The *In Vitro* and *In Vivo* Activity of the Microtubule Disruptor STX140 Is Mediated by Hif-1 Alpha and CAIX Expression

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Abstract. *Tumor neo-angiogenesis is regulated, in part, by the hypoxia-inducible gene HIF1. Evidence suggests HIF1 associates with polymerized microtubules and traffics to the nucleus. This study investigated the role of HIF1 in mediating the antitumor activity of two steroid-based sulfamate ester microtubule disruptors, STX140 and STX243, in vitro and in vivo. The effects of STX140, STX243 and the parental compound 2-methoxyestradiol (STX66) on HIF1α and HIF2α protein expression were assessed in vitro in MCF-7 and MDA-MB-231 cells cultured under hypoxia. More pertinently, their effects were examined on HIF1-regulated genes in vivo in mice bearing MCF-7 or MDA-MB-231 tumors. The level of mRNA expression of vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), phosphoglycerate kinase (PGK), ATP-binding cassette sub-family B member 1 (ABCB1) and carbonic anhydrase IX (CAIX) was quantified by Real-time Polymerase Chain Reaction (RT-PCR). Despite inhibiting nuclear HIF1α protein accumulation under hypoxia in vitro, STX140 and STX243 did not significantly regulate the expression of four out of five HIF1α-regulated genes in vitro and in vivo. Only CAIX mRNA expression was down-regulated both in vitro and in vivo. Immunoblot analysis*

showed that STX140 and STX243 reduced CAIX protein expression in vitro. These compounds had no effect on HIF2α translocation. The potential for inhibition of CAIX by STX140 and STX243 was examined by docking the ligands to the active site in comparison with a known sulfamate-based inhibitor. Microtubule disruption and antitumor activity of STX140 and STX243 is most likely HIF1-independent and may, at least in part, be mediated by inhibition of CAIX expression and activity.

Angiogenesis, the creation of new blood vessels, is induced in tumors by secretion of growth factors, such as vascular endothelial growth factor (VEGF). Great amount of research into targeting angiogenesis has been undertaken to discover new therapeutic agents for cancer therapy. 2-Methoxyestradiol (STX66; Figure 1, compound I) has shown promise *in vitro* by being an anti-angiogenic and anti-proliferative agent (1, 2) and has progressed to numerous clinical trials (3). However, 2-methoxyestradiol has poor *in vivo* efficacy because of poor bioavailability (3, 4).

2-Methoxyestradiol-3,17-*O,O-bis*-sulfamate (STX140; Figure 1, compound II) and 2-ethylestradiol-3,17-*O,O-bis*-sulfamate (STX243; Figure 1, compound III) are a new class of steroid A-ring-modified anticancer compounds (5). They compete with colchicine for tubulin binding and disrupt interphase microtubules, leading to cell-cycle arrest and apoptosis *in vitro* and *in vivo* (6-9). They have excellent oral bioavailability *in vivo* (10) and inhibit angiogenesis *in vitro* and *in vivo* (10, 11). STX140 and STX243 can cause endothelial cell apoptosis *in vitro* (7, 8, 11, 12), but this may not fully explain their anti-angiogenic activity. The hypothesis that the anti-angiogenic activities of microtubule disruptors are mediated by inhibition of hypoxia inducible factor 1 (HIF1) was proposed by Mabjeesh *et al.* (13).
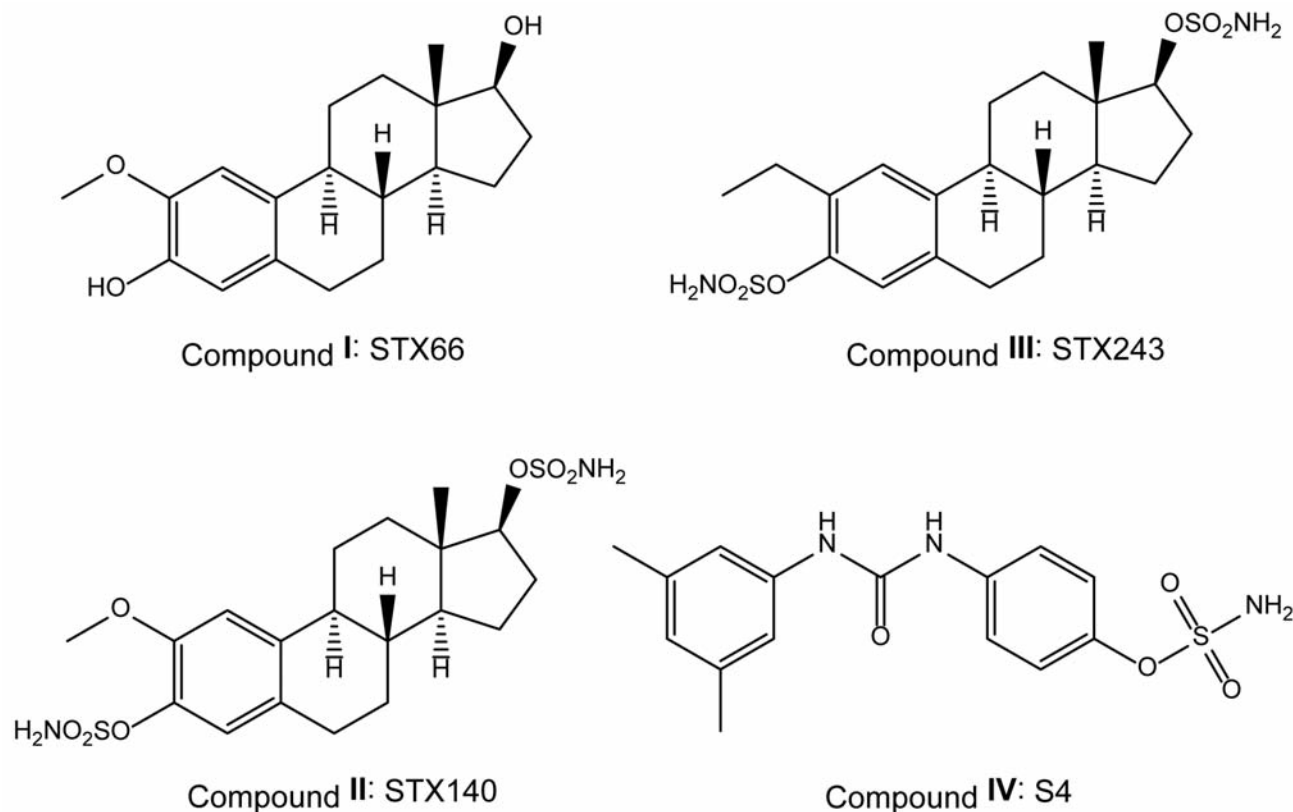
Figure 1. *Structures of study compounds. I: 2 Methoxyestradiol, STX66; II: 2-methoxyestadiol-3,17-O,O-bis-sulfamate, STX140; III: 2-ethylestradiol-3,17-O,O-bis-sulfamate, STX243; IV: S4.*

