

A New Micronized Formulation of 2-Methoxyestradiol-*bis*-sulfamate (STX140) is Therapeutically Potent against Breast Cancer

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Abstract. *There is a continued need for orally bioavailable anticancer compounds that exhibit good efficacy against breast cancer. STX140, a derivative of 2-methoxyestradiol (2-MeOE2), has been shown to have excellent oral bioavailability and significantly reduces tumor growth. A new micronized formulation of STX140 has now been developed and its pharmacokinetics (PK) in rats and effect on MDA-MB-231 breast cancer growth in nude mice was investigated. Materials and Methods: For the PK studies, female Wistar rats were treated orally with STX140 in two separate vehicles (10% tetrahydrofuran (THF) in propylene glycol (PG) or 0.5% methyl cellulose (MC) in saline) and plasma samples taken for high performance liquid chromatography analysis over 48 h. For the tumor efficacy studies, female nude mice were inoculated with MDA-MB-231 breast cancer cells and then treated orally with a range of doses of STX140. Results: The PK studies demonstrated that the THF/PG vehicle resulted in a greater oral bioavailability of STX140 compared to the 0.5% MC vehicle. However, this was not translated to the tumor efficacy studies where STX140 at 20 mg/kg in either vehicle caused a significant reduction in tumor volume. Conclusion: The new micronized formulation of STX140 is orally bioavailable and efficacious at inhibiting MDA-MB-231 breast tumor growth.*

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Breast cancer is the most common cancer among women, with a lifetime risk of developing it at 12% and a risk of death of 5% (1). Anticancer compounds that exhibit potent anti-angiogenic activity, are cytotoxic, and have good oral bioavailability would be of significant importance. There is significant potential for the use of estrogen derivatives for the treatment and prevention of breast cancer. The natural estrogen metabolite, 2-methoxyestradiol (2-MeOE2) has demonstrated some efficacy in a variety of *in vitro* and *in vivo* tumor models (2). However, when tested in the clinic it has failed to translate good laboratory potential into therapeutic application (3). This is most likely due to poor oral bioavailability as 2-MeOE2 is metabolised by 17 β -hydroxysteroid dehydrogenase (17 β -HSD) type 2 (4) which is found in abundance in the stomach lining (5). Recently a Phase I clinical trial was halted due to the complete absence of 2-MeOE2 detected in patient plasma despite a dose of 3000 mg per day being administered (3).

Consequently the need for estrogen derivatives that have greater oral bioavailability compared to 2-MeOE2 is essential. One such compound is 2-methoxyestradiol-3,17-*O,O*-*bis*-sulfamate (2-MeOE2*bis*MATE, STX140) which was discovered as part of a program exploring non-estrogenic steroid sulfatase (STS) inhibitors closely related to estrone-3-*O*-sulfamate (EMATE). It was found that sulphamoylation of the 17-hydroxyl group protects these steroidal derivatives from metabolism by 17 β -HSD Type 2 *in vivo* (4). This led to a new class of A-ring modified anticancer compounds, which in addition to inactivating the STS enzyme, are potent inhibitors of cell proliferation *in vitro* (6-9) and reduce the growth of nitrosomethylurea (NMU)-induced tumors *in vivo* (10).

The *in vitro* proliferation of a range of cancer cell lines (MCF-7, MDA-MB-231, CAL51, A2780, LNCaP) are inhibited by STX140 to a greater extent than by 2-MeOE2 (6, 7, 11). The growth of MDA-MB-435 xenograft tumors implanted in a nude mouse model was decreased by 86% by STX140 (20 mg/kg, orally daily) treatment (12). At the same dose, 2-MeOE2 had no significant effect. Furthermore, STX140 treated tumors still remained significantly smaller than the controls four weeks after cessation of treatment (12). There are a number of reasons why, in addition to its inherently higher anti-proliferative activity, STX140 is more potent than the parent compound 2-MeOE2 *in vivo*. Firstly, estrogen sulfamates are able to bind to erythrocytes and escape first pass liver metabolism (13). Secondly, C-17 sulfamate is not a substrate for 17 β -HSD type 2 dehydrogenase and therefore it is not oxidised to an inactive metabolite (4). Finally, sulfamoylation of the 3- and 17-hydroxyl groups blocks inactivating conjugation which delivers rapidly cleared metabolites such as sulphate derivatives. These factors contribute towards the 85% oral bioavailability of STX140 from a single 10 mg/kg dose in rats which compares to 0% observed for identically dosed 2-MeOE2 (12).

