toxic side effects of conventional chemotherapeutics. Cephalosporins have proven to be highly versatile triggers for the enzymatic activation of such prodrugs.

Results: A cephem prodrug of taxol (PROTAX) was synthesized by substituting the C-3' position of cephalothin with 2'-(γ-aminobutyryl) taxol. Hydrolysis of PROTAX by β-lactamase rapidly released 2'-(γ-aminobutyryl) taxol (k_{cat}/K_M = (1.4 ± 0.1) x 10^5 s^{-1} M^{-1}), which yielded taxol following intramolecular displacement. PROTAX is inactive in a microtubule assembly assay in vitro but has similar activity to taxol following prolonged activation with β-lactamase. PROTAX is ~10-fold less toxic than taxol against SK-BR-3 breast tumor cells *in vitro* but has activity approaching that of taxol following prolonged activation with a fusion protein comprising β-lactamase fused to a tumor-targeting antibody fragment.

Conclusions: Tubulin polymerization activity is abolished and cytotoxicity is reduced in the PROTAX prodrug compared to taxol. Activation of PROTAX by β-lactamase followed by self-immolation restores the activity of PROTAX to that of free taxol.

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Introduction

The use of enzyme-activatable prodrugs in conjunction with antibody–enzyme fusion proteins or conjugates is a promising strategy for enhancing the anti-tumor efficacy of antibodies and minimizing the toxicity of chemotherapeutics (reviewed in [1,2]). Indeed, targeted prodrugs have proven to be much more efficacious than corresponding free drugs against human tumor xenografts in nude mice [3–5].

Our humanized anti-$p185^{HER2}$ antibody, humAb4D5–8 [6] was developed as a potential therapy for human cancers which overexpress $p185^{HER2}$ and has recently completed phase II clinical testing for the treatment of metastatic breast cancer. This humanized antibody is now being used as a building block to design potentially more potent immunotherapeutics. To this end we developed a fusion protein (dsFv3–β-lactamase) comprising a disulfide-stabilized Fv fragment of humAb4D5–8 fused to β-lactamase, RTEM-1, for use in targeted therapy in conjunction with cephem-based prodrugs [7].

β-Lactamase is an attractive choice of enzyme for prodrug activation because of its high catalytic efficiency and broad substrate specificity. Furthermore, there are no known similar activities, competing substrates or inhibitors endogenous to man. Cephalosporins have proven to be highly versatile substrate triggers in the construction of enzyme-activatable prodrugs including vinca alkaloids [3,8,9], nitrogen mustard drugs [10–12], a carboplatin analog [13] and doxorubicin [7,14,15]. Additional cephalosporin prodrugs have been described in the patent literature, including 5-fluorouracil, 6-mercaptopurine, 6-thioguanine, methotrexate and mitomycin C [16]. The cephem moiety is attached to a group on the chemotherapeutic agent chosen so that the prodrug is less toxic than the drug. Fully active drug (or drug analog) is released after β-lactamase-mediated cleavage of the β-lactam ring.

Taxol is an attractive drug for combining with humAb4D5–8 since $p185^{HER2}$ is overexpressed in ~25 % of primary human breast and ovarian cancers [17,18] and taxol itself is approved in the United States for the treatment of these cancers. Targeted prodrugs offer a way of potentially reducing the toxicities of taxol which include myelosuppression, mucositis and peripheral neuropathy [19,20]. In addition, taxol is only sparingly soluble and is delivered in polyoxyethylated castor oil (Cremaphor EL) which has its own associated toxicities including hypersensitivity [19,20]. The biological activity of taxol in promoting and stabilizing the assembly of microtubules requires a free C-2' hydroxyl (reviewed in [21]). In contrast the C-7 hydroxyl of taxol can be modified with retention of biological activity [21]. These structure-function properties of taxol have been used by others in the

