2-Methoxyoestradiol-3,17-O,0-bis-sulphamate and 2-deoxy-d-glucose in combination: a potential treatment for breast and prostate cancer

SLC Tagg1, PA Foster1, MP Leese2, BVL Potter2, MJ Reed1, A Purohit1 and SP Newman*,1

1Oncology Drug Discovery and Women’s Health Group, Faculty of Medicine, Imperial College London, St Mary’s Hospital, London W2 1NY, UK; 2Medicinal Chemistry and Sterix Ltd, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

Drug combination therapy is a key strategy to improve treatment efficacy and survival of cancer patients. In this study the effects of combining 2-methoxyoestradiol-3,17-O,0-bis-sulphamate (STX140), a microtubule disruptor, with 2-deoxy-d-glucose (2DG) were assessed in MCF-7 (breast) and LNCaP (prostate) xenograft models in vivo. In mice bearing MCF-7 xenografts, daily p.o. administration of STX140 (5 mg kg−1) resulted in a 46% (P<0.05) reduction of tumour volume. However, the combination of STX140 (5 mg kg−1 p.o.) and 2DG (2 g kg−1 i.p.) reduced tumour volume by 76% (P<0.001). 2-Methoxyoestradiol-3,17-O,0-bis-sulphamate also reduced tumour vessel density. 2-Deoxy-d-glucose alone had no significant effect on tumour volume or vessel density. A similar benefit of the combination treatment was observed in the LNCaP prostate xenograft model. In vitro the degree of inhibition of cell proliferation by STX140 was unaffected by oxygen concentrations. In contrast, the inhibition of proliferation by 2DG was enhanced under hypoxia by 20 and 25% in MCF-7 and LNCaP cells, respectively. The combination of STX140 and 2DG in LNCaP cells under normoxia or hypoxia inhibited proliferation to a greater extent than either compound alone. These results suggest that the antiangiogenic and microtubule disruption activities of STX140 may make tumours more susceptible to inhibition of glycolysis by 2DG. This is the first study to show the benefit of combining a microtubule disruptor with 2DG in the two most common solid tumours.

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LNCaP cells also required 1% sodium pyruvate. Cells were maintained in a humidified incubator at 37°C under 5% CO₂.

For hypoxic experiments an Innova CO-48 CO₂ humidifier incubator (Newbrunswick Scientific, St Albans, UK) was used, oxygen was maintained at 1% and CO₂ at 5% in a humidified atmosphere at 37°C.

**Proliferation assay**

MCF-7 and LNCaP cells were seeded at 3000 and 5000 cells per well, respectively, in 96-well plates. To ensure that cells had adhered to the plate, compounds were not added until 4h after the seeding of the cells. After 72–96h cell numbers were measured by the addition of 10 μl of Alamar blue (Biosource, Nivelles, Belgium) for 2h and, subsequently, fluorescence was quantified using a Fluostar Optima (BMG Labtech, Offenburg, Germany) Plate reader (544 nm excitation; 590 nm emission).

**Measurement of ATP**

MCF-7 and LNCaP cells were seeded at 3000 and 5000 cells per well, respectively, in 96-well plates. Compounds were added 4h after seeding of cells. The ATPlute assay procedure was carried out according to the manufacturer’s instructions (Perkin-Elmer, Beaconsfield, UK). The luminescence emitted from the ATP-dependent luciferase reaction was measured using a Fluostar Optima Plate reader.

**Cell cycle/apoptosis analysis**

Cells were plated in T-25 flasks so that after 24 h they were 60–70% confluent and were then treated with the compounds for a further 48h. Control cells were untreated or treated with tetrahydrofuran (THF) vehicle only. To harvest cells for flow cytometric DNA analysis, cells were washed with PBS before being trypsinised (0.25% trypsin, 0.05% EDTA). The medium containing non-adherent cells was also collected and pooled with the trypsinised cells. The cells and PBS-washings were pelleted by centrifugation at 1500 rpm and washed twice with PBS.

For cell cycle analysis the cells were then fixed in cold 70% ethanol, treated with 100 μg ml⁻¹ RNase for 5 min, stained with 50 μg ml⁻¹ propidium iodide and analysed using a flow cytometer (FACScan, Becton Dickinson, Cowley, UK).

For quantification of apoptosis the cells were re-suspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at 1 × 10⁶ cells ml⁻¹. Cells were then stained with fluorescein-conjugated Annexin V (BD Biosciences, Cowley, UK) antibody and propidium iodide (5 μg ml⁻¹) before flow cytometric analysis. Apoptotic cells are defined as cells positive for Annexin V and negative for propidium iodide.

**In vivo tumour xenograft model**

Intact, athymic, female and male MF-1 nude mice (nu/nu) were purchased from Harlan (Bicester, Oxon, UK) at 5 weeks of age (∼20–25 g in weight). All efforts were made to minimise both suffering and the number of animals used. Experiments were carried out under the UK Animals (Scientific Procedures) Act 1986 and complied with institutional guidelines. Animals were kept in a 12 h light/12 h dark cycle and given food and water ad libitum. Five million cells (MCF-7 or LNCaP), in ice-cold Matrigel (BD Biosciences), were inoculated s.c. into the right flank of the animals. Once the tumours reached 100–150 mm³ in volume, mice were randomly divided into four treatment groups (n = 5):

1. Vehicle (10% THF; 90% propylene glycol (PG)), 100 μl p.o. (oral gavage) + saline i.p. 200 μl.
2. Vehicle p.o. + 2DG (2 g kg⁻¹) i.p. 200 μl.