In a recent study on STX140, 10% tetrahydrofuran (THF) 90% propylene glycol (PG) was used as the oral dosing vehicle (8, 12). This is not suitable for evaluating the compound for toxicity and efficacy or for human clinical trials. Therefore, a new micronized formulation of STX140, using a methyl cellulose vehicle (MC), was developed and evaluated in an MDA-MB-231 xenograft breast cancer model. Chemically, MC is a methyl ether of cellulose, arising from substituting the hydrogen atoms of some of cellulose's hydroxyl groups -OH with methyl groups -CH₃, forming -OCH₃ groups. There have been few reports of the effects of MC viscosity on oral drug bioavailability and toxicity. Therefore, two different viscosities of MC, 25 centipoise (cP) and 400 cP, were tested as vehicles to determine whether the viscosity of MC has any effect on the efficacy and pharmacokinetics of STX140 *in vivo*. The THF:PG vehicle was also tested to examine any possible pharmacokinetic differences.

Materials and methods

Chemicals and reagents. The following reagents were obtained from the suppliers listed: analytical reagent grade toluene and high-performance liquid chromatography (HPLC) grade methyl-tert-butyl ether, methanol and water (Fisher Scientific UK Ltd., Loughborough, Leics, UK); isoflurane (Abbot Ltd., Maidenhead, Berks, UK); propylene glycol and ammonium sulfate (Sigma-Aldrich Co. Ltd., Gillingham, Dorset, UK); tetrahydrofuran (Riedel-de Haën, Seelze, Germany). Full details of the synthesis of STX140 have been published by Leese and colleagues (14) and the structure of STX140 is shown in Figure 1. Micronization of STX140 was

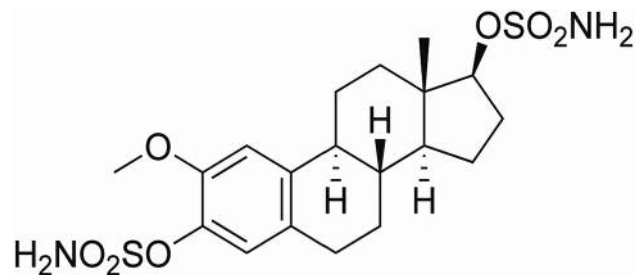


Figure 1. Chemical structure of STX140 (2-MeOE2bisMATE).

performed by a jet mill under nitrogen in a glove box. The purpose of the micronization step was to obtain batches of STX140 with particle size distribution of 5 μ m (D50%). The powder was dispersed in water Tween 80 and analyzed on a Mastersize Microplus (Malvern Instruments, Orsay Cedex, France) to confirm particle size distribution.

HPLC analysis. STX140 was separated from its putative metabolites, 2-MeOE2, 2-MeOE2-3-O-sulfamate and 2-MeOE2-17-O-sulfamate, using a modified version of a reversed-phase HPLC method described previously (15). An 1100 autosampler (Agilent, Massy, France), photodiode array detector and solvent delivery system were used. The agents were separated from endogenous plasma components by an isocratic mobile phase consisting of 58% methanol in 0.02 M ammonium sulphate. Sodium azide (1 mM) was added to the mobile phase in order to decrease microbial growth. The extracted samples were reconstituted in the mobile phase and aliquots of 100 μ l were injected on to a C3-phenyl column (250x5 mm, 5 μ m). STX140 was analysed with a photodiode array detector with detection at 285 nm. The method was validated by spiking plasma with STX140 (960 ng/ml). Plasma calibration curves were found to be linear from 40 to 9000 ng ml⁻¹.