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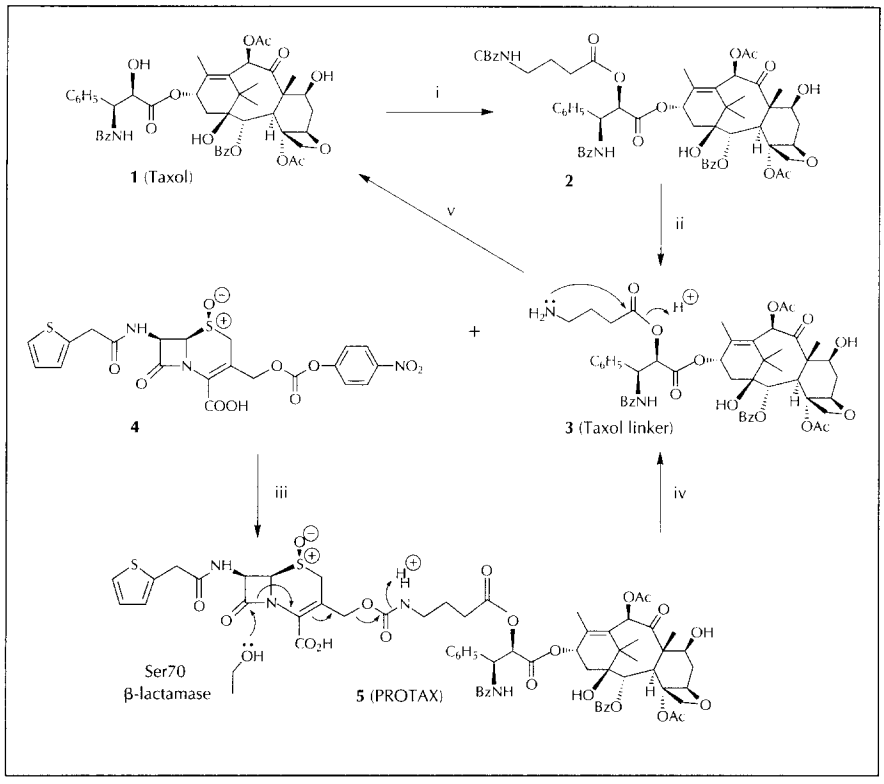

Fig. 1. Synthesis and activation of PROTAX. PROTAX was synthesized from taxol and subsequently activated to taxol using the following conditions: (i) dicyclocarbodiimide, N-CBz-γ-aminobutyric acid; (ii) 10 % Pd/C, formic acid, MeOH; (iii) 0.1 N $NaHCO_3$; (iv) β-lactamase or dsFv3–β-lactamase in PBS, pH 7.4; (v) PBS, pH 7.4. Compound **4** [9] was synthesized as previously described [7].

synthesis of water-soluble derivatives [22–24] including prodrugs that are efficiently activated in human serum [25]. Here we describe the synthesis and preliminary characterization of PROTAX, a cephem prodrug of taxol that is activatable by β-lactamase.

Results and discussion

Chemical synthesis of PROTAX

The activated cephem sulfoxide, compound **4**, has been coupled to a primary amine in a vinca alkaloid [9] or in doxorubicin [7,14] to create corresponding cephem prodrugs. We synthesized compound **4** [7] with minor modifications of the original method [9] but were not successful in coupling it to the C-2' hydroxyl of taxol (compound **1**). It was possible, however, to modify the C-2' hydroxyl of taxol with an aminobutyryl linker to give compound **3**, the taxol linker [26], and couple this to compound **4** to yield compound **5**, the taxol prodrug, PROTAX (Fig.1).

Activation of PROTAX by β-lactamase

PROTAX is rapidly hydrolyzed by both free β-lactamase and the tumor-targeting fusion protein, dsFv3–β-lactamase, to release taxol linker: $k_{cat}/K_M = (1.4 \pm 0.1) \times 10^5\ s^{-1}\ M^{-1}$ and $(6.3 \pm 1.3) \times 10^4\ s^{-1}\ M^{-1}$, respectively. The second activation step (reaction v in Fig. 1) involving self-immolation of taxol linker to release free taxol is slow compared to the first enzymatic cleavage step (reaction iv), as judged by HPLC (Fig. 2). Reaction v is approximately 50 % complete after 6 h and is approaching completion after 16 h.