**MATERIALS AND METHODS**

**Compounds**

2-Deoxy-D-glucose (Figure 1, B) was obtained from Sigma (Poole, UK). 2-Methoxyoestradiol-3,17-O-bis-sulphamate (Figure 1, C) was synthesised as described previously (Leese et al, 2006). 2-Methoxyoestradiol-3,17-O-bis-sulphamate yielded spectroscopic and analytical data in accordance with its structure.

**Cell culture**

MCF-7 and LNCaP cells were obtained from the American Tissue Culture Collection (LGPC Promochem, Teddington, UK). Cells were cultured in the following medium obtained from Sigma: RPMI 1640 with 10% foetal bovine serum, 1% L-glutamine, 1% MEM non-essential amino acids and 1% sodium bicarbonate solution.
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Inhibition of proliferation by 2DG and STX140

The growth-inhibitory effects of 2DG and STX140, used alone and in combination, were compared in MCF-7 and LNCaP cells, under both normoxia and hypoxia in vitro (Figure 3A and B). The growth inhibition was determined after 72 h. Compared with normoxic untreated controls, STX140 (0.5 μM) inhibited cell proliferation by 45 and 48% in MCF-7 cells (P < 0.01, Figure 3A) under normoxia and hypoxia, respectively, and by 65% in LNCaP cells (P < 0.001, Figure 3B) under both normoxia and hypoxia. Under normoxic conditions, in both cell types, 2DG alone (8 mM) inhibited cell proliferation by 50%. Under hypoxic conditions 2DG inhibited cell proliferation by 70 and 75% in MCF-7 and LNCaP cells, respectively, a significant increase in efficacy relative to normoxic conditions (P < 0.01). Compared with STX140 at 0.5 μM, 2DG (8 mM) alone is significantly more effective at inhibiting tumour cell proliferation in both cell types (MCF-7: P < 0.01 and LNCaP: P < 0.05) under hypoxia, but not under normoxia (Figure 3A and B). The addition of STX140 (0.1–0.5 μM) did not further enhance the inhibition of cell proliferation seen with 8 mM 2DG in MCF-7 cells either in normoxia or in hypoxia (Figure 3A). However, improved efficacy was seen when combining the two agents in vitro in the LNCaP cells. The combination of 0.1 μM STX140 and 8 mM 2DG was more potent (P < 0.05) than either compound alone under normoxia at these concentrations. In contrast, this result was not seen under hypoxic conditions with the same concentrations. However, 8 mM 2DG plus either 0.5 or 1.0 μM STX140 was more potent (P < 0.05 and **P < 0.01, respectively) than either compound alone under hypoxia at these concentrations; this result was not seen under normoxia with the same concentrations.

Cell cycle/apoptosis

To understand the possible mechanisms for STX140/2DG-mediated cell death, both the cell cycle state and mechanism of cell death were assessed by FACS analysis (Figure 4A and B). Earlier studies showed that STX140 induced cell cycle arrest and apoptosis in a range of tumour cell lines (Day et al, 2003; Raobailkady et al, 2005; Newman et al, 2007). In both cell types 2DG alone in normoxia had little effect compared with untreated controls, although a reduction in the S-phase population was seen in LNCaP cells (**AP < 0.001). In LNCaP cells under hypoxia, 2DG alone significantly (P < 0.05) reduced the number of cells in G1 and G2/M compared with normoxic and hypoxic controls and with 2DG alone in normoxia. Despite this, no significant increase in cells undergoing apoptosis was detected. In MCF-7 cells under hypoxia a small increase was seen in the G2/M and apoptotic cell population compared with normoxia alone (**P < 0.01). Similar data were seen for STX140 in hypoxia compared with STX140 in normoxia in MCF-7 cells (*P < 0.05 and **P < 0.01). However, 2DG had little effect in MCF-7 cells under hypoxia and the extent of apoptosis measured was the same as seen for hypoxia alone. In the LNCaP cell line STX140 alone and STX140 with 2DG under hypoxia induced the greatest extent of apoptosis seen in this study; approximately 20% of cells were undergoing apoptosis (**P < 0.01). In contrast, the greatest extent of apoptosis observed in the MCF-7 line was only 10%, and the combination of hypoxia, 2DG and STX140 was less effective (**P < 0.01) than STX140 alone in hypoxia and 2DG combined with STX140 in normoxia (**P < 0.001 vs normoxia control).

