A Comparison of Two Orally Bioavailable Anti-cancer Agents, IRC-110160 and STX140

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Abstract. *This study characterises two recently developed anti-cancer agents in vitro and in vivo, 2-methoxyoestra-1,3,5(10), 16-tetraene-3-carboxamide (IRC-110160) and STX140. Materials and Methods: Hormone-dependent (MCF-7), hormone-independent (MDA-MB-231) and P-glycoprotein overexpressing (MCF-7$_{DOX}$) cells were used for proliferation experiments. For the tumour efficacy studies, female nude mice were inoculated with MDA-MB-231 cells. Results: IRC-110160 is a potent inhibitor of both MCF-7 and MDA-MB-231 cell proliferation. Furthermore, the potency of IRC-110160 was unaffected by the over-expression of the P-glycoprotein drug efflux pump. IRC-110160 and 2-methoxyoestradiol-3,17-O,O-bis-sulfamate (STX140) induced apoptosis in a similar timeframe in the MDA-MB-231 cell line, but only STX140 caused G2/M arrest in these cells. In the MDA-MB-231 xenograft model 300 mg/kg p.o. (daily) of IRC-110160 and 20 mg/kg p.o. STX140 (daily) both completely inhibited tumour growth; however some toxicity was observed with IRC-110160. After 28 days of daily dosing STX140 (20 mg/kg p.o.) had minimal effect on the white blood population of mice with tumours. The masking of STX140 from white blood cells may be due to its interaction with carbonic anhydrase II (CAII) in the red blood cells. In contrast to STX140, IRC-110160 does not inhibit CAII. These studies highlight the activity of two orally bioavailable anti-cancer agents one of which, STX140, may offer a significant clinical advantage over existing drugs as a common dose limiting factor, haemotoxicity, may be minimised.*

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While much progress has been made in the treatment of ER-positive breast cancer, such as the recent introduction of aromatase inhibitors, fewer efficacious options are available for hormone-independent breast cancer. The taxanes, paclitaxel (Taxol) and docetaxel (Taxotere), are routinely used in late stage metastatic breast cancer and have also been successfully trialled in the adjuvant and neoadjuvant setting for early breast cancer (1). Although initially responsive, many tumours quickly become resistant to taxane therapy and the disease progresses. *In vivo* this resistance is largely due to the expression of the P-glycoprotein pump (2) and/or the over-expression of the β type III tubulin isoform (3). In addition, haemotoxicity and peripheral neuropathy limit the effectiveness of the taxanes. Therefore, new compounds with the anti-tumour properties of the taxanes but with an improved clinical profile need to be developed for the treatment of breast cancer.

One such potential agent is the natural metabolite of oestradiol, 2-methoxyoestradiol (2-MeOE2), which over the last decade has shown promise both *in vitro* and *in vivo* (4, 5, 6). 2-MeOE2 is an anti-angiogenic / anti-proliferative agent *in vitro* (7, 8) and inhibits the *in vivo* growth of xenografts (5, 9, 10). However, the *in vivo* efficacy of 2-MeOE2 is poor, with comparatively high oral or intra-peritoneal doses of 75 and 150 mg/kg per day respectively, being required to reduce the growth of melanoma or myeloma tumours in rodent models (5, 10). In Phase I trials a clinical benefit was shown only in two patients who were receiving 1600-3200 mg/day 2-MeOE2 orally. The trial was stopped early due to extremely low plasma concentrations of 2-MeOE2 even at 3000 mg/day (11). One possible explanation is provided by the observation that 2-MeOE2 is oxidised to the inactive 2-MeOE1 by 17β-HSD Type 2 (12). The gastro-intestinal tract expresses 17β-HSD Type 2 (13), and this may inactivate 2-MeOE2 before it enters the blood stream. This is supported by data from a recent Phase I trial in which a daily oral dose

of 1000 mg 2-MeOE2 was given to 24 patients with advanced metastatic breast cancer (14). Metabolism studies showed that 80-95% of the 2-MeOE2 was oxidised to 2-MeOE1 and furthermore 80-90% of both 2-MeOE2 and 2-MeOE1 were present as inactive glucuronide or sulphate conjugates. Additional evidence for the poor oral bioavailability of 2-MeOE2 comes from the work of Ireson *et al*. (15) who demonstrated that 2-MeOE2 could not be detected in the plasma of rats one hour after administration of a single oral dose of 2-MeOE2 (10 mg/kg). The problems of poor bioavailability and rapid metabolism associated with this drug may be overcome by synthesising analogues resistant to conjugative and metabolic inactivation (16).

2-Methoxyoestra-1,3,5(10), 16-tetraene-3-carboxamide (IRC-110160, ENMD-1198) is structurally related to 2-MeOE2, and may have improved *in vitro* and *in vivo* efficacy relative to 2-MeOE2. The potential anti-angiogenic and anti-tumour activities of 2-methoxyoestradiol-3,17-*O,O-bis*-sulfamate (STX140) have been well documented (16-26).

In this study, for the first time, IRC-110160 and STX140 are compared both *in vitro* and *in vivo* for their possible anti-cancer efficacy. Furthermore, due to the observed *in vivo* potency of STX140, the potential blood toxicity of STX140 was assessed in a preliminary experiment *in vivo*.