Cephem prodrugs including PROTAX are prone to slow non-enzymatic hydrolysis (a property of their

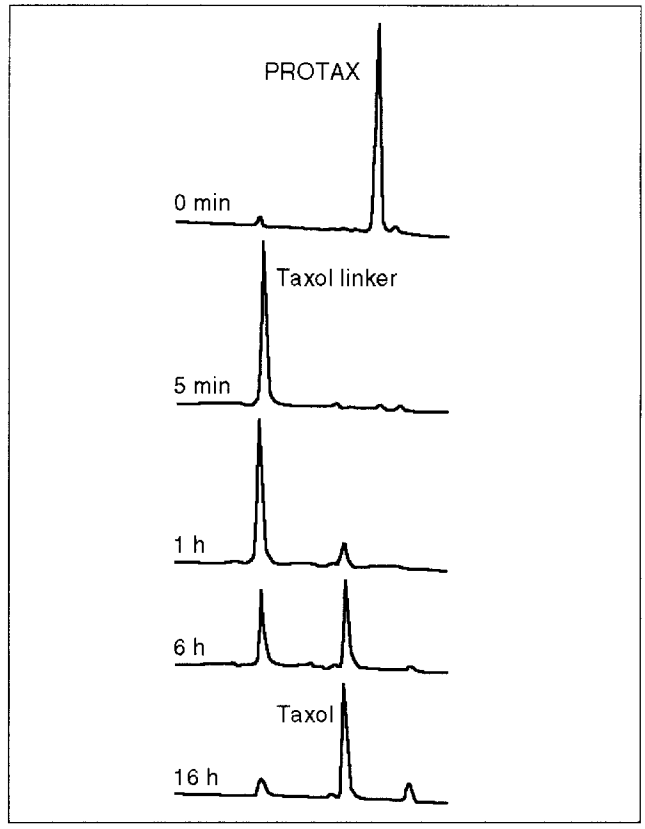

Fig. 2. HPLC analysis of PROTAX activation. PROTAX (70.4 μM) was hydrolyzed at 37 °C in the presence of 180 nM β-lactamase in PBS, pH 7.4, for varying times prior to analysis by reverse phase HPLC (C_{18} column, Vydac, Hesperia, CA) using 30–90 % (v/v) acetonitrile, 0.1 % (v/v) TFA in H_2O as the mobile phase. Peaks were identified as taxol-linker or taxol on the basis of electrospray mass spectrometry: taxol *m/e* 939.5 (M^+), expected 939.4; taxol linker *m/e* 854.4 ($M+H^+$), expected 853.9.

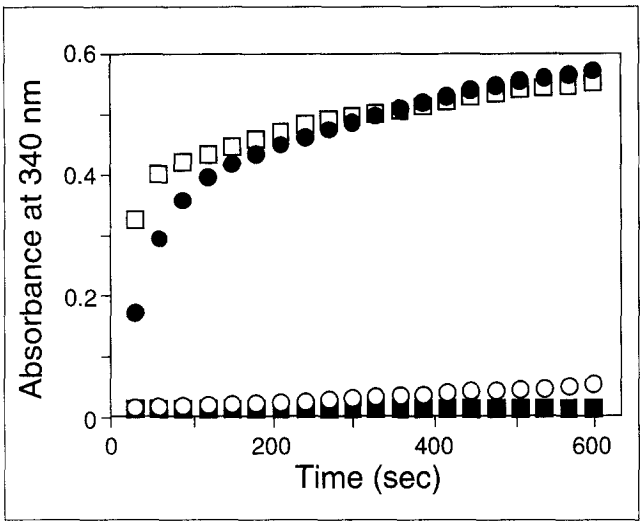

Fig. 3. Microtubule-assembly assay. Polymerization of bovine brain tubulin (1 mg ml^{-1}) at (35 ± 1) °C was monitored by following the absorbance at 340 nm in the presence of 11 μM taxol (□), or 11 μM PROTAX before (○) or after (●) activation with 180 nM β-lactamase for 18 h, or in the absence of further additions (■).