HIF1 is a heterodimer consisting of a constitutively expressed β-subunit and an α-subunit, the expression of which is tightly regulated by intracellular oxygen concentration. Under normoxia, HIF1α is rapidly and continuously degraded by ubiquitination and proteosomal degradation (14, 15). Under hypoxic conditions, HIF1α accumulates and is translocated to the nucleus, where it forms an active complex with HIF1β and activates transcription of about 60 target genes (16). Hypoxia stimulates the transcription of many genes such as those for glucose transporters (*e.g.* GLUT1), growth factors (*e.g.* VEGF), survival proteins and enzymes [*e.g.* carbonic anhydrase IX (CAIX), phosphoglycerate kinase (PGK)] and ATP binding cassette, subfamily B, member 1 (ABCB1; multidrug-resistant protein) (17-20). HIF1α activity is also controlled in an oxygen-independent manner through regulation of two main signaling pathways: the phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) pathways (16), which can also regulate the activity of the CAIX enzyme (21). HIF1 is increased in human cancer as a result of the physiological prevention of degradation of the HIF1α subunit in response to

intratumoral hypoxia and because of genetic alterations that activate oncogenes and inactivate tumor suppressor genes, making HIF1α an attractive target for cancer therapies (22).

2-Methoxyestradiol, like other microtubule-disrupting agents, interferes with the crosstalk between cancer cells and endothelial cells, which contributes to the anti-tumor and anti-angiogenic activity of this compound (13). It has also been shown that 2-methoxyestradiol inhibits nuclear accumulation of HIF1alpha, which leads to the inhibition of HIF1 transcriptional activity and to the subsequent down-regulation of VEGF expression. This suggests that microtubule disruption may be required for HIF1α inhibition (13). However, Mabjeesh *et al.* failed to demonstrate this in an *in vivo* tumor model because of the technical challenge of staining for HIF1α in sections from xenografts. Escuin *et al.* hypothesized that all microtubule disruptors may inhibit angiogenesis *via* HIF1, by inhibiting the synthesis and the nuclear accumulation of HIF1α (23). Intriguingly, microtubule disruption can significantly down-regulate nuclear translocation of HIF1α protein and this can therefore directly affect HIF1 transcriptional activity (24, 25).

To date, however, none of the results obtained from *in vitro* studies has been confirmed in *in vivo* xenograft tumor models and, furthermore, the effects of microtubule disruptors on HIF1-regulated genes have not been studied. It is also not clear as to whether HIF1 inhibition can occur at therapeutically relevant concentrations of these agents (26).

More recently, HIF2α, the other oxygen-labile α-subunit that can form a HIF1 complex with the stable β-subunit, has been demonstrated to promote cell-cycle progression and increase target gene transcription in hypoxic renal clear carcinoma cells (27, 28). The actions of HIF1α and HIF2α are distinct and differ according to cell type. Although HIF2α involvement in the survival of hypoxic breast cancer cell lines remains controversial (29, 30), the expression of both α-subunits seems essential for the growth of most tumors. Consequently, it is important to investigate whether microtubule disruptors inhibit HIF1 activity by reducing nuclear accumulation of HIF2α.

In the present study, we investigated the effects of two new microtubule disruptors, STX140 and STX243, on HIF1α and HIF2α expression and translocation into the nucleus and, more pertinently, on HIF1-regulated genes in MCF-7 and MDA-MB-231 cells. In addition, *in vivo* tumor models were used to explore the mechanistic role of HIF1 in mediating the anti-angiogenic activities of these compounds. Computational modeling was used to explore docking of ligands to CAIX in comparison with those of known activity.

Materials and Methods

Drug synthesis. STX66 was synthesized as previously described (31). STX140 and STX243 were synthesized by reaction of the parent 2-methoxyestradiol and 2-ethylestradiol respectively with sulfamoye chloride in dimethyl acetamide solution (5, 32). The compounds were tested alongside the known commercially available tubulin agent, paclitaxel (Sigma, Poole, UK).

Molecular modeling. Models of STX140, STX243, the known CAIX inhibitor S4 [Figure 1, compound IV, (33)] and acetazolamide were built and minimized in Discovery Studio (BIOVIA, San Diego, CA, USA). These compounds were docked into the 'A' chain of the 3IAI crystal structure of the CAIX catalytic domain (34). This structure has an acetazolamide molecule, a glycerol molecule, and several water molecules in the substrate-binding site. For the dockings, acetazolamide and glycerol were removed, with the substrate-binding site being defined as a sphere of 5Å radius centered on the centroid of the acetazolamide molecule: the centroid of the docked compounds was required to lie within this sphere. Each compound was docked 25 times using GOLD (35). The position of the docked compounds were assessed by visual inspection and the docking scores recorded.

Cell culture and cell treatment. MCF-7 estrogen receptor-positive (ER+ve) human breast cancer cells, MDA-MB-231 ER-negative (ER−ve) human breast cancer cells were obtained from the American Type Culture Collection (LGC Promochem, Teddington, UK). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, 1% L-glutamine (200 mM), 1% non-essential amino acids (100 X) and 1% bicarbonate (7.5%) from Sigma and maintained in a humidified incubator under 5% CO_2 atmosphere at 37°C. For experiments using hypoxic conditions, cells were incubated under 1% O_2 and 5% CO_2 atmosphere at 37°C.

Xenograft models. Athymic, female MF1 nude mice ($nu^{-/-}$) were purchased from Harlan (Bicester, Oxfordshire, UK) at 5 weeks of age (~20-25 g in weight). A total of $5×10^6$ MCF-7 or $2×10^6$ MDA-MB-231 cells were inoculated subcutaneously into the flanks of the animals. When tumors reached 100 to 150 mm³, mice were randomly divided into four treatment groups of five mice: vehicle (10% tetrahydrofuran: 90% propylene glycol), STX66 (40 or 75 mg/kg), STX140 (20 mg/kg) or STX243 (40 mg/kg). All compounds were given orally daily for 28 days for mice bearing MCF-7 xenografts and daily over 21 days for mice bearing MDA-MB-231 xenografts. A separate group of mice with MCF-7 xenografts were also treated intravenously twice weekly with paclitaxel at 15 mg/kg. Throughout the study, mice were weighed and tumor measurements were taken weekly. Tumor volumes were calculated using the formula (length × width²/2). At the conclusion of dosing (day 28 for mice with MCF-7 xenografts or day 21 for mice with MDA-MB-231 xenografts), the animals were euthanized and tumors removed for mRNA isolation. All experiments were carried out under conditions that complied with UK Home Office Animals (Scientific Procedures) Act 1986 and local institutional ethical guidelines. For ethical reasons, animals were removed from the study if the mean tumor diameter exceeded 1.5 cm.

