Novel in silico-designed estradiol analogues are cytotoxic to a multidrug-resistant cell line at nanomolar concentrations

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Abstract

Purpose 2-Methoxyestradiol (2ME) is a promising anti-cancer agent that disrupts the integrity and dynamics of the spindle network. In order to overcome the pharmacokinetic constraints of this compound, a panel of sulphamoylated estradiol analogues were in silico-designed by our laboratory. In this study, we analysed the potential of each analogue to induce cell death on a panel of cancer cell lines. Moreover, the mechanism of action of the most effective compounds was determined.

Methods Cytotoxicity screening of the compounds and intermediates was performed on five different cancer cell lines to determine IG_{50} values. An in vitro tubulin polymerization assay was done to determine the effect of the drugs on tubulin polymerization while their intracellular effects on the microtubule network were assessed by immunofluorescence microscopy.

Results IG_{50} calculations showed that the sulphamoylated analogues induce cytotoxicity at nanomolar concentrations in all cell lines, including the P-glycoprotein pump overexpressing multidrug-resistant uterine sarcoma cell line. The non-sulphamoylated compounds were only cytotoxic at micromolar ranges, if at all. The sulphamoylated compounds inhibited pure tubulin polymerization in a dose-dependent manner and induced microtubule destruction in cells after 24-h exposure.

Conclusion Results revealed that the novel sulphamoylated 2ME derivatives have potential as anti-cancer drugs, possibly even against chemoresistant cancer cells. These compounds disrupt the intracellular microtubule integrity which leads to mitotic block of the cells.

Keywords Multidrug resistance · 2-Methoxyestradiol analogue · Anti-mitotic · Cytotoxicity · Microtubule dynamics · Tubulin

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Introduction

Microtubule disrupting agents have been a cornerstone of anti-cancer treatment for the past three decades. Spindle poisons can be further classified based on their effect on microtubule structure and dynamics. These classes include microtubule stabilizers such as the taxanes and microtubule depolymerizers such as nocodazole, vinca alkaloids and colchicine [1]. Disadvantages of these agents include high side effect profiles, as well as the development of drug resistance caused by the overexpression of efflux pumps or various mutations in the tubulin gene, among others [2].

2-Methoxyestradiol (2ME) is an endogenous metabolite of 17 β-estradiol. It has undergone phase I and phase II clinical trials as an anti-mitotic and anti-angiogenic agent in various cancer types [3]. Kamath et al. [4] demonstrated that 2ME suppressed microtubule dynamics at low concentrations, while higher doses resulted in microtubule

depolymerization. 2ME binds the tubulin colchicine binding site resulting in cells blocked at metaphase that will ultimately undergo apoptosis [5, 6]. Advantages of 2ME over classic microtubule poisons include a better tolerated side effect profile, preferential sparing of non-malignant cells, and its inability to function as a substrate of the P-glycoprotein efflux pumps [7, 8]. A shortcoming of the compound is its rapid 17 β-hydroxysteroid dehydrogenase mediated metabolism resulting in a poor pharmacokinetic profile [9].

We have previously designed a panel of 3′ sulphamoylated 2ME analogues in silico (Fig. 1a) by modifying the 2′ and 17′ positions with moieties known to improve the anti-mitotic activity and increase the half-life of the compounds [10, 11]. This panel was analysed using AutoDockTools4 with the prepare_ligand4py script to determine which compounds bound best with the tubulin colchicine binding site and with carbonic anhydrase IX (CA IX) [10]. CA IX is overexpressed in numerous tumour cells, lending a growth advantage to malignant cells within the acidic and hypoxic microenvironment [12]. Selective inhibition of CA IX could potentially be useful in manipulating the extracellular tumour milieu and curtailing metastatic properties. Additionally, due to its steroid structure, 2ME is lipophilic, and thus poorly soluble in aqueous medium [13].

The aim of this study was to assess the cytotoxic effects of these in silico-designed compounds on various cancer cell lines including a multidrug-resistant (MDR) sarcoma cell line. In addition, the compounds demonstrating the best cytotoxic profiles were analysed regarding their effect on in vitro microtubule polymerization activity as well as on intracellular microtubule dynamics.

Materials and methods

Cell lines, culture methods and chemicals

Estrogen and progesterone receptor-negative MDA-MB-231 breast cancer cells, human cervical adenocarcinoma (HeLa) cells, K-RAS-mutated human alveolar basal epithelial cancer cells (A549), MCF7 estrogen positive breast cancer cells, human uterine sarcoma (MES-SA) and the P-glycoprotein pump (PgP) overexpressing MDR-derivative MES-SA/DX5 (uterine sarcoma) cells were used for cytotoxic studies. All cells were obtained from the American Tissue Culture Collection (ATCC).