Materials and Methods

Drug synthesis. 2-Methoxyoestra-1,3,5(10), 16-tetraene-3-carboxamide (IRC-110160, Figure 1A) was synthesised as described in US2005/203075. 2-Methoxyoestradiol-3,17-*O,O-bis*-sulfamate (STX140, Figure 1B) was synthesised as described previously (22). Spectroscopic and analytical data were obtained in accordance with the compounds' structure. The purity of the compounds was confirmed through the use of high-performance liquid chromatography (HPLC).

Cell culture. MCF-7_{WT} (ER+ve) and MDA-MB-231 (ER-ve) breast cancer cells were obtained from the American Type Culture Collection (LGC Promochem, Middlesex, United Kingdom) and MCF-7_{DOX} cells were kindly donated by Dr. G.L. Scheffer (Department of Pathology, Free University Hospital, Amsterdam, The Netherlands). Cells were maintained in Dulbecco's Modified Eagle's Medium containing phenol red, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids and 0.075% sodium bicarbonate. (Sigma, Dorset, United Kingdom). All cells were cultured at 37˚C under 5% CO_2 in a humidified incubator. The Resistance Factor (RF) values were calculated from the IC_{50} values (RF=IC_{50} in drug resistant cell line / IC_{50} in parental$_{WT}$ cell line) for cell proliferation after 96 h of compound treatment (0.1 nM to 10 μM), using the CellTiter96 Aqueous one assay reagent microtiter plate assay (Promega, Hampshire, United Kingdom). All compounds were dissolved at 10^{-2} M in tetrahydrofuran (THF) for *in vitro* experiments (10^{-6} to 10^{-1}% final THF concentration).

STS enzyme activity. Cells were incubated overnight with [6,7-^{3}H] E1S (5 pmol, $7x10^5$ dpm, 60 Ci/mmol; Perkin-Elmer LS, Wellesley, MA). The product E1 was separated from E1S by toluene partition using [4-^{14}C] E1 to monitor procedural losses, and radioactivity was measured by liquid scintillation counting (27). The STS inhibitor, EMATE, was used as a positive control.

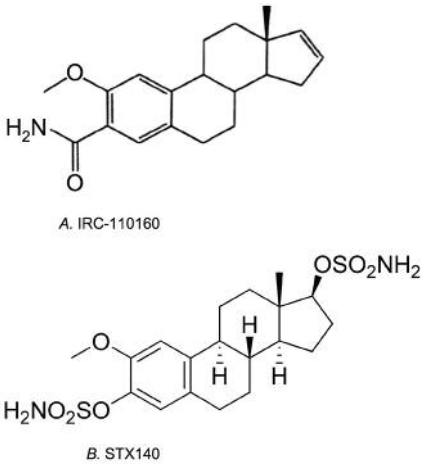

Figure 1. *Structures: A: 2-Methoxyoestra-1,3,5(10),16-tetraene-3-carboxamide (IRC-110160). B: 2-Methoxyestradiol-3,17-O,O-bis-sulfamate (STX140).*

Carbonic anhydrase II activity. An adaptation of a colorimetric assay developed by Armstrong *et al*. (28) was used which measures the carbonic anhydrase-catalysed hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol, which has an absorption peak at 384 nm. Briefly, 7 U/ml final concentration of human CAII, in the presence or absence of inhibitors (1 nM to 10 μM), and 1 mM final concentration of *p*-nitrophenol acetate were mixed in quadruplicate, in a 96-well plate, in a reaction buffer of 0.05 M Tris, pH 7.6, and final volume of 0.18 ml. After incubation at 20˚C for 30 min, the absorbance at 405 nm was measured. The percentage inhibition was defined relative to the maximal activity which was measured without inhibitor. IC_{50} values were obtained from a graph of percent inhibition versus inhibitor concentration and calculated using Prism (Graphpad Software, CA). Acetazolamide was used as a positive control.

Cell cycle/apoptosis analysis. Cells were plated at 60-70% confluency in T-25 flasks. After 24 h they were treated with compound for a further 24 h, 48 h or 72 h. Control cells were untreated or treated with 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). Media 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 RNase for 5 minutes, stained with 50 μg/ml propidium iodide and analysed using a flow cytometer (FACScan, Becton Dickinson, Cowley, United Kingdom).

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 $CaCl_2$) at $1x10^6$ cells/ml. Cells were then stained with fluorescein-conjugated Annexin V (BD Biosciences, Cowley, United Kingdom) antibody and propidium iodide (5 μg/ml) before flow cytometric analysis. Apoptotic cells are defined as cells positive for Annexin V and negative for propidium iodide.

Table I. *Cell proliferation IC_{50} values*.

Compound	MDA-231	MCF-7_{WT}	MCF-7_{DOX}	RF
IRC-110160	0.30	0.30	0.24	0.80
STX140	0.29[a]	0.25[b]	0.38[b]	1.52

[a]Data presented for comparative purposes previously published by Newman *et al*. (17), [b]Newman *et al*. (32). n=3.

Tubulin polymerisation. The effects of test compounds (IRC-110160 and STX140) on *in vitro* polymerisation of purified bovine brain tubulin (Cytoskeleton, Denver, CO) were measured by turbidometry. Tubulin (1 mg/ml) in MES buffer (0.1 M MES pH 6.5, 0.5 mM $MgCl_2$, 1 M monosodium glutamate and 1 mM GTP) was incubated with or without test compounds (10 μM, 1% v/v ethanol) for 5 min at 37˚C. Tubulin assembly was stimulated by adding paclitaxel (10 μM). The change in the absorbance was continuously monitored at 350 nm for 15 min at 37˚C.