Immunoblotting. MCF-7 and MDA-MB-231 were seeded at ~$2.5×10^5$ per T-25 flask in 5 ml medium and incubated at 37°C, with 5% CO_2 in a humidified incubator. After 24 h either STX140 (0.5 μM), STX243 (0.5 μM) or STX66 (0.5 μM) were added to the cells which were incubated for a further 18 h under normoxia then for 6 h under hypoxia (atmosphere of 1% O_2 and 5% CO_2). Protein was prepared from treated cells using radioimmuno-precipitation assay lysis buffer (Sigma) and a Bradford assay was undertaken to determine protein concentration.

To prepare nuclear extracts, cells were seeded at ~ $1×10^6$ per T-75 flask in 18 ml of medium and incubated at 37°C with 5% CO_2 in a humidified incubator. After 24 h, the compounds were added and the cells were incubated for a further 18 h under normoxia then for 6 h under hypoxia (1% O_2 and 5% CO_2 atmosphere). At the end of treatment, the medium was removed and the cells were washed with 10 ml ice-cold PBS, and centrifuged for 5 min at 600 ×g at 4°C. The pellets were resuspended in 500 μl ice-cold lysis buffer (20 mM Tris pH 8, 20 mM NaCl, 0.5% NP40, 1 mM Dithiothreitol and 1 μl/ml protease inhibitor cocktail from Sigma) and left for 5 min on ice to lyse the cells. The supernatant, referred to as the cytoplasmic fraction, and the nuclear pellets were obtained by centrifugation at 600 ×g 1 min at 4°C. The nuclear pellets were then lysed with 100-200 μl of ice-cold buffer C (20 mM Hepes pH 8, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM Dithiothreitol and 1 μl/ml protease inhibitor cocktail from Sigma). The proteins were isolated from the nuclei by centrifugation at 16,000 × *g* for 10 min at 4°C. Supernatants containing the cytoplasmic fraction or nuclear proteins were frozen at −80°C until used and the protein concentrations were determined by a Bradford assay with the Bradford reagent (Sigma).

Following determination of protein concentrations, equal amounts of either nuclear or whole cell protein extracts (50 μg) were loaded into all wells and separated by electrophoresis through a 412% NuPAGE Bis-Tris gel (Invitrogen Ltd., Paisley, UK) and transferred to Hybond-P membrane (GE Healthcare, Bucks, UK). To further ensure equal protein loading and successful transfer, the membranes were stained and visualized with Ponceau S (Sigma) before proceeding with immunodetection. Membranes with uneven transfer of proteins were rejected.

Detection was carried-out using primary antibodies to human HIF1α (ref. 610959; BD Biosciences, Erembodegen, Belgium), PI-3K (ref. sc1637; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HIF2α (ref. NB100122SS; Novus Biological, Littleton, CO, USA). Alkaline phosphatase-conjugated anti-rabbit IgG (ref. 7054; Cell Signaling Technology, Boston, MA, USA) or anti-mouse (ref 7056; Cell Signaling) secondary antibodies were used for detection. Digital images and band intensities were quantified using Kodak 1D software, version 3.5 (Eastman Kodak Company, Rochester, New York, USA). The results shown are representative of three separate experiments.

Real-time Polymerase Chain Reaction (RT-PCR). For tumor samples, 20 to 40 mg of tissue were excised from tumor xenografts and transferred to 2 ml of RNAlater solution (Ambion, Inc., Austin, TX, USA). The tissue sample was transferred to 600 μl of RNeasy lysis buffer (Qiagen, Ulm, Germany) plus 1% β-mercaptoethanol (Promega Ltd., Southampton, UK) and homogenized. The homogenate was centrifuged (3 min at 10,000×g) and the RNA was isolated from the supernatant using the RNeasy kit (Qiagen) and stored at −80˚C. For the *in vitro* samples, cells were lysed with 350 μl of RNeasy lysis buffer (Qiagen) plus 1% β-mercaptoethanol (Promega) and homogenized with the QIAshredder columns (Qiagen). The RNA was isolated from the homogenate using the RNeasy kit (Qiagen) and stored at −80˚C.

A 5-μg aliquot of each RNA sample was reverse transcribed in a final volume of 33 μl to generate a cDNA using the First-Strand cDNA Synthesis kit (GE Healthcare) and stored at −20˚C. Reverse-transcription PCRs were performed in a Rotor Gene 2000 Real-time Cycler (Corbett Research, Cambridge, UK) with 2 μl cDNA in a final volume of 25 μl using Taqman Universal PCR Master Mix (Applied Biosystems, CA, USA). Primers and hydrolysis probes for *VEGF$_{165}$* (isoform A, Ref. Hs00900057_m1), *GLUT1* (Ref. Hs00197884_m1), phospho-glycerate kinase (PGK; Ref. Hs99999906_m1), *CAIX* (Ref. Hs00154208_m1) and *ABCB1* (Ref. Hs00184491_m1) genes and for the internal control human large ribosomal protein (RPLO; Ref. 4310879E) were synthesized by Applied Biosystems. Human-specific primers were used because human cancer cell lines were inoculated into the flanks of the mice to generate the xenografts. The conditions used were: 95˚C for 10 min followed by 40 cycles of 95˚C for 30 s, 60˚C for 45 s and 72˚C for 45 s. The relative Ct values were calculated using the software rotorgene 6 (Corbett Research, Cambridge, UK) and the comparative Cts were calculated as:

$$\frac{\text{amplification efficiency of target gene}^{-(\text{target Ct of control} - \text{target Ct of sample})}}{\text{amplification efficiency of reference gene}^{-(\text{reference Ct of control} - \text{reference Ct of sample})}}$$

Statistics. *In vitro* experiments were carried-out in triplicate and data presented are representative of three or more different experiments. All data shown are the mean±SEM. Student's *t*-test was used to assess significance.