***In vivo* pharmacokinetic and metabolism studies.** Male Sprague Dawley rats (220-250g) were purchased from Janvier (Le Genest-St Isle, France) and housed in a dedicated animal facility. The rats received food and water *ad libitum*, and were maintained in positive pressure isolators under a 12 h light-dark cycle. A solution of 10 mg/kg in 5 ml/kg of vehicle (propylene glycol: THF, 9:1 v v⁻¹ or 0.5% methyl cellulose cP 400 in saline) was given by oral gavage. After treatment, the rats were sampled at defined times (15, 30 min and 1, 3, 8, 24 and 48 h) blood was taken by cardiac puncture centrifuged at 3500 rpm for 15 min at 4°C. The supernatant was mixed with the appropriated solvent (cold acetonitrile + 0.1% formic acid) to precipitate the proteins, after a further centrifugation, the samples were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) or frozen at -80°C pending analysis. There were two reasons for selecting 10 mg/kg for both oral and intravenous dosing. Firstly, a single dose of EMATE administered by these routes has been shown to inhibit rat liver sulfatase by at least 99% (14). Secondly, administration of this dose was found to elicit sufficiently high levels of the agents for their detection in plasma.

Pharmacokinetic analysis. The pharmacokinetic parameters were calculated using WinNonlin software (Pharsight Corporation, Mountview, CA, USA). The area under the curve (AUC) was calculated using the linear trapezoidal method, with extrapolation of the terminal phase to infinity.

In vivo tumor model. All the *in vivo* experiments were carried out under conditions that complied with the institutional requirements. The MDA-MB-231 cells (2×10^6) in 100 μ l media were injected subcutaneously (*s.c.*) into the flank of female MF-1 nu/nu mice (Harlan, Bicester, Oxon, UK). Oral administration of vehicle only (either 0.5% 25 cP methyl cellulose (MC), 0.5% 400 cP MC, or 10% THF: 90% PG) or STX140 (80 mg/kg, 20 mg/kg, 10 mg/kg) in 0.5% MC or STX140 (20 mg/kg) in THF:PG was initiated when the tumors reached approximately 100 mm³ in volume. The animals were dosed daily for a total of 28 days. Each treatment group comprised six animals. The mouse weights were recorded at weekly intervals throughout the study. The tumor measurements were taken every week using electronic callipers and the volumes of the tumors were determined using the following equation: $length \times width^2/2$ ($l \times w^2/2$).

Statistics. A one-way ANOVA followed by a Bonferroni's multiple comparison test was performed to determine statistical significance on data sets. All the values are represented as the mean \pm standard errors of the mean (S.E.M.).

Results

Figure 2 shows the plasma concentrations of STX140 in rats after a single 10 mg/kg dose using the two different vehicles. The THF:PG vehicle gave the largest AUC of 341.41 μ g/min/ml whereas the micronized STX140 in 0.5% MC gave a lower AUC of 158.18 μ g/min/ml. Therefore, the 0.5% MC vehicle was just under half as effective at delivering the STX140 into rodent plasma *in vivo*.

Effect of STX140 in 0.5% MC (25 cP) on MDA-MB-231 tumor growth. Mice bearing MDA-MB-231 breast cancer xenografts were orally treated with of STX140 (80, 20 or 10 mg/kg) in a vehicle of 0.5% MC (25 cP) or STX140 (20 mg/kg) in a vehicle of 10% THF 90% PG. All the dosing regimes, except 80 mg/kg, caused a significant ($p < 0.001$) reduction in tumor volume after 21 days dosing (Figure 3a) with the 20 mg/kg doses with either vehicle being the most efficacious. The animals treated with 80 mg/kg STX140 lost weight rapidly during the first 7 days of dosing and were culled due to over 10% body weight loss at this time-point (Figure 3b). No significant weight loss was observed in the other groups.

Effect of STX140 in 0.5% MC at 400 cP on MDA-MB-231 tumor growth. The mice bearing MDA-MB-231 xenografts were treated with STX140 (80, 20 or 10 mg/kg) in a vehicle of 0.5% MC (400 cP) or STX140 (20 mg/kg) in a vehicle of 10% THF 90% PG. STX140 at 20 mg/kg in either vehicle significantly inhibited tumor growth (Figure 4a). However, at 10 mg/kg, STX140 did not have a significant effect. The animals orally dosed with 80 mg/kg STX140 lost weight during the first 7 days and were terminated at this time-point (Figure 4b).