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco®) supplemented with 10 % heat-inactivated foetal calf serum (Hyclone), 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich). Phosphate-buffered saline (PBS) was purchased from Gibco®. Cells were cultured at 37 °C in a 5 % CO_2 humidified atmosphere in a Forma Scientific water-jacketed incubator (Ohio, USA).

The non-commercially available in silico-designed sul-phamoylated 2ME analogues were synthesized by Ithemba (PTY) Ltd Pharmaceuticals (Gauteng, South Africa). They were dissolved in dimethyl sulfoxide (DMSO) to make a

10 mM stock solution and stored at −20 °C.

All other reagents not specifically mentioned were purchased from Sigma-Aldrich.

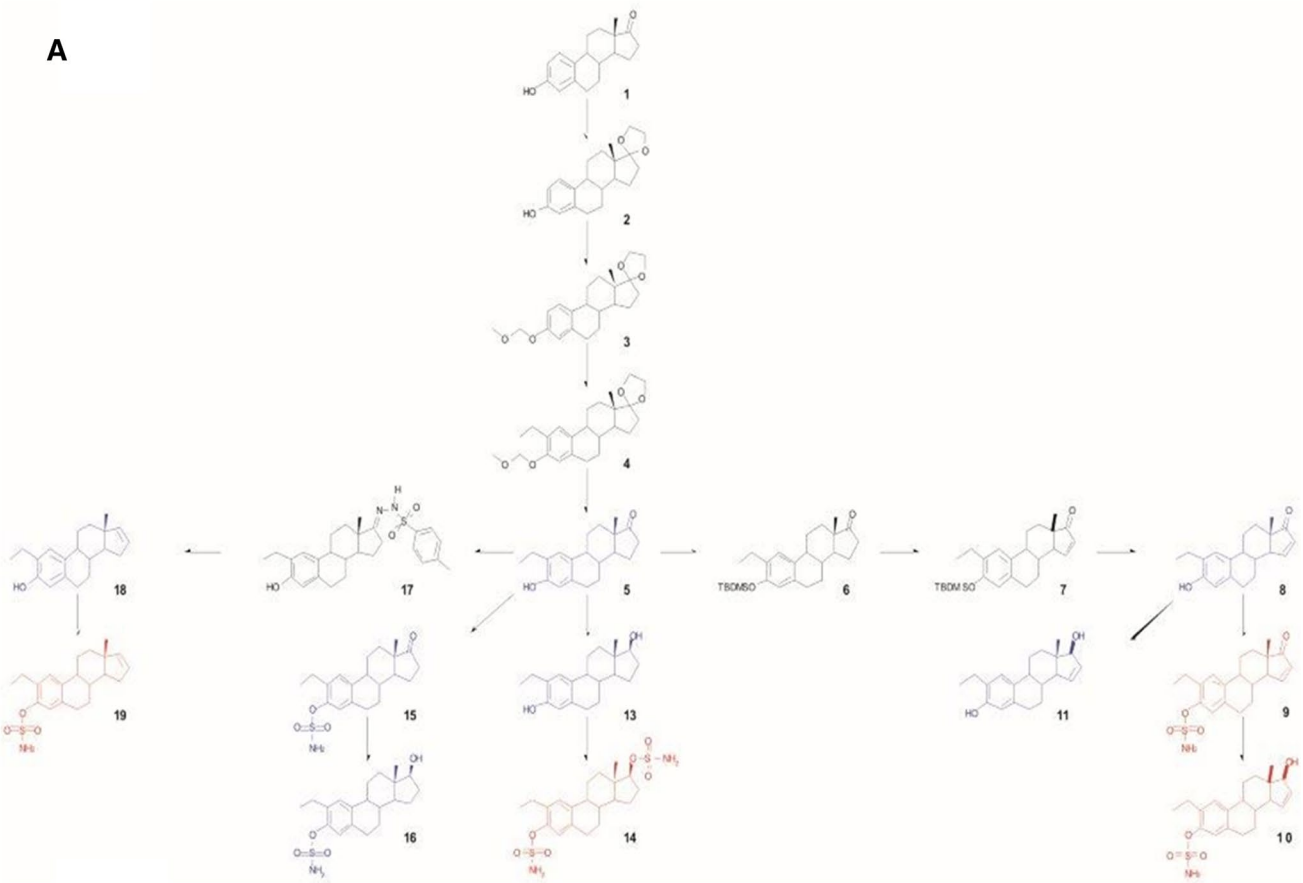
Cytotoxicity quantification

The cytotoxic effects of the generated compounds were quantified by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). HeLa, MDA-MB-231, A549, MES-SA and MES-SA/DX5 cell were seeded in 96-well clear bottom black polystyrene microplates (Greiner) based on their optimal seeding densities. After attachment, cells were exposed to the 17 compounds in a dose dilution series ranging between 19 and 10,000 nM for 48 h. DMSO (0.05 % v/v) was used as a negative control (100 % cell viability) and 1 % SDS as a positive control (complete cell death). Upon termination, wells were washed with PBS followed by incubation in 0.5 mg MTT in RPMI without phenol red (Gibco®) at 37 °C for 4 h. The formazan dye crystals were solubilized with an acidic isopropanol solution (isopropanol, 0.1 N hydrochloric acid, 10 % triton X-100). Absorbance was read on a FLUOstar spectrophotometer (BMG Labtechnologies) at 570 nm. Each experimental plate was run in triplicate and the experiment repeated three times. The concentration of the drug at which 50 % growth inhibition occurred over a given time period (IG_{50}) was calculated in all cell lines.

In vitro tubulin polymerization