Angiogenesis assay. The effect of the compounds on *in vitro* vessel formation was assessed using an angiogenesis kit (TCS Cellworks). For this assay endothelial cells were cultured in a 24-well plate within a matrix of human diploid fibroblasts of dermal origin in optimised medium supplied by TCS Cellworks. The co-cultured cells were incubated throughout the experiment at 37˚C under 5% CO2 in a humidified incubator. On day 1 the culture medium was removed and replaced with medium containing the compounds under investigation. On days 4, 7 and 9 the medium was replaced with fresh medium containing the compounds. Each compound was tested in triplicate. On day 11, the cells were washed (PBS) and 70% ethanol (1 ml) was added to each well for 30 min to fix the cells. After fixation the cells were washed with blocking buffer (1 ml, PBS + 1% BSA) and stained for CD31 in accordance with the manufacturers instructions (TCS Cellworks).

The extent of vessel formation was then quantified electronically using the previously validated method of Newman *et al*. (17).

Xenograft models. Female MF-1 nu/nu mice were injected subcutaneously (*s.c.*) in the flank with $2x10^6$ MDA-MB-231 cells and dosing commenced once the tumours had reached 100 mm^3 approximately. All experiments were carried out under conditions that complied with institutional guidelines. Daily oral dosing of IRC-110160 (100 and 300 mg/kg) was compared with STX140 (20 mg/kg *p.o.* daily) for 28 days. IRC-110160 was dissolved in 10% THF / 90% propylene glycol (PG) and STX140 was dosed as a micronized suspension in 0.5% methylcellulose. The micronised suspension in 0.5% methylcellulose is equally as efficacious as the previously published 10% THF / 90% PG solution of STX140 (15), when administered orally at the same concentrations (29). In both studies animal weights and tumour measurements were taken regularly. Tumour volume (V), in mm^3, was determined using the following equation: $length \times width^2/2$ ($l \times w^2/2$). Results are expressed as a percentage of the tumour volume at day of measurement (Vn) over the volume at day 0 (Vo).

Blood analysis. Due to the *in vivo* potency of STX140 relative to IRC-110160, further pre-clinical studies were undertaken to ascertain whether STX140 caused significant blood toxicity which can limit the applicability of many microtubule disruptors. Female MF-1 nu/nu mice were injected subcutaneously (*s.c.*) in the flank with $2x10^6$ MCF-7 cells and daily dosing of STX140 (20 mg/kg, *p.o.*) commenced once the tumours reached 100 mm^3. After 28 days dosing the animals were anesthetised and the blood collected by cardiac puncture. The white blood cell (WBC), red blood cell (RBC) and platelet numbers were then quantified by FACS analysis. Although immuno-compromised, nude mice have been used previously for the study of myelotoxicity (30) and many different WBC types have been found in their blood (31).

Table II. *Enzyme inhibition IC_{50} values*.

Compound	CAII IC_{50} (nM)	STS IC_{50} (nM)
Acetazolamide	10-20	-
EMATE	-	20
IRC-110160	>10000	>10000
2-MeOE2	>10000	>10000
STX140	379[c]	30[d]

[c]Data presented for comparative purposes previously published by Ho *et al*. (33), [d]Leese *et al*. (22). n=3.

Statistics. All *in vitro* experiments were carried out in triplicate and data presented are representative of one of three such experiments. All errors shown are the mean±SD. Student's *t*-test was used to assess the significance of the differences in cell proliferation *in vitro*. For xenograft data one-way ANOVA followed by a Bonferroni's multiple comparison test was performed to determine statistical significance on most data sets. Where only two groups are compared a Student's *t*-test was applied. All values are represented as the mean±standard errors of the mean (SEM). Statistics were calculated using Prism 3 for Mac (Graphpad Software inc., CA).

Results

Cell proliferation assays. IRC-110160 and STX140 inhibited both oestrogen receptor-positive (MCF-7_{WT}) and oestrogen receptor-negative (MDA-MB-231) breast cancer cell proliferation in the sub-micromolar range. The IC_{50} values (250-300 nM) were not substantially different between the two compounds, and between the two cell lines. To assess whether IRC-110160 may be a substrate for the P-glycoprotein drug efflux pump, the IC_{50} for proliferation was attained in a variant MCF-7 cell line, MCF-7_{DOX}, which over-expresses active P-glycoprotein (32), and the RF value calculated. IRC-110160 was equipotent in both cell types with an RF value of 0.8, indicating that this compound is unlikely to be a substrate for the P-glycoprotein pump (Table I).

Enzyme assays. At 10 μM 2-MeOE2 (Panzem) and IRC-110160 did not inhibit either the STS enzyme or the CAII enzyme (Table II). In contrast, as previously reported, (33, 22) STX140 had inhibitory action against these enzymes.