Results

HIF1α immunoblotting. To investigate the role of HIF1α in mediating the anti-angiogenic activities of STX140 and STX243, HIF1A expression was initially examined *in vitro*. ER+ve MCF-7 and ER-ve MDA-MB-231 breast cancer cells were treated with STX140, STX243 or STX66 under normoxia for 18 h and then under hypoxia for 6 h, according to the protocol described by Escuin *et al.* (23). All the compounds were initially used at 0.5 μM to compare their efficacy. Nuclear extracts and cytoplasmic fractions were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antibody to HIF1α. An increase of HIF1A expression was observed in the nuclei of the cells cultured under hypoxia (2.4-fold increase in MCF-7 cells and 1.37-fold increase in MDA-MB-231 cells, $p<0.05$, Figure 2A) compared to the HIF1α expression in nuclei of cells cultured under normoxia (Figure 2A). This indicates that 6 h hypoxia is enough to induce HIF1α expression. When both cell lines were treated with STX140 or STX243 (Figure 2A), nuclear HIF1α expression was lower than that in untreated cells cultured under hypoxia ($p<0.05$) and the steady-state level of HIF1α protein was similar to that in cells cultured under normoxia. In addition, no down-regulation of HIF1α protein expression was seen for both cell lines when treated with 0.5 μM STX66 (Figure 2A). These results show that STX140 and STX243 block nuclear accumulation of HIF1α in ER+ve and ER-ve breast cancer cells. Immunoblotting assays of the cytoplasmic fractions showed that HIF1α protein was not expressed or only very weakly-expressed in the cytoplasm of both cell types under hypoxia and normoxia (Figure 2B). HIF1α was detected at 120 kDa in the cytoplasm of cells treated with STX140, STX243 or STX66 (Figure 2B).

In vitro gene expression. To examine the effects of STX140, STX243 and STX66 on HIF1 activity, the transcription of five HIF1-regulated genes was studied in MCF-7 and MDA-MB-231 cells treated with STX140, STX243 at 0.5 μM or STX66 at 0.5 μM and 5 μM under normoxia for 18 h and then under hypoxia for 6 h. Total RNA was extracted and reverse transcribed. The expression of *CAIX, ABCB1, VEGF$_{165}$, PGK* and *GLUT1* mRNA was quantified by RT-PCR (Figure 3). Although *CAIX, GLUT1, PGK* and *VEGF$_{165}$* were overexpressed under hypoxia compared to their expression level in cells cultured under normoxia, showing that 6 h hypoxia is enough to enhance their
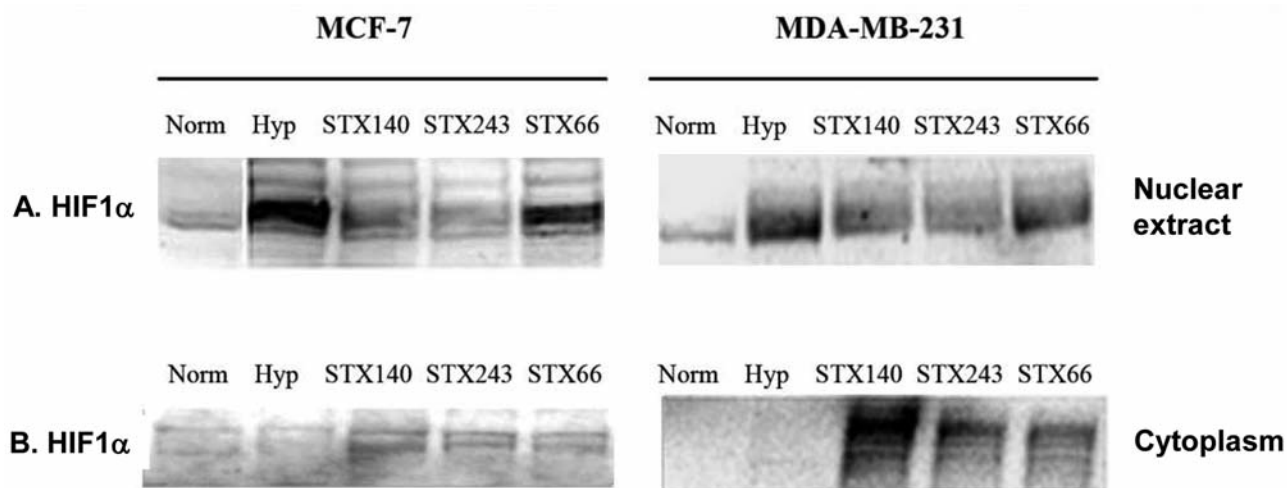
Figure 2. *Hypoxia-inducible factor-1α (HIF1α) protein expression in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with 0.5 μM STX140, STX243 or STX66 under normoxia for 18 h and then under hypoxia (1% O_2, 5% CO_2) for 6 h. Equal amounts of each nuclear extract (A) or cytoplasmic fraction (B) were resolved by SDS-PAGE and blotted for HIF1α. The immunoblot shown is representative of three separate experiments and was quantified using Kodak 1D software. A: MCF-7 nuclear extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (241%); 3: STX140 (162%*); 4: STX243 (105%*); 5: STX66 (216%). MDA-MB-231 nuclear extracts, lane 1: control normoxia (100% intensity); 2: control, hypoxia (147%); 3: STX140 (128%*); 4: STX243 (123%*); 5: STX66 (144%). B: MCF-7 cytoplasmic extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (75%); 3: STX140 (146%*); 4: STX243 (134%*); 5: STX66 (122%*). MDA-MB-231 cytoplasmic extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (67%); 3: STX140 (259%*); 4: STX243 (173%*); 5: STX66 (162%*). *p<0.05, compared to control.*

expression, only *CAIX* mRNA expression was significantly down-regulated by treatment with STX140 or STX243 in both cell lines (Figure 3). The mRNA expression of the other genes studied was neither significantly down-regulated nor up-regulated by treatment with the compounds tested and *ABCB1* expression was not significantly induced by 6 h hypoxia in either cell line.

In vivo gene expressions. To analyze whether STX140, STX243 and STX66 had an effect on HIF1-regulated genes *in vivo*, the expression of *CAIX, ABCB1, GLUT1, PGK* and *VEGF* was analyzed in tissue taken from the MCF-7 and MDA-MB-231 xenograft models. STX140 at 20 mg/kg (*p.o.* daily) and STX243 at 40 mg/kg (*p.o.* daily) significantly inhibited both MCF-7 and MDA-MB-231 xenograft growth (*p*<0.05; Figure 4A). In contrast, STX66 at 40 mg/kg against MDA-MB-231 xenografts or 75 mg/kg (*p.o.* daily) had no effect on tumor growth in both models. Paclitaxel was efficacious at 15 mg/kg (*i.v.* twice weekly) when tested in the MCF-7 model (Figure 4A). The mRNA level for $VEGF_{165}$ was not affected by any treatment in either xenograft model and the transcription level was similar to that in xenografts from untreated mice (Figure 4B). The transcription levels of the HIF1-regulated genes *CAIX, ABCB1, GLUT1* and *PGK* were also quantified for each sample. Only *CAIX* mRNA expression was significantly down-regulated by STX140 in both xenograft models (*p*<0.05) and was also significantly

down-regulated by STX243 in the MDA-MB-231 xenograft model (*p*<0.05) (Table I). *PGK* mRNA expression was significantly increased in MCF-7 xenografts treated with STX140 (*p*<0.05). Treatment with paclitaxel did not affect any of the HIF1-regulated genes studied (Table I). Although STX66 did not affect the growth of tumors in either model, it down-regulated *CAIX* in the MDA-MB-231 xenograft model (*p*<0.05).