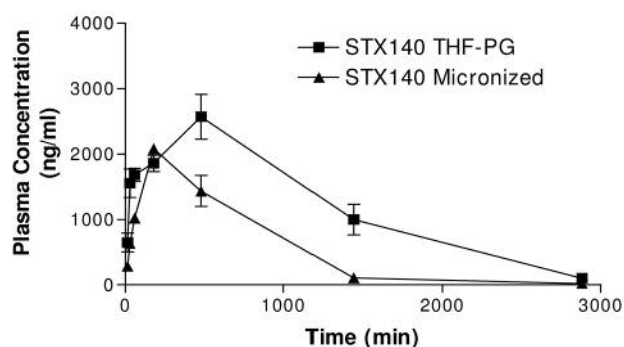
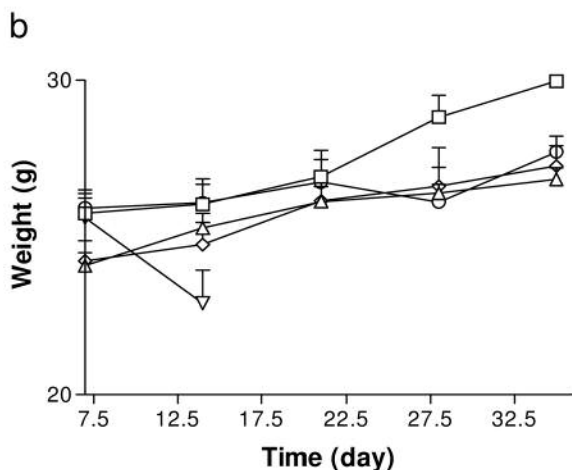
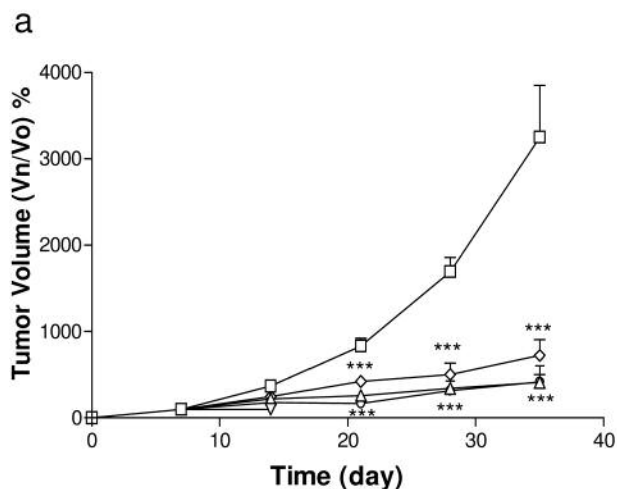


Figure 2. Concentration of STX140 in rat plasma after a single 10 mg/kg oral dose. STX140 was formulated either in 10% THF: 90% PG or as micronized in 0.5% MC (400 cP). Rat plasma samples were evaluated using HPLC-MS/MS analysis. The concentration of STX140 was quantified using linear calibration curves in the range of 10 to 2,000 ng/ml.

Discussion

The THF/PG vehicle gave a greater overall plasma concentration of STX140 compared to the 0.5% MC vehicle. The AUC of STX140 when in the THF/PG vehicle was approximately twice that measured when in 0.5% MC (400 cP). Therefore, in this pharmacokinetic model the THF/PG vehicle led to a greater bioavailability compared to the 0.5% MC. This finding was primarily due to the lower concentration of STX140 found in the plasma after oral administration when given in 0.5% MC. The peak plasma concentration of STX140 was different in the two vehicles used. The micronized formulation reached maximal concentration after 180 minutes compared to after 480 minutes for the THF/PG formulation. Furthermore, STX140 in the THF/PG vehicle maintained plasma concentration for a longer time-period, still being detected after 48 hours. Additionally, no significant metabolism of STX140 was observed after oral administration, indicating that it is not a pro-drug of 2-MeOE2.