In vitro tubulin polymerization assays were performed as previously described [14]. Pure bovine tubulin was prepared as described [15]. Microtubule assembly was carried out in half-area 96-well black plates (Greiner). Pure tubulin was added to each well at a final concentration of 30 µmol/L in MEM buffer (100 mM 2-[N-morpholino] ethanesulfonic acid (MES), 1 mM $MgCl_2$, 1 mM EGTA, pH 6.75) with 10 micromol/L 40,6-diamidino-2-phenylindole (DAPI) and variable concentrations of the novel sulphamoylated compounds. 2-Methoxyestradiol-3,17-O,O-bis-sulphamate

A



B

Cell line	HeLa	MDA-MB-231	A549	MCF7	MES-SA	MES-SA/DX5
	IG50 (nM)					
Compound						
2MEbisMATE	175	383	960	195	220	474
Compound 1	>10k	3600	>10k	>10k	>10k	>10k
Compound 2	>10k	8600	>10k	>10k	>10k	>10k
Compound 3	>10k	>10k	>10k	>10k	>10k	>10k
Compound 4	>10k	>10k	>10k	>10k	>10k	>10k
Compound 5	>10k	>10k	>10k	>10k	>10k	>10k
Compound 6	>10k	>10k	>10k	>10k	>10k	>10k
Compound 7	1825	6300	933	450	1607	1983
Compound 8	4988	9800	1980	1600	3883	2610
Compound 9	176	229	147	280	277	309
Compound 10	75	203	100	150	77,5	146
Compound 11	6375	8450	4075	8330	8900	>10k
Compound 13	3983	4550	>10k	>10k	8600	>10k
Compound 14	313	421	395	210	208	405
Compound 15	170	254	128	100	113	167
Compound 16	208	185	471	143	110	239
Compound 17	9900	9250	8750	>10k	>10k	>10k
Compound 19	208	392	290	147	232	255

Fig. 1 a Synthesis pathway of the novel in silico-designed 2-methoxyestradiol analogues [11]. **b** IG_{50} values calculated from the cytotoxic effects of the generated compounds in various neoplastic cell lines. Those with no cytotoxic effects below 10,000 nM are denoted by '>10 k.' Values depict nanomolar (nM) concentrations. All experiments are conducted in triplicate

(2MEbisMATE) and an azaindole derivative CMO2 [14] were used as positive controls. Following a 10-min incubation, tubulin assembly was initiated by injection of 1 mmol/L guanine triphosphate (GTP) and 5 mmol/L $MgCl_2$. Fluorescence of microtubule-bound DAPI was monitored as a function of time at 37 °C using a microplate reader (FLUOstar OPTIMA, BMG Labtechnologies) using excitation and emission wavelengths of 360 and 450 nm, respectively. Fluorescence signal at time 0 was subtracted from each of the subsequent fluorescence readings. Each compound was assayed in triplicate.