In vitro study of HIF2α expression. The effects of STX140 and STX243 on HIF2 expression and HIF2α translocation into the nucleus were investigated. MCF-7 and MDA-MB-231 cells were treated with STX140, STX243 or STX66 at 0.5 μM under normoxia for 18 h and then under hypoxia for 6 h. Whole-cell protein extracts and nuclear extracts were prepared, then 50 μg proteins were loaded and HIF2α expression was detected by immunoblotting with a human antibody against HIF2α. HIF2α protein was detected at 118 kDa. Immunoblot analysis showed that HIF2α expression in nuclear extracts of both cell lines was not affected by any treatment and was similar to that in untreated cells (Figure 5A). HIF2α protein expression in whole-cell extracts from MCF-7 and MDA-MB-231 cells was not increased under hypoxia (Figure 5B). The steady-state level of HIF2α protein was higher in MDA-MB-231 cells treated under hypoxia with STX140 and STX243 (*p*<0.05) than that in untreated cells cultured under both normoxia and hypoxia (Figure 5B).
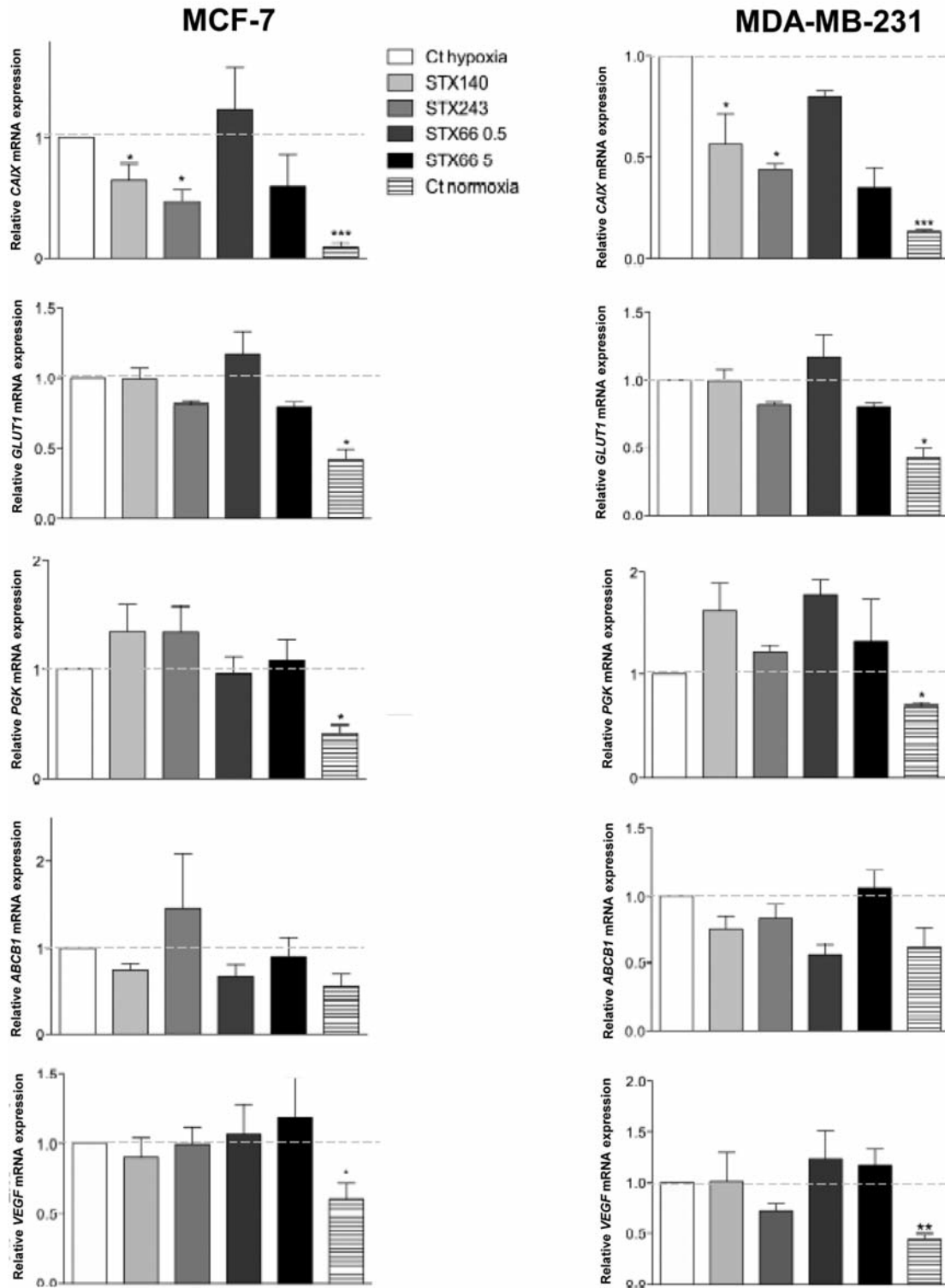
Figure 3. *Vascular endothelial growth factor (VEGF)₁₆₅, ATP-binding cassette sub-family B member 1 (ABCB1), glucose transporter 1 (GLUT1), Phosphoglycerate Kinase (PGK1) and Carbonic Anhydrase IX (CAIX) mRNA expression in MCF-7 and MDA-MB-231 cells. MCF-7 cells and MDA-MB-231 cells were seeded in T25 flasks, and treated with 0.5 μM STX140, STX243 or STX66, or with 5 μM STX66 under normoxia for 18 h and under hypoxia (1% O_2, 