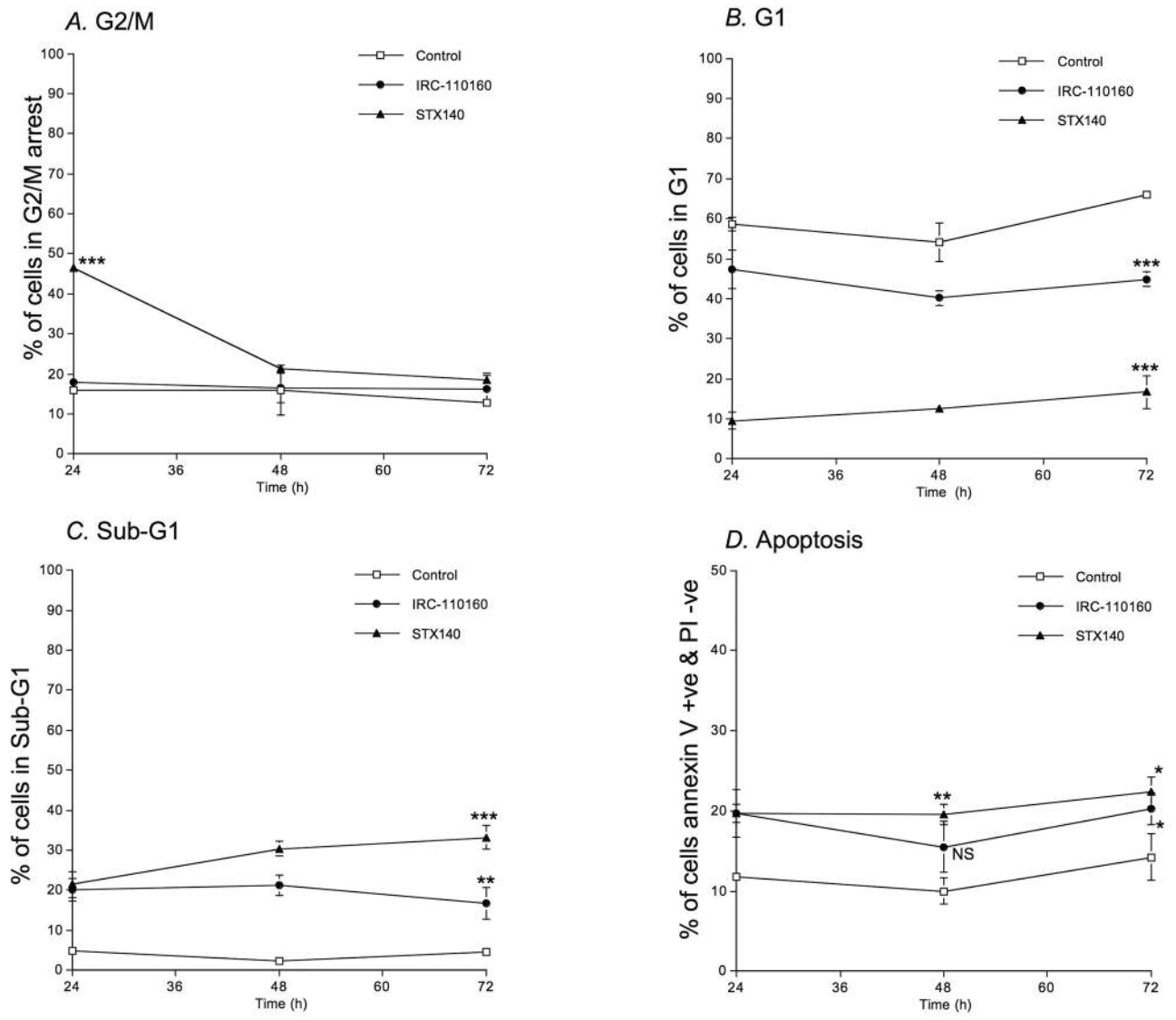

Figure 2. *Cell cycle arrest and apoptosis in MDA-MB-231 cells. Cells were exposed to either IRC-110160 or STX140 at their IC_{50} values for cell proliferation, 300 nM and 290 nM respectively. A. The number of cells in G2/M arrest was assessed by flow cytometery, only 24 h exposure to STX140 induced significant G2/M cell cycle arrest compared to control (***p<0.001). B. Both IRC-110160 and STX140 significantly reduced the number of cells in the G1 phase of the cell cycle, with STX140 being significantly (p<0.001) more effective than IRC-110160. C. The number of cells in the sub-G1 phase indicates cells which are dying and possibly undergoing apoptosis. Both IRC-110160 and STX140 significantly (***p<0.001, **p<0.01) increase the sub-G1 population at all time points studied. D. To identify cells actually undergoing apoptosis, cells were then harvested and stained with fluorescein-conjugated Annexin V antibody and propidium iodide before analysis using flow cytometer and CellQuest Pro software. In response to IRC-110160 and STX140 there was a significant (**p<0.01, *p<0.05) increase in the rate of apoptosis compared to untreated cells at all time points studied, except with IRC-110160 at 48 h which did not reach significance (NS).*

Cell cycle/apoptosis analysis. STX140 induces cell cycle arrest and apoptosis in a range of tumour cell lines when used at concentrations greater than its IC_{50} value for proliferation in the corresponding cell line (24, 21, 18). In this study the ability of IRC-110160 and STX140 to affect the cell cycle and induce apoptosis in MDA-MB-231 cells, when used at their IC_{50} for the inhibition of proliferation (290 and 300 nM respectively), was compared. After 24 h 46.5% ± 1.5 SD of cells exposed to STX140 were in G2/M arrest, no G2/M arrest was seen at any time point with IRC-110160 (Figure 2A). IRC-110160 caused a small reduction in the proportion of cells in G1 relative to untreated cells at all time points studied (Figure 2B; 10% ± 4.7 SD @ 24 h, 14% ± 1.8 SD @ 48 h and 21% ± 1.9 SD @ 72 h). However, STX140 caused a significantly ($p<0.001$) greater reduction in the proportion of cells in G1 compared to IRC-110160 (Figure 2B; 49% ± 2.1