Immunofluorescence microscopy

This method has been described by Paturle et al. [16]. HeLa and MDA-MB-231 Cells (60,000 cells) were seeded on 12-mm-round coverslips and grown overnight before being exposed to the relevant novel compounds for 24 h. DMSO was used as the negative vehicle control. Colchicine (0.5 μM) was used as a positive control for microtubule depolymerization, 1 μM vinblastine as an inhibitor of microtubule polymerization and 1 μM paclitaxel as a microtubule stabilizer. Exposed cells were permeabilized with OPT buffer (80 mmol/L pipes, 1 mol/L EGTA 1 mol/L $MgCl_2$, 0.5 % triton X-100, and 10 % glycerol, pH 6.8) at 37 °C and fixed in methanol at −20 °C. Cells were incubated in a primary antibody cocktail consisting of L4 rabbit anti-detyrosinated tubulin (glu-tubulin) and YL½ rat anti-tyrosinated tubulin [16] at 1/4,000 in PBS-tween 0.1 % and 0.3 % bovine serum albumin. After rinsing in PBS-tween 0.1 %, cells were incubated with the secondary antibodies Alexa Fluor® 488 anti-rabbit (Invitrogen), Cy3 anti-rat (Jackson ImmunoResearch Laboratories) and Hoechst at 1/1,000 in PBS-tween 0.1 % containing 0.3 % BSA. Rinsed slides were mounted and viewed with a Zeiss Axio Imager Z1 microscope controlled by Axiovision software (Carl Zeiss) using a X63 oil objective. Images were captured using an Ocra R2 N/B camera (Hamamatsu). These images were examined for tubulin integrity and the ratio of tyrosinated to detyrosinated tubulin.

Results

Cell cytotoxicity

The IG_{50} of all 17 in silico-designed compounds on the various cell lines were determined (Fig. 1b). The sulphamoylated compounds 9, 10, 14, 15, 16 and 19 showed IG_{50} values at nanomolar concentrations after 48-h exposure in all the cell lines exposed. Notably, similar values were obtained for the standard cancer cell lines and the MDR carcinoma cell line, MES-SA/DX5, suggesting that these compounds may possibly overcome drug resistance. The non-sulphamoylated intermediates did not show significant cytotoxicity, even at 10 μM, suggesting that the sulphamoyl group confers the observed cytotoxicity.

In vitro tubulin polymerization

To understand how the cytotoxic compounds may cause cell death their effect on microtubule polymerization was analysed. The rate of tubulin polymerization was measured in vitro in the absence (negative control) or presence of increasing concentrations of the sulphamoylated compounds. Results showed that all six sulphamoylated compounds inhibited microtubule polymerization although not all to the same extent. Specifically, compound 19 inhibited polymerization even at 5 μM, while compound 15 was less active with only 25 μM showing a complete inhibitory effect (Fig. 2a).

Cellular microtubule dynamics

Fluorescence microscopy was used to visualize the integrity and dynamic qualities of the microtubule network. This technique is based on the substrate properties of the tubulin enzymes active in the tubulin tyrosination cycle. Tyrosinated tubulin staining is indicative of dynamic microtubules, whereas stabilized tubules are mostly detyrosinated. HeLa and MDA-MB-231 cells were exposed to the IG_{50} concentrations of compounds 9 and 19. Control results (DMSO) demonstrated structurally intact, dynamic spindle fibres (red) stained with an anti-tyrosinated antibody (Fig. 2b). Paclitaxel induced prominent stabilization of the microtubule network compared with vinblastine which induced crystal formation and complete microtubule obliteration. Colchicine treatment resulted in rounded cells, with only fragmented detyrosinated microtubules remaining. Compound 9- and compound 19-treated cells showed disrupted microtubules, rounded cells and a decreased cell density similar to colchicine-treated cells. HeLa cells exposed to compound 9 appear to be blocked at metaphase, with numerous mitotic spindles being visible.

Discussion

Sulphamate side chains on estrogenic structures are predicted to increase the molecules' bioavailability by bypassing the first-pass hepatic metabolism [17]. This has been attributed to the reversible binding of the sulphamine moiety to carbonic anhydrase II (CA II) in erythrocytes. Additionally, maximal anti-tubulin activity is achieved with the substitution of unbranched chains at position 2′ of the A-estradiol ring [18]. Certain modifications of the D ring have also been shown to increase the anti-proliferative activity of 2ME while decreasing its metabolic breakdown