5% CO_2) for 6 h. Total mRNA was then prepared, reverse-transcribed and the relative quantity of VEGF₁₆₅, ABCB1, GLUT1, PGK1 and CAIX were determined by RT-PCR. mRNA expression of untreated cells after 6 h hypoxia of each experiment was given the value 1. *p<0.05, **p<0.01, ***p<0.001, compared to control (Ct).*
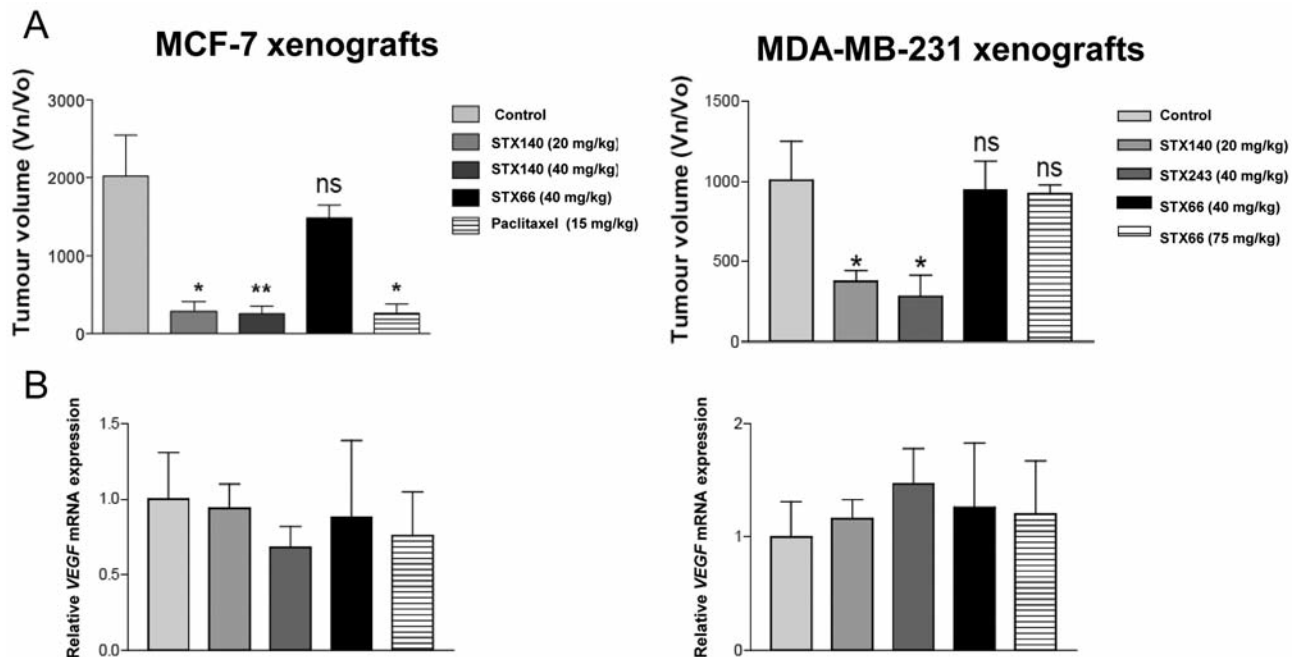
Figure 4. *A: MCF-7 and MDA-MB-231 xenograft models. Mice were treated orally for 7 days per week with STX140, STX243, STX66 at the doses indicated. Mice bearing MCF-7 xenografts were also treated intravenously once weekly with paclitaxel at 15 mg/kg. Results show average tumor volumes at the end of dosing (28 days for MCF-7 and 21 days for MDA-MB-231 xenografts, n=4-9 tumors); data are means±SEM. *p<0.05, **p<0.01, compared to control. Volume at day measured/Volume at day 0 (Vn/Vo). B: VEGF$_{165}$ mRNA expression in MCF-7 and MDA-MB-231 xenografts treated with STX140, STX243, STX66 or paclitaxel. Total mRNA was prepared from 20 to 40 mg of tissues excised after 21 days treatment for mice with MDA-MB-231 xenografts and after 28 days treatment for mice with MCF-7 xenografts. mRNA was reverse-transcribed and quantified by RT-PCR. Three to four tumor samples (n=3 or 4) were submitted to a minimum of three independent amplifications, and each amplification was performed in duplicate. The results presented are the average of the relative Ct values from all of the experiments relative to vehicle-dosed animals (control).*