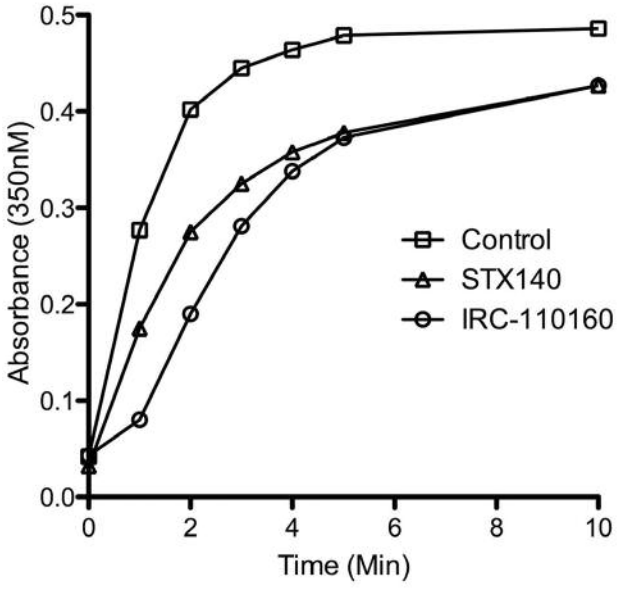

Figure 3. *In vitro tubulin assembly was measured by turbidity at 350 nm. Tubulin was incubated with or without test compounds (20 μM) for 5 min at 37˚C. Tubulin assembly was stimulated by adding paclitaxel (10 μM). The change in the absorbance was continuously monitored at 350 nm for 10 min at 37˚C.*

SD @ 24 h, 41% ± 0.5 SD @ 48 h and 49% ± 4.1 SD @ 72 h). Both compounds significantly ($p<0.01$) increased the proportion of cells in the sub-G1 phase by about 4-fold (Figure 2C), indicating that these compounds may induce apoptosis. To confirm the induction of apoptosis the extent of annexin V flipout, an early event during apoptosis, was quantified. Only cells staining positive for annexin V and negative for propidium iodide were counted as apoptotic. In response to IRC-110160 and STX140 there was an approximate 2-fold increase in the rate of apoptosis compared to untreated cells at all time points studied, except with IRC-110160 at 48 h which did not reach significance (Figure 2D).

Tubulin polymerisation. The inhibition of paclitaxel-stimulated tubulin polymerisation *in vitro* is commonly observed with many microtubule destabilising agents, such as colchicine and the vinca alkaloids. Previously, it has been shown that STX140 inhibited the paclitaxel-stimulated tubulin polymerisation, as measured by changes in turbidity (17). In this study IRC-11016 and STX140 inhibited paclitaxel-stimulated tubulin polymerisation to a similar extent (Figure 3). The vehicle alone, ethanol, had no effect on the paclitaxel-stimulated polymerisation of tubulin (data not shown).

Inhibition of in vitro angiogenesis. To assess whether IRC-110160 was potentially anti-angiogenic a co-culture model was used in which endothelial cells are co-cultured with fibroblasts in a specially formulated medium. The pro-angiogenic factor VEGF (2 ng/ml) was used to further stimulate vessel formation in this assay, and the capacity of the compounds to inhibit the VEGF-stimulated angiogenesis was assessed.

The representative high-resolution scans of the wells show that 100 nM STX140, and to a lesser extent IRC11060 (50 and 100 nM) and 50 nM STX140 inhibited VEGF-stimulated vessel formation (Figure 4A). Quantification of the scans, using a previously validated method (17, 20), shows that 100 nM STX140 inhibited vessel formation by 80% ± 10 SD (Figure 4B), in contrast 100 nM IRC-110160 only inhibited vessel formation by 29% ± 6.5 SD.

Xenograft studies. Hormone-independent MDA-MB-231 breast xenograft tumours in untreated mice increased by over 2000% in size (2224% ± 820 SEM) over the study period; in contrast, tumours in mice treated with STX140 (20 mg/kg p.o; daily) or IRC-110160 (300 mg/kg *p.o.*; daily) were significantly smaller ($p<0.001$) and had only increased in size by 418% ± 306 SEM and 314% ± 227 SEM, respectively. Daily oral dosing with 100 mg/kg of IRC-110160 was significantly ($p<0.05$) less effective with tumours increasing in size by 986% ± 416 SEM over the study period (Figure 5A). Despite the lack of any significant weight loss (Figure 5B) 2 of 6 animals in the high dose IRC-110160 (300 mg/kg) died on study after 14 and 21 days dosing, the cause of death could not be determined. No deaths were seen in any other cohorts.