parent cephalosporins [27]), which probably sets an upper limit on the ratio of toxicity of prodrug to drug. The toxicity profile of PROTAX *in vivo* may be reduced because of slow self-immolation if this allows clearance of circulating taxol linker prior to conversion to the active compound, taxol. However, slow self-immolation of taxol linker may also limit the anti-tumor efficacy of PROTAX. The solubilities of PROTAX and taxol in 1 % (v/v) DMSO in PBS at 20 °C are ~20μg ml^{-1} and < 5 μg ml^{-1}, respectively (M.L.R., unpublished data). It may be possible to optimize the therapeutic benefit of PROTAX and enhance the solubility by judicious modification of the linker.

Effect of PROTAX on microtubule assembly

Taxol promotes the polymerization of bovine brain tubulin [21], whereas PROTAX has virtually no effect on the assembly of microtubules (Fig. 3). In contrast, PROTAX activated overnight in the presence of β-lactamase has very similar activity to free taxol in promoting microtubule assembly. Brief activation (15 min) of PROTAX with β-lactamase did not enhance its ability to promote tubulin polymerization (not shown). This was anticipated from the lack of microtubule-assembly activity of taxol linker (M.L.R., unpublished data) and its slow conversion to taxol (Fig. 2).

Cytotoxicity of PROTAX

PROTAX is ~10-fold less toxic than taxol against the breast tumor cell line, SK-BR-3, following 6 h exposure to these cytotoxic agents (Fig. 4a). In contrast, in the presence of the targeting fusion protein, dsFv3–β-lactamase, the potency of PROTAX approaches that of free taxol for corresponding 6 h exposures. Following brief exposure (1 h) to dsFv3-β-lactamase the cytotoxic activity of PROTAX against SK-BR-3 cells is enhanced to a level that is approximately half that of taxol (Fig. 4b). This enhancement is greater than that anticipated from the slow kinetics of the self-immolation reaction (Fig. 2). Enhanced cellular uptake of taxol-linker and taxol compared to PROTAX warrants investigation as a possible explanation for this observation.

Significance

The efficacy of taxol in breast and ovarian cancers has led to its approval for treatment of these diseases despite its poor solubility and numerous toxicities. The solubility of taxol has previously been enhanced by the synthesis of water-soluble derivatives [22–24] including prodrugs [25] that are efficiently activated in human serum. Here we have extended the scope of taxol prodrugs by the synthesis of PROTAX, which is the first reported enzyme-activatable prodrug of taxol. Thus PROTAX is the first taxol prodrug that is potentially targetable to a tumor using an antibody–enzyme fusion protein.