Effect of STX140 and 2DG in vivo

As no previous studies have investigated in vivo the combination of a microtubule disruptor and 2DG in breast and prostate cancer, the efficacy of STX140 and 2DG was assessed in the MCF-7 (ER-positive, breast) and LNCaP (AR-positive, prostate) xenograft models.
models. In the breast cancer model (MCF-7) at the end of dosing (day 42), vehicle-treated tumours had increased in size by 1165 ± 73% relative to the tumour starting volumes on day 14. Growth of MCF-7 tumours was significantly inhibited by STX140 (5 mg kg⁻¹ p.o.; daily), tumours having increased in size by 664 ± 133% (P < 0.05). In mice treated with the combination of STX140 (5 mg kg⁻¹ p.o.; daily) and 2DG (2 g kg⁻¹ i.p.; daily) tumours increased in size only by 328 ± 82% (P < 0.001). The inset figure reveals that no weight loss occurred, indicating the potential absence of toxicity with these treatments (Figure 5A).

Figure 5B shows representative sections cut from the MCF-7 xenografts stained with an endothelial specific marker, von Willebrand’s factor, to assess vessel density. 2-Methoxyoestra
diol-3,17-β-sulphamate (5 mg kg⁻¹ p.o.; daily) and STX140 (5 mg kg⁻¹ p.o.; daily) combined with 2DG (2 g kg⁻¹ i.p.; daily) caused a reduction in the staining for endothelial cells (indicated by arrows). 2-Deoxy-d-glucose (2 g kg⁻¹ i.p.; daily) alone had no effect (data not shown). Conventional H&E staining revealed that the reduction in von Willebrand’s staining is not because of a decrease in cell density (Figure 5C).

In the prostate cancer model (LNCaP) at the end of dosing (day 28), vehicle-treated tumours had increased in size by 614 ± 162% relative to the tumour starting volumes on day 0. The combination of STX140 (5 mg kg⁻¹ p.o.; daily) with 2DG (2 g kg⁻¹ i.p.; daily) significantly (P < 0.001) inhibited tumour growth compared with STX140 alone. No weight loss occurred, indicating the absence of toxicity with these treatments (data not shown) (Figure 6).

**DISCUSSION**

Liu et al. (2001) recently proposed that 2DG combined with a traditional chemotherapeutic agent may offer a new strategy for cancer therapy. They hypothesised that 2DG would target the
slowly proliferating cells at the hypoxic centre of the tumour, which are highly dependent on glycolysis, and a chemotherapeutic agent would target the rapidly proliferating cells towards the tumour rim. In our study the combination of STX140 with 2DG was a potent inhibitor of tumour growth, in both breast and prostate cancer xenograft models in vivo. The excellent efficacy of
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therapy with 2DG had no significant effect on tumour growth. Unlike the MCF-7 model, no efficacy was seen with STX140 alone, further highlighting the benefit of combining the two agents. Although some studies do report weight loss in response to 2DG (Maschek et al, 2004), our data, in both models, concur with those of Gupta et al (2005), who reported no weight loss in response to 2DG.

The dose of STX140 used in these combination studies was approximately a quarter of the optimal dose so far identified (Foster et al, 2008b), and as such, more work needs to be undertaken to optimise the dosing of this combination treatment. Furthermore, this work indicates that the addition of 2DG to a chemotherapeutic regime may allow for lower doses of chemotherapy to be used, thereby reducing chemotherapy-related toxicity.

To investigate how the combination of 2DG and STX140 may target tumour cells, a series of in vitro experiments were undertaken; in these studies a hypoxic incubator was used to try and model the inner core of the tumour. The MCF-7 and LNCaP cell lines were equally sensitive to 2DG alone in vitro, with approximate IC_{50} values of 8 mM being obtained for both cell lines in normoxia. This value is in agreement with other studies that have shown IC_{50} values in the range of 4 – 12 mM in a variety of cell lines (Kaplan et al, 1990; Aft et al, 2002; Lampidis et al, 2006). The relatively high IC_{50} values for the inhibition of proliferation reflect the fact that 2DG has to compete with glucose in the culture medium for uptake into the cells. When the cells were grown under hypoxic conditions their sensitivity to 2DG significantly increased. These data suggest that under hypoxia the cells become more reliant on glycolysis and are therefore more sensitive to 2DG. This is supported by the significant reduction in cellular ATP levels seen in response to hypoxia alone, and the further reduction in ATP levels observed when hypoxia is combined with 2DG. Even in normoxia both these cell lines appear to be reliant on glycolysis to some extent, as 2DG alone in normoxia reduces ATP production by over 50% compared with normoxic controls. The combination of STX140 with 2DG only enhanced the inhibition of cell proliferation seen with 2DG alone, in the LNCaP cell line, at low doses of STX140 in normoxia and only at higher doses of STX140 in hypoxia. This result was supported by the significant increase in apoptosis seen with STX140 and 2DG under hypoxia compared with other treatments in the LNCaP cells, but not in the MCF-7 cells. These in vitro data further support the hypothesis of Liu et al. (2001), as the chemotherapeutic agent, STX140, is equally efficacious in normoxia and hypoxia but 2DG is more efficacious in the hypoxic environment, which is hypothesised to reflect the centre of the tumour.

This study for the first time shows the benefit of combining 2DG with an antiproliferative compound in vivo in the two most common cancers. This work supports the continued investigation into the use of 2DG combined with chemotherapeutic drugs for the treatment of cancer.

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