Blood analysis. To further assess STX140 a second xenograft study was initiated to facilitate the collecting of blood samples at the end of study to evaluate the potential blood toxicity of STX140. IRC-110160 was not included in this study due to relatively high doses required to match the activity of STX140, and because of the possible toxicity observed at the 300 mg/kg dose. After 28 days daily oral dosing with 20 mg/kg STX140, blood was collected by cardiac puncture and subsequently analysed by FACS. Figure 6 shows there was a slight reduction (34% ± 17 SD) in the total WBC count at the end of study in response to daily dosing with STX140. RBC and platelet counts were not altered by dosing with STX140. The blood composition of mice bearing tumours (control) and non-tumoured mice was not significantly different. Tumour growth data will be published elsewhere.

Discussion

The disappointing clinical studies with 2-MeOE2 are primarily due to its potential for metabolism and subsequent inactivation by 17β-HSD Type 2 at the C-17 position in the D-ring and concomitant conjugation (14). These clinical findings are supported by studies undertaken by our group

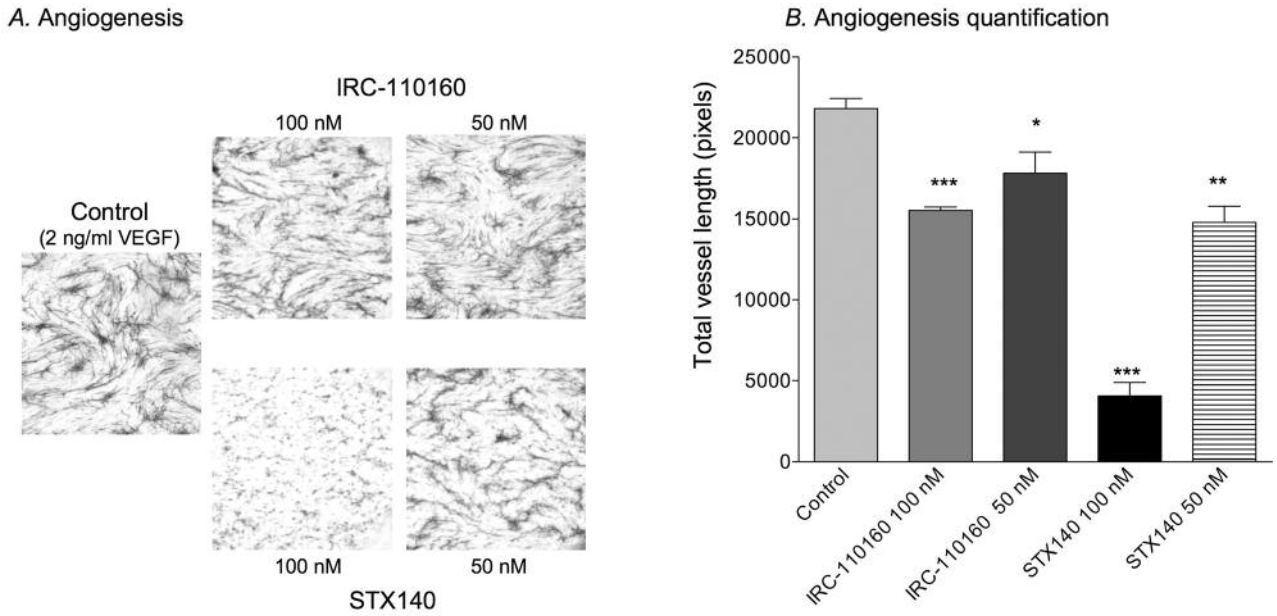

Figure 4. *Vessel formation in an in vitro model of angiogenesis. A. Representative high resolution scans showing the effects of the indicated compounds on vessel formation by co-cultures of endothelial cells and fibroblasts. Cells were exposed to compounds for 11 days and the tubules were stained using an antibody for CD31. B. Co-cultures of endothelial cells and fibroblasts were exposed to compounds for 11 days. The tubules were stained using an antibody for CD31 and quantified by high resolution scanning and subsequent image processing to quantify the number of pixels per well representing tubules. Values are means of triplicate determinations: bars; SD. ***p<0.001, **p<0.01 and *p<0.05 vs. control (2 ng/ml VEGF).*