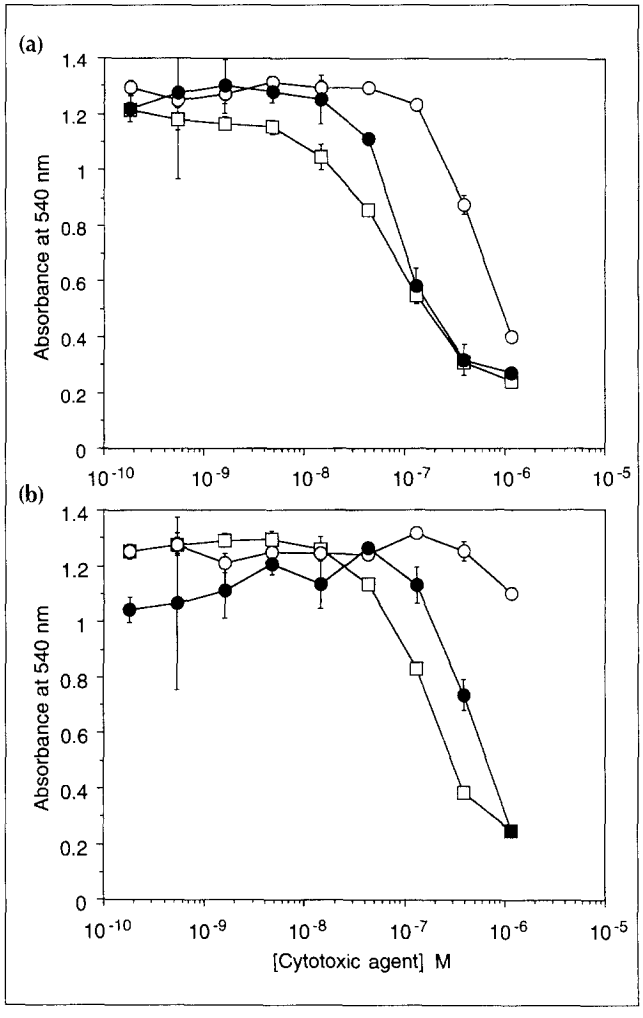

Fig. 4. *In vitro* cytotoxic effect of taxol and PROTAX against SK-BR-3 cells. Tumor cells were incubated with taxol (□) or with PROTAX in the absence (○) or presence (●) of 180 nM dsFv3–β-lactamase for **(a)** 6 h or **(b)** 1 h at 37 °C. The cells were cultured in fresh media for a total culture time of 72 h prior to crystal violet staining. Data shown are the mean ± SD of duplicate samples.

PROTAX comprises taxol linked by the C-2' hydroxyl to a cephalosporin via an aminobutyryl linker. Taxol is released from PROTAX following β-lactamase-mediated hydrolysis and self-immolation. PROTAX broadens the utility of cephems as triggers for prodrugs by demonstrating the efficacy of coupling through a hydroxyl group on a drug and of using a self-immolative linker to synthesize a prodrug.

PROTAX is ~10-fold less toxic than free taxol against SK-BR-3 cells *in vitro*, but is equally active following prolonged activation. In contrast, a related cephem prodrug of a vinca alkaloid is only five-fold less toxic than the parent free drug [8] against tumor cells *in vitro* but is highly efficacious in a tumor xenograft model *in vivo* [3]. These data encourage the evaluation of the anti-tumor efficacy of PROTAX in conjunction with the dsFv3-β-lactamase targeting fusion protein *in vivo*.

Materials and methods

Chemical synthesis

2'-N-(Carbobenzyloxy)-γ-aminobutyryl taxol (compound 2)
N-CBz-γ-aminobutyric acid (50 mg, 0.21 mmol) and dicyclohexylcarbodiimide (40 mg, 0.19 mmol) were added to a solution of taxol (compound **1**, Fluka, Ronkonkoma, NY) (50 mg, 0.059 mmol) in anhydrous acetonitrile (10 ml). The reaction mixture was stirred at room temperature (r.t.) for 48 h. The solvent was evaporated and product dissolved with EtOAc/hexane (55:45) and purified by flash chromatography to give compound **2** (42 mg, 66 %): white solid; R_f = 0.37 (EtOAc/hexane, 60:40). All ^{1}H NMR spectra were obtained using a Varian VXR-300 spectrometer at 25 °C using DMSO-D6 (contains 0.05 % (v/v) TMS) as a solvent. Proton shift assignments were facilitated by ^{1}H-^{1}H correlated spectroscopy (COSY). 9.10 (1H, d, $^3J_{HH}$=8.5 Hz, C3'HN**H**), 8.0–7.0 (20H, m, Ar–H), 7.10 (1H, CbzNH), 6.28 (1H, s, C10–H), 5.83 (1H, m, C13–H), 5.54 (1H, d, $^3J_{HH}$=8.8Hz), 5.42 (1H, d, $^3J_{HH}$=7.1Hz, C2–H), 5.35 (1H, C2'-H), 4.99 (2H, s, PhCH_2O), 4.91 (1H, d, $^3J_{HH}$=8.31 Hz, C5–H), 4.11 (1H, m, C7–H), 4.02 (2H, m, C20–H), 3.59 (1H, d, $^3J_{HH}$=7.0 Hz, C3–H), 3.01 (2H, m, CbzNHC**H**$_2$), 2.42 (2H, t, $^3J_{HH}$=7.57Hz, C**H**$_2$COOC2'H), 2.24 (4H, m, C6–H, CH_3COOC4), 2.10 (3H, s, CH_3COOC10), 1.78 (3H, C18–H), 1.70 (1H, C14–H), 1.67 (2H, m, CbzNHCH$_2$C**H**$_2$), 1.50 (5H, m, C6–H, C19–H, C14–H), 1.03 (3H, s, C16–H), 1.00 (3H, s, C17–H). FAB HRMS *m/e* 1073.4284 (M+H$^+$) calculated for $C_{59}H_{64}N_2O_{17}$: 1073.4313.