Table I. *ATP-binding cassette sub-family B member 1 (ABCB1), phosphoglycerate kinase (PGK), glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX) mRNA expression in MCF-7 and MDA-MB-231 xenografts from mice dosed with STX140, STX243, STX66 or paclitaxel. Mice bearing MCF-7 xenografts were dosed daily with STX140 at 20 mg/kg, STX243 at 40 mg/kg, or STX66 at 40 mg/kg p.o. or weeklywith paclitaxel at 15 mg/kg i.v. . Mice bearing MDA-MB-231 xenografts were dosed daily with STX140 at 20 mg/kg, STX243 at 40 mg/kg, STX66 at 40 mg/kg or STX66 at 75 mg/kg p.o. Total mRNA was prepared from 20 to 40 mg of tissues excised after 21 days dosing for mice with MDA-MB-231 xenografts and after 28 days dosing for those with MCF-7 xenografts. mRNA was reverse transcribed and quantified by RT-PCR. Three to four tumor samples (n=3 or 4) were submitted to a minimum of three independent amplifications, and each amplification was performed in duplicate. The results presented are the average of the Ct values from all of the experiments relative to vehicle-dosed animals (control).*

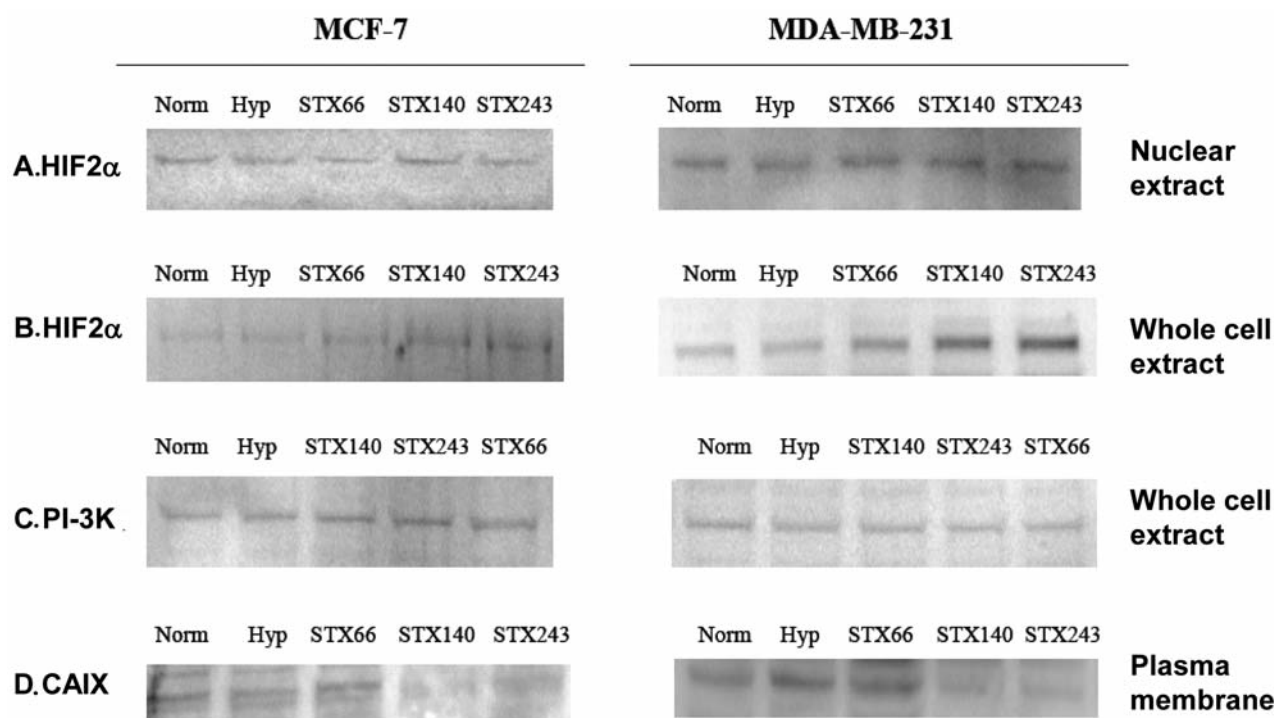| | Gene expression | | | | | | | | | | | |
| | *ABCB1* | | | *PGK* | | | *GLUT1* | | | *CAIX* | | |
	Average	SDTEV	*t*-Test	Average	SDTEV	*t*-Test	Average	SDTEV	*t*-Test	Average	SDTEV	*t*-Test
MCF-7												
Control	1.00	1.90		1.00	0.32		1.00	0.45		1.00	0.23	
STX140, 20 mg/kg	0.61	0.71	0.70	1.52	0.18	0.04	0.64	0.28	0.24	0.42	0.08	0.05
STX243, 40 mg/kg	0.14	0.07	0.40	0.97	0.21	0.91	0.66	0.26	0.25	0.46	0.22	0.10
STX66, 75 mg/kg	0.33	0.50	0.50	1.08	0.20	0.65	0.76	0.24	0.39	0.58	0.21	0.18
Paclitaxel, 15 mg/kg	0.22	0.26	0.40	1.05	0.18	0.78	0.89	0.13	0.66	0.71	0.50	0.39
MDA-MB-231												
Control	1.00	1.20		1.00	0.33		1.00	0.36		1.00	0.75	
STX140, 20 mg/kg	0.59	0.09	0.71	1.64	0.50	0.78	0.77	0.21	0.27	0.25	0.06	0.01
STX243, 40 mg/kg	0.80	0.17	0.84	1.48	0.53	0.13	0.77	0.20	0.53	0.47	0.30	0.02
STX66, 40 mg/kg	0.87	1.11	0.53	1.26	0.69	0.53	1.41	0.29	0.11	0.55	0.16	0.03
STX66, 75 mg/kg	0.26	0.20	0.54	1.45	0.55	0.21	1.23	0.07	0.23	0.72	0.34	0.71

Figure 5. *Hypoxia-inducible factor-2α (HIF2α), Phosphatidylinositol-3 Kinase (PI-3K) and carbonic anhydrase IX (CAIX) protein expression in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with 0.5 μM STX140, STX243 or STX66 under normoxia for 18 h and then under hypoxia (1% O$_2$, 5% CO$_2$) for 6 h. Nuclear extracts (A) and total protein extracts (B) were prepared and analyzed by SDS-PAGE and immunoblotting with the antibody to HIF2α or PI-3K (C). Cells were given an additional 48 h incubation without compound and plasma membrane fractions were prepared and analyzed by SDS-PAGE and immunoblotting with the antibody to CAIX (D). A: MCF-7 nuclear extracts, lane 1: control normoxia (100% intensity), 2: control hypoxia (99%); 3: STX66 (101%); 4: STX140 (101%); 5: STX243 (102%). MDA-MB-231 nuclear extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (101%); 3: STX66 (102%); 4: STX140 (104%); 5: STX243 (103%). B: MCF-7 total protein extracts; lane 1: control normoxia (100% intensity); 2: control hypoxia (102%); 3: STX66 (108%); 4: STX140 (119%); 5: STX243 (123%). MDA-MB-231 total protein extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (99%); 3: STX66 (108%); 4: STX140 (121%*); 5: STX243 (134%*). C: MCF-7 total protein extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (100.2%); 3: STX140 (100.3%); 4: STX243 (99.6%); 5: STX66 (100.3%). MDA-MB-231 total protein extracts, lane 1: control normoxia (100% intensity); 2: control hypoxia (99.8%); 3: STX140 (99.2%); 4: STX243 (98.8%); 5: STX66 (99.3%). D: MCF-7 plasma membrane fraction, lane 1: control normoxia (100% intensity); 2: control hypoxia (101.3%); 3:STX66 (103%); 4: STX140 (61.2%*); 5: STX243 (74.2%*). MDA-MB-231 plasma membrane fraction; 1: control (100% intensity); 2: control hypoxia (104%); 3: STX66 (99.23%); 4: STX140 (67.3%*); 5: STX243 (67.4%*). *p<0.05, compared to control.*

In vitro study of PI-3K expression. As previous results showed that STX140 and STX243 down-regulate *CAIX* mRNA expression without affecting the other HIF1-regulated genes studied, the role of PI-3K protein in mediating the down-regulation of *CAIX* mRNA expression was investigated. MCF-7 cells and MDA-MB-231 cells were treated with STX140, STX243 or STX66 under normoxia for 18 h and then under hypoxia for 6 h. Cells were lysed and the protein extracts were analyzed by SDS-PAGE and immunoblotted with a human antibody against PI-3K. A band was detected at 85 kDa, the correct size for the α-subunit of PI-3K, in MCF-7 and MDA-MB-231 cells regardless of treatment (Figure 5C). No changes were observed in PI-3K expression.

In vitro study of CAIX expression. MCF-7 and MDA-MB-231 cells were treated with 0.5 μM STX140, STX243 or STX66

under normoxia for 18 h followed by 6 h under hypoxia. Plasma membrane fractions were prepared and 50 μg of protein were analyzed by immunoblotting. No changes in CAIX expression were observed (data not shown). This may be due to the long half-life of CAIX protein (38 h) masking the changes observed in *CAIX* mRNA expression after compound treatment for just 24 h. Therefore, the initial experiment was repeated but the cells were given an additional compound-free, 48 h incubation, for any changes in *CAIX* mRNA expression to be reflected at the protein level. The results showed that CAIX protein expression is inhibited in cells treated with STX140 and STX243 (Figure 5D, *p*<0.05).