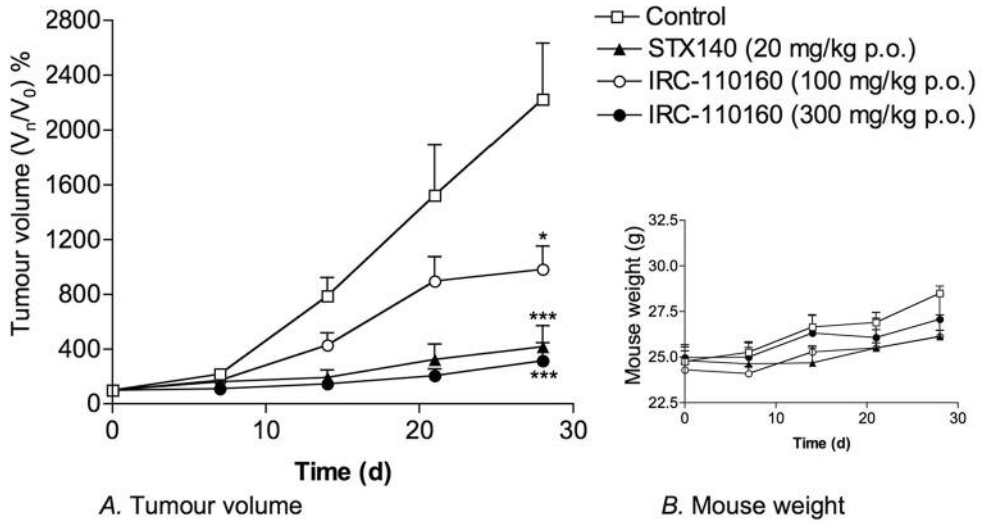

Figure 5. *MDA-MB-231 xenograft studies. A. IRC-110160 (100 and 300 mg/kg, p.o.) and STX140 (20 mg/kg. p.o.) inhibited MDA-MB-231 xenograft tumour growth in vivo. Data represents mean±SEM n=6. *p<0.05 and ***p<0.001 vs. vehicle control (10% tetrahydrofuran (THF) / 90% propylene glycol (PG); p.o.). 20 mg/kg STX140 and 300 mg/kg IRC-110160 were significantly (p<0.05) more efficacious than 100 mg/kg IRC-110160. B. The effect of compounds on animal weight. No significant weight loss was recorded with any of the treatments. However, despite the lack of any significant weight loss 2 of 6 animals in the high dose IRC-110160 (300 mg/kg) died on study after 14 and 21 days dosing, the cause of death could not be determined. No deaths were seen in any other cohorts.*

which show 2-MeOE2 to be poorly bioavailable (15), rapidly metabolised and inactivated by 17β-HSD2 (12), and to only have weak activity *in vitro* (20) and *in vivo* (18). These data suggested a need for protective modifications of 2-MeOE2 to generate new anti-cancer agents with improved oral bioavailability and efficacy.

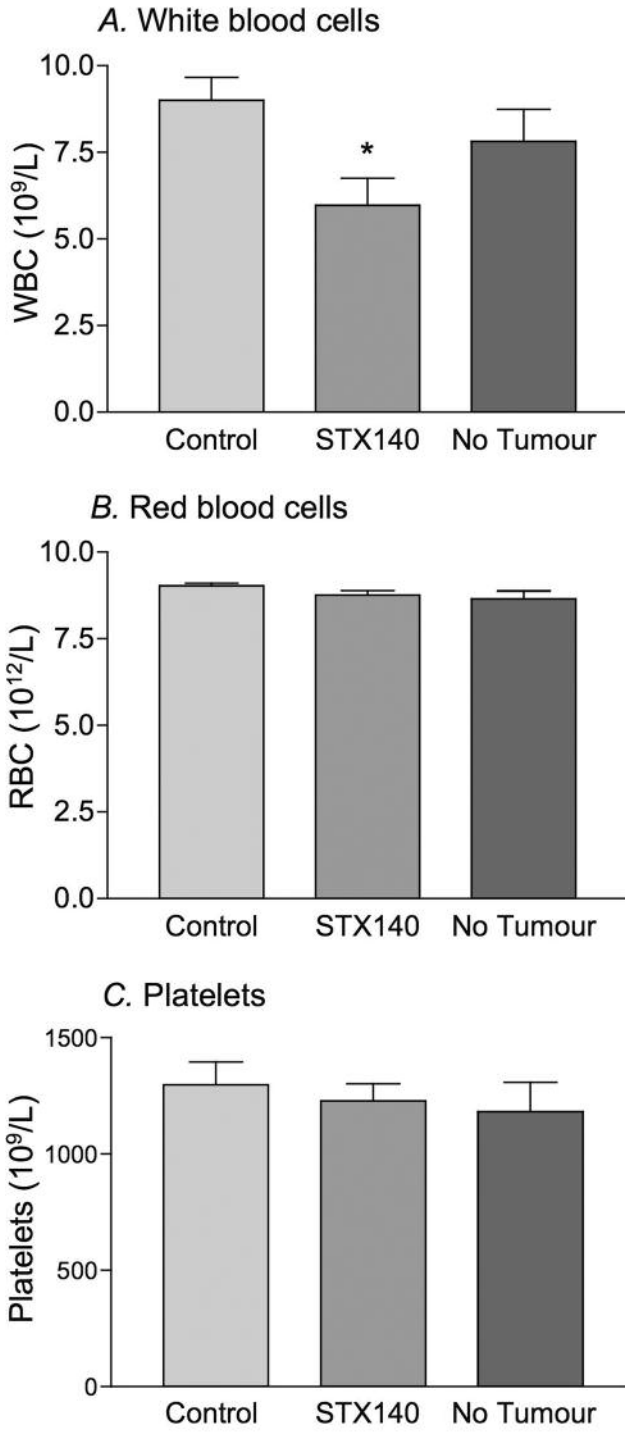

Figure 6. *Blood analysis. To further assess STX140 a second xenograft study was initiated to facilitate the collecting of blood samples at the end of study to evaluate the potential blood toxicity of STX140. After 28 days daily oral dosing with 20 mg/kg STX140, blood was collected by cardiac puncture and subsequently analysed by FACS. A. STX140 caused a 34% (± 17 SD) reduction in the total WBC count at the end of study in response to daily dosing with STX140. RBC (B) and platelet counts (C) were not altered by dosing with STX140. The blood composition of mice bearing tumours (control) and non-tumoured mice was not significantly different.*

One such compound is STX140, which was one of a series of compounds developed to overcome the oestrogenicity of the original steroid sulphatase (STS) inhibitor, EMATE (27). This led to a new class of A-ring modified anti-cancer compounds which, in addition to inhibiting STS, were potent inhibitors of cell proliferation *in vitro* and NMU-induced tumour growth *in vivo* (34). The *in vitro* and *in vivo* activity, and mechanisms of the anti-angiogenic / anti-proliferative agent STX140 have been well documented (15-26, 32). Another 2-MeOE2-like compound which is currently in clinical trials is 2-methoxyoestra-1,3,5(10), 16-tetraene-3-carboxamide (IRC-110160, ENMD-1198), although to date there is very little published data on this agent.