2'-(γ-Aminobutyryl) taxol formate (compound 3)
CBz-aminobutyryl taxol (compound **2**) (68 mg, 0.063 mmol) was dissolved in MeOH (50 ml). Formic acid (3.5 ml) plus a catalytic amount of 10 % Pd/C were then added and the reaction mixture stirred at r.t. for 4 h. The resulting mixture was filtered and concentrated under vacuum. The resulting residue (50 mg compound **3**) was then used immediately for the synthesis of compound **5**.

Cephalothin-2'-(γ-aminobutyryl) taxol prodrug (compound 5)
Compound **3** (50 mg) was resuspended in tetrahydrofuran (40 ml) and compound **4** (100 mg, 0.19 mmol, synthesized as previously described [7]) was added to the solution followed by $NaHCO_3$ (0.1 N, 8 ml). After stirring at r.t. for 15 min the reaction was terminated with HCl (0.1 N, 9 ml). The product was extracted with EtOAc and purified on a C_{18} column (Rainin, Microsorb 80-220C_5) with 40 % (v/v) acetonitrile, 0.1 % (v/v) trifluoroacetic acid (TFA) in H_2O as the mobile phase. The eluted HPLC pool was lyophilized to give the taxol prodrug, compound **5** (68 mg, 81 %): white solid. ^{1}H NMR spectra were obtained as described above: 9.31 (1H, d, $^3J_{HH}$=7.3 Hz, C3'HN**H**), 8.0–7.0 (16H, m, PhH, SCH), 7.19 (1H, CH_2OCN**H**), 6.96 (2H, m, SCH**CH**C**H**), 6.28 (1H, s, C10–H), 5.80 (1H, m, C13–H), 5.53 (1H, d, $^3J_{HH}$=8.7Hz, C3'–H), 5.40 (1H, d, $^3J_{HH}$=6.9Hz, C2–H), 5.34 (1H, C2'–H), 5.07 (1H, cyclo-NCOC**H**CH), 4.92 (2H, s, C**H**$_2$OCONH), 4.90 (1H, C5–H), 4.61 (1H, cyclo-NCOCHC**H**), 4.09 (1H, m, C7–H), 4.00 (2H, m, C20–H), 3.90 (1H, d, $^2J_{HH}$=15.5Hz, S(O)C**H**HC), 3.78 (1H, d, $^2J_{HH}$=15.5Hz, S(O)CH**H**C), 3.50 (1H, C3–H), 2.97 (2H, m, OCNHC**H**$_2$), 2.40 (2H, t, $^3J_{HH}$=7 Hz, CH_2COOC2'), 2.22 (1H, C6–H), 2.22 (3H, s, CH_3COOC4), 2.09 (3H, s, CH_3COOC10), 1.77 (2H, m, C14–H), 1.64 (2H, m, C**H**$_2$CH$_2$COOC2'), 1.49 (1H, C6–H), 1.49 (3H, s, C19–H), 1.01 (3H, s, C16–H), 0.99 (3H, s, C17–H); FAB HRMS *m/e* 1335.4006 calculated for $C_{66}H_{70}N_4O_{22}S_2$: 1335.4002.