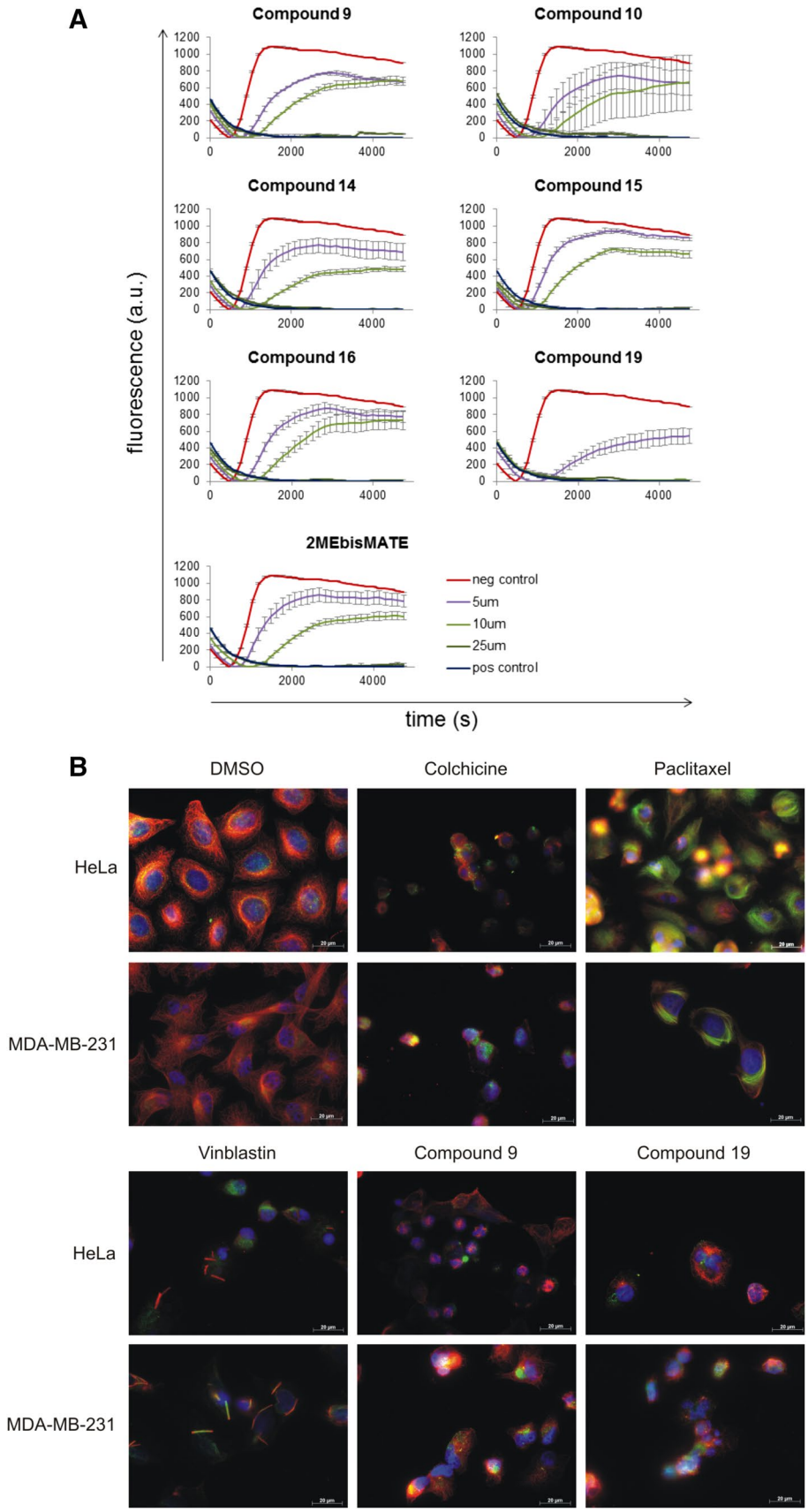


Fig. 2 a The effect of six sulphamoylated 2ME derivatives on tubulin polymerization. DMSO was used as a negative control, while CMO2 was used as a positive control [14]. Graphs depict each of the compounds' effect on tubulin polymerization (y-axis) over time (x-axis) and include a DMSO negative control (*red line*) and a CMO2 positive control. Data points show the average of three experiments with the *error bars* representing the SEM. The compounds inhibited tubulin polymerization in a dose-dependent manner, with compound 19 demonstrating the most pronounced effect. **b** Double immunofluorescence micrographs of HeLa and MDA-MB-231 cells (×63 magnification) after 24-h treatment with compounds 9 and 19. *Green staining* indicates nondynamic detyrosinated tubulin, whereas the *red* fibres indicate dynamic tyrosinated microtubules. Compound-treated cells resulted in abrogated microtubule networks and a morphology similar to cells treated with colchicine. HeLa cells exposed to compound 9 showed a block in metaphase, with mitotic spindles visible. Paclitaxel-treated cells demonstrated microtubule stabilization. Nuclei are stained *blue* with DAPI. The *scale bar* denotes 20 μm, as indicated

[7]. Of particular note is the dehydration at the metabolically active 17′ position which confers increased cytotoxicity to the modified analogues [19].