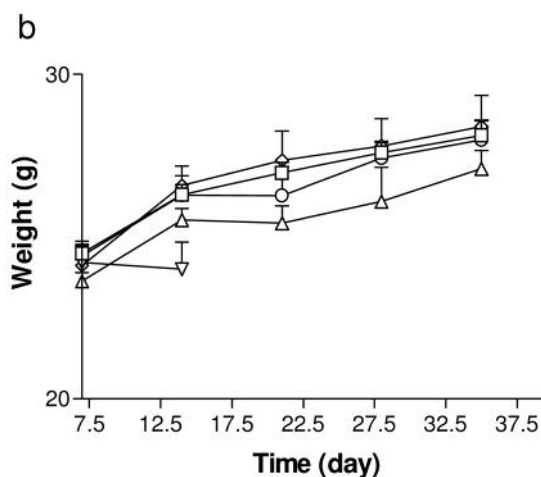
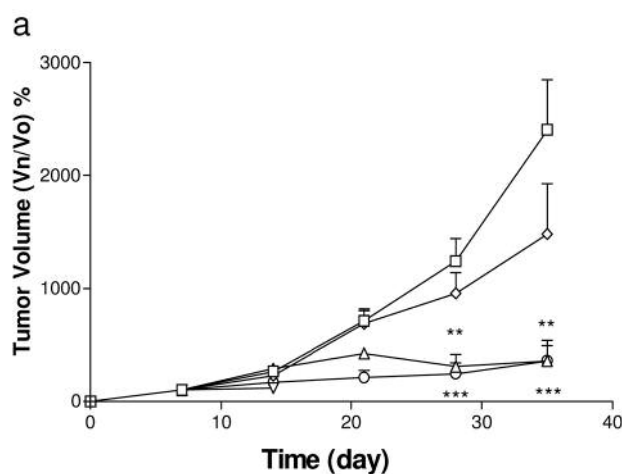
As the pharmacokinetics suggested that the oral efficacy of the micronized formulation of STX140 was not as effective as the previously used THF/PG vehicle, it was necessary to assess the ability of this formulation to reduce the growth of MDA-MB-231 breast tumors in nude mice. The highest dose of STX140 (80 mg/kg) caused rapid weight loss and fatalities after a week of dosing. STX140, in 0.5% MC at 25 cP and 400 cP, administered at 20 mg/kg caused a significant reduction in tumor growth which was mimicked by the 20 mg/kg dose of STX140 in THF/PG. This is in contrast to the pharmacokinetic studies that indicated that the 0.5% MC vehicle compromised oral efficacy. One possible solution to this paradox is that the pharmacokinetics of STX140 differs in rats and mice and, unfortunately, to date, no studies have been performed on the oral bioavailability of STX140 in mice.



- Vehicle (0.5% MC)
- ▽— STX140 (80 mg/kg) MC
- △— STX140 (20 mg/kg) MC
- ◇— STX140 (10 mg/kg) MC
- STX140 (20 mg/kg) THF:PG

Figure 3. The effect of STX140 in low viscosity 0.5% MC (25 cP) on MDA-MB-231 tumor growth in female nude mice (a) and on animal weight (b). Data represents mean±S.E.M. ***p<0.001 compared to control, n=6.

The differences in MC viscosity also gave some unexpected results. At 25 cP, the lower viscosity, STX140 at 10 and 20 mg/kg gave similar tumor reduction. However, at 400 cP, STX140 at 10 mg/kg was not as effective as at 20 mg/kg. This suggests that the higher viscosity of MC has a detrimental effect on the oral efficacy of STX140 in a MC vehicle. A previous study, using HPLC techniques, on the effects of MC on naproxen release from tablets demonstrated that a reduction in the concentration of MC resulted in an increase in the



- Vehicle (0.5% MC)
- ▽— STX140 (80 mg/kg) MC
- △— STX140 (20 mg/kg) MC
- ◇— STX140 (10 mg/kg) MC
- STX140 (20 mg/kg) THF:PG

Figure 4. The effect of STX140 in high viscosity 0.5% MC (400 cP) on MDA-MB-231 tumor growth in female nude mice (a) and on animal weight (b). Data represents mean±S.E.M. ***p<0.001, p<0.01 compared to control, n=6.

percentage of drug release (16). Therefore, it is possible that the reduction in the MC viscosity led to a greater bioavailability of the micronized STX140. Furthermore, it is likely that the greater viscosity of the MC alters the solubility and/or the diffusion rate of STX140, collectively known as the solution characteristics.

To conclude, a new micronized formulation of STX140 is highly orally efficacious and is effective at inhibiting the growth of breast cancer tumors. It is envisaged that this compound will enter clinical trials in 2008.

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