Expression and purification of β-lactamase RTEM-1 and dsFv3–β-lactamase fusion protein

The dsFv3–β-lactamase fusion protein and β-lactamase were secreted from *E. coli* strain 27C7 containing expression plasmids pRZ1 and pRZ3, respectively, grown for 32 h at 30 °C in an aerated 10 liter fermentor and purified as previously described [7]. Purified proteins were buffer exchanged into PBS and their concentration estimated from the absorbance at 280 nm (ϵ_{280}): dsFv3–β-lactamase, ϵ_{280} = 1.2 x 10^5 cm^{-1} M^{-1} [7]; β-lactamase ϵ_{280} = 2.94 x 10^4 cm^{-1} M^{-1} [28]. Purified proteins were flash frozen in liquid nitrogen and stored at –70 °C until required.

Kinetic procedure

Kinetic assays were performed in 10 % (v/v) DMSO in PBS, pH 7.4, at (25 ± 0.2) °C using either 0.2 cm or 1 cm path length quartz cuvettes and a Kontron Uvikon 860 spectrophotometer. Initial rates of hydrolysis of 8–34 μM PROTAX with 4.76 nM dsFv3–β-lactamase or 11–112 μM PROTAX with 3.7 nM β-lactamase were determined by monitoring the reduction in absorbance at 260 nm over ~2.5 min and using the experimentally determined extinction coefficient for PROTAX: $\Delta\epsilon_{260}$ = 1.03 x 10^4 cm^{-1} M^{-1}. Estimates of k_{cat}/K_M ratios were made from the slope of the linear plots of initial reaction rates versus substrate concentrations [29] using a non-linear least squares fit (KaleidaGraph version 3.0.5, Synergy Software, Reading, PA).

Microtubule assembly assay

Bovine brain tubulin (1 mg ml^{-1}; Cytoskeleton, Santa Barbara, CA) was incubated with 11 μM taxol, or 11 μM PROTAX before or after activation with 180 nM β-lactamase for 18 h, or buffer alone (80 mM PIPES (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, 1 % (v/v) DMSO) at (35 ± 1) °C. Polymerization of tubulin was monitored by following the absorbance at 340 nm.

In vitro *cytotoxicity assay*

SK-BR-3 breast carcinoma cells (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium/Ham's nutrient F-12 (50:50) supplemented with 2 mM glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Gibco BRL, Grand Island, NY), and 10 % bovine fetal serum (Hyclone, Logan, UT)

at 37 °C, 5 % CO_2 and pH 7–7.4. Cells were seeded at 1.2 x 10^4 cells per well in 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and allowed to attach for a minimum of 4 h. Test media consisting of 0–1.2 μM taxol or 0–1.2 μM PROTAX and ≤ 0.1 % (v/v) DMSO in the absence or presence of 180 nM dsFv3-β-lactamase was added to the wells. Plates were incubated at 37 °C, 5 % CO_2, for 24 h, washed twice with warm medium, and incubated for a further 48 h. The assay was terminated by staining with 0.5 % (w/v) crystal violet in MeOH, and the absorbance was read at 540 nm (SLT 340 ATTC plate reader, SLT Lab Instruments, Salzburg, Austria). Very similar results were obtained using methods that detect only viable cells: alamar blue assay, MTT staining, or direct cell counting (data not shown). No enhancement of the cytotoxic activity of taxol against SK-BR-3 cells was observed in the presence dsFv3–β-lactamase (data not shown).

Supplementary material available

Supplementary materials include 1D and 2D NMR spectra for compounds **2** and **5** plus HPLC traces for compound **5**.

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