2-Methoxyestradiol-3,17-O,O-bis-sulphamate (2MEbisMATE) was designed with the above concepts in mind and is currently undergoing clinical trials as STX140 [20]. 2MEbisMATE has a positive prolife for anti-cancer and anti-angiogenic activities. It was therefore used as a base to design a range of molecules that had improved cytotoxicity and, more importantly, selective CA IX activity [10]. The CA IX enzyme is overexpressed within the hypoxic tumour milieu and thus would theoretically localize and concentrate the compound to the tumour itself, leading to a decrease in systemic toxicity and non-cancerous (normal) tissue damage.

The novel sulphamoylated compounds showed up to a tenfold increase in cytotoxicity when compared to previously reported 2ME IG_{50} values in various cells lines [21]. However, the intermediary non-sulphamoylated compounds 1–6 showed little to no cytotoxicity on any cell line. It therefore seems that cytotoxicity is conferred by the addition of a sulphamoylated side chain at position 3′ of the steroid structure. Our data also suggest that cytotoxicity is not influenced to a great extent by the various selected conformations at position 16′ and 17′ of the D ring. The addition of a carbonyl group (compound 15) or a sulphur side chain at position 17′ (compound 14) results in minor differences in the IG_{50}. Dehydration at the 16′ position together with the hydroxyl group at 17′ also makes little difference to the cytotoxicity (compounds 10 and 16).

From a galenic point of view, steroids are usually hard to solubilize due to their hydrophobic scaffold [13]. With the addition of sulphamate moieties, the compound's solubility in aqueous media should increase. With more soluble compounds, use of better tolerated excipients becomes a possibility.

We observed very little difference in cytotoxicity between cell lines including the MDR sarcoma cell line. This suggests that these compounds may be able to circumvent drug resistance induced by the activity of PgP efflux pumps [22]. MCF7 and MDA-MB-231 cells were similarly sensitive to the sulphamoylated compounds indicating that these compounds act independently of the estrogen and/or progesterone receptors to induce cell death.

The anti-proliferative effect of 2ME has been shown to stem, at least partially, from its ability to inhibit tubulin assembly through its interaction with the protein at the colchicine site [9, 10, 23]. The inhibition of pure tubulin polymerization in the in vitro assay indicates that the sulphamoylated compounds are also able to impact tubule formation. Furthermore, microscopy confirmed that these compounds affect cellular microtubule dynamics similarly to cells treated with colchicine which induces complete destruction of microtubules. This is in contrast to the spindle stabilization observed in cells treated with paclitaxel. As with traditional spindle poisons, disruption of the microtubules by the novel compounds halt the cells at metaphase, with the inability to pass the spindle assembly checkpoint resulting in the induction of apoptosis.

In conclusion, the cytotoxic effects of a panel of novel sulphamoylated and non-sulphamoylated 2ME analogues were demonstrated using a variety of tumour cell lines including a MDR sarcoma cell line. The sulphamoylated compounds showed increased cytotoxic effects when compared to the non-sulphamoylated intermediates, implicating the important role of the sulphamine side chain in the modification of 2ME as a more effective anti-cancer agent. The effect of the compounds on microtubule dynamics could not however account for all the cytotoxicity, as the IG_{50} is not strictly correlated to the depolymerizing effect (compare similar IG_{50} values of compounds 9 and 19 with their in vitro inhibition of tubulin assembly). As these compounds were in silico-designed to optimize binding to CA IX which is highly expressed in the hypoxic tumour milieu, further studies investigating the ability of these compounds to specifically localize to such environments would be beneficial. Additionally, these compounds show promise to overcome cancer cell multidrug resistance, including hypoxia-induced resistance. Further investigations will allow us to understand how the efflux pump transport system is avoided and if these compounds will also be able to induce cell death in MDR cancers in vivo.

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Conflict of interest The authors declare that they have no conflict of interests.

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