The aim of this study was to compare the two structurally related compounds, IRC-110160 and STX140, both *in vitro* and *in vivo*. Both compounds are potent inhibitors of oestrogen receptor-positive and negative breast cancer cell lines *in vitro*, and they are approximately 10-fold more potent than 2-MeOE2 (20). Furthermore, like STX140 (32), IRC-110160 does not appear to be a substrate for the P-glycoprotein drug efflux pump, a clinical mechanism of resistance to many chemotherapeutic drugs.

STX140 is thought to primarily act by binding to tubulin causing cell cycle arrest and subsequent apoptosis of tumour cells. Recent work by Foster *et al*., has clearly demonstrated both cell cycle arrest and apoptosis occurring in cells from xenografts from mice dosed with STX140 (18). The key role of apoptosis was further highlighted by the inverse correlation between tumour size and the number of cells undergoing apoptosis (18). In the current study both compounds induced a modest increase in apoptosis, with approximately 20% of cells actively undergoing apoptosis at any one time point, in contrast to the background rate of apoptosis of 10% in untreated cells. As previously reported STX140 induced G2/M cell cycle arrest in the MDA-MB-231 cells after 24 h (18, 21). The rapid subsequent decease in the number of cells arrested in G2/M may be a reflection of the lower concentrations of STX140 used in this study. In addition to the induction of apoptosis, STX140 dramatically reduced the number of cells in G1. Despite the observed increase in apoptosis in response to IRC-110160 there was no apparent cell cycle arrest at the time points studied and only a modest decrease in the number of cells in G1. These discrepancies may be a result of the two compounds acting/targeting different pathways within the cell. However, in this study both compounds disrupted the taxol-stimulated polymerisation of tubulin, suggesting like STX140 (17), IRC-110160 may also bind to tubulin. This is not surprising given the structural similarities to other known microtubule disrupting compounds (10, 17, 35). Further studies will need to be undertaken to ascertain the precise mechanism of action of IRC-110160.

The potential differences between the two compounds are further highlighted by their *in vitro* anti-angiogenesis

activity. Previous studies with 2-MeO2-like compound's have shown a correlation between their anti-proliferative activity in tumour cell lines and their *in vitro* anti-angiogenic activity (17, 20). However, in this study despite similar potency in the two breast cancer cell lines, STX140 was significantly more potent in an *in vitro* model of angiogenesis compared to IRC-110160. These data suggest that IRC-110160 has a different spectrum of activity against endothelial cells compared to STX140. Further studies will need to be undertaken to investigate this finding. This may be potentially important, as work by Chander *et al*. showed the *in vitro* anti-angiogenic activity was a good indicator of the more clinically relevant *in vivo* anti-angiogenic potential of a compound (19).

In the oestrogen receptor-negative MDA-MB-231 xenograft model a 15-fold greater dose of IRC-110160 was required to see the equivalent efficacy observed with 20 mg/kg STX140 when both compounds are administered daily by the oral route. The difference between the *in vitro* and *in vivo* data may reflect: 1) The potentially poor anti-angiogenic activity of IRC-110160. 2) The failure of IRC-110160 to inhibit CAII and therefore the tumour associated, CAIX (33). 3) The oral bioavailability of IRC-110160 may be significantly less than the 85% observed with STX140 (15), or a combination of these factors. As this is an oestrogen receptor -negative model the failure of IRC-110160 to inhibit STS is unlikely to account for its relatively poor efficacy.

One major limiting factor with the use of taxanes and other chemotherapeutic drugs for anti-cancer therapy is the extensive myelotoxicity they induce (1, 36). This limits how often they can be administered and gives time for the tumour to recover, the pro-angiogenic burst to occur and for drug-resistance to develop, between treatment schedules. Efforts to give lower doses more often (metronomic dosing; 37) are hampered as the dose given may only be anti-angiogenic and no longer target the tumour cells directly. In this study daily (x 28) oral dosing of STX140 only resulted in a modest decrease in the total WBC count and had no significant effect on the RBCs or platelets. A more detailed blood analysis needs to be undertaken to identify which WBC cell population may be affected by STX140. The relative lack of myelotoxicity induced could be due to interaction of STX140 with CAII, which allows the uptake of STX140 into RBCs which highly express CAII. This may mask STX140 from the WBC population. These results indicate that a more regular dosing schedule of STX140 may be possible in the clinic and thus circumvent many of the drawbacks of current chemotherapeutic regimes.

In summary we show for the first time a detailed comparison of two anti-cancer compounds, IRC-110160 and STX140. IRC-110160 is a potent *in vitro* inhibitor of tumour cell proliferation, induces apoptosis, does interact with tubulin and shows good anti-tumour activity *in vivo*. We also show for the first time that the well characterised compound STX140 may have a superior pre-clinical profile to IRC-110160 and further, may have reduced myelotoxicity for an agent of this class.

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