Modeling results. Compound S4 is a small-molecule sulfamate ester derivative that inhibits CAIX with a K_i of 7 nM (33). STX140, STX243, S4 and acetazolamide were

docked into the 3IAI crystal structure of the CAIX catalytic domain (34). This structure has an acetazolamide molecule in the substrate-binding site, with the ligand sulfonamide group coordinated to the zinc ion in the binding site. With the acetazolamide removed, our docking protocols were able to recreate the observed crystallographic docking pose for this ligand (data not shown), giving confidence for the later studies. It was expected that the structurally related sulfamate group of the docked compounds would bind in a similar manner (35-37) and this indeed proved to be the case (Figure 6). All three compounds docked in the same region of the binding site: the docked poses of the two STX compounds were almost identical and the two sulfamates overlaid with the sulfonamido group of the acetazolomide. For each of the three compounds, all 25 docked poses were approximately the same. The docking scores of the best-ranked poses were 45.45 (S4), 14.35 (STX140) and 16.24 (STX243), with the higher score suggesting better binding.

Discussion

Angiogenesis, the creation of a new network of blood vessels supplying nutrients and oxygen, plays an essential role in tumor growth, tumor progression and metastasis. Therefore, angiogenesis inhibitors offer a potential therapy for cancer. Many anti-angiogenic agents identified have led to the development of a number of novel cancer therapies, such as Avastin (bevacizumab). Optimized sulfamoylated compounds based on 2-methoxyestradiol, such as STX140 and STX243, were derived from our earlier evidence that the addition of a sulfamate group can have profound effects on activity (39). They are potent inhibitors of *in vitro* and *in vivo* angiogenesis (10, 11, 40). Previous studies also showed that they are microtubule disruptors and bind to the colchicine site on tubulin, as does the parent steroid 2-methoxyestradiol. Nevertheless, the mechanisms linking microtubule disruption, anti-angiogenic activity and cell death, remain largely unknown. Escuin *et al*. demonstrated that the anti-angiogenic activity of microtubule disruptors occurs after the disruption of microtubules and is mediated by the post-transcriptional down-regulation of the pro-angiogenic transcription factor HIF1α protein (23). This was confirmed only with a non-cancer *in vivo* model for 2-methoxyestradiol when mice were dosed with 100 mg/kg 2-methoxyestradiol (41). So far, no studies have been carried out with *in vivo* cancer models.

Although the protocol described by Escuin *et al*. (23) was followed, the results obtained differ from their findings, since no diminution in nuclear HIF1α accumulation was seen in either of the cell lines treated with 2-methoxyestradiol (STX66). This may be due to the lower concentration used. The *in vitro* experiments were undertaken with 0.5 and 5 μM 2-methoxyestradiol, but using higher concentrations is
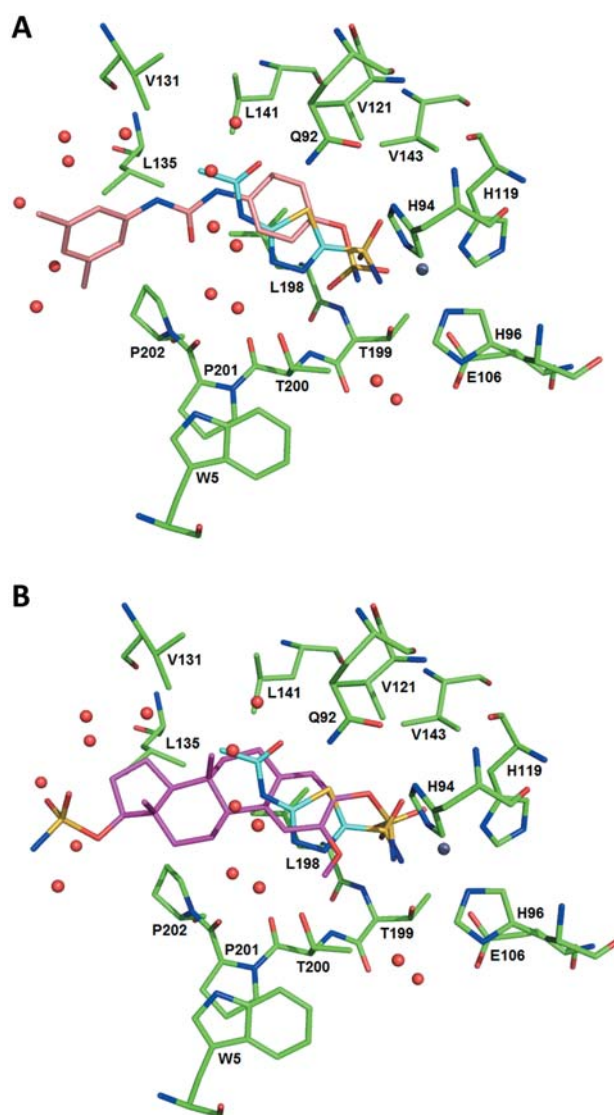

Figure 6. *S4 (A) and STX140 (B) docked into the 3IAI 'A' chain crystal structure of CAIX. Protein carbon atoms are shown in green: the residues are labelled. The crystal structure acetazolamide is shown with cyan carbon atoms. The grey sphere is a zinc ion and the red spheres are the oxygen atoms of water molecules. The docked S4 is shown with light brown carbon atoms and the docked STX140 is shown with purple atoms.*

clinically irrelevant as its poor bioavailability prevents it reaching plasma concentrations higher than 0.1 μM (3, 4, 10, 42). However, Chua *et al*. have shown that doses of 50 μM and above are required to affect HIF1α expression (43). STX140 and STX243 did inhibit nuclear HIF1α accumulation in MCF-7 and MDA-MB-231 cells, supporting the theory that microtubule disruptors inhibit nuclear HIF1α accumulation. STX140 and STX243 increased cytoplasmic HIF1α protein expression, suggesting they may increase

HIF1α stability or inhibit its nuclear translocation. Cytoplasmic HIF2α expression was increased by STX140 or STX243 treatment, whereas nuclear HIF2α accumulation was unchanged, indicating that HIF2α translocation is not inhibited by STX140 or STX243.

To test whether the inhibition of nuclear accumulation of HIF1α, observed *in vitro*, mediated anti-angiogenic activity of microtubule disruptors by down-regulating HIF1 transcriptional factor activity, mRNA expression was measured for a cohort of HIF1-regulated genes. Uniquely, and in addition to samples from *in vitro* experiments, cancer xenograft tissue was also examined to test the relevance of this mechanism *in vivo*. No HIF1 immunochemistry was carried-out on *in vivo* samples because the cellular composition of tumors is heterogeneous and cells may be necrotic. It is also known that tumor oxygen levels oscillate over both hours and days, causing periodic fluctuations of HIF expression (44). The study of mRNA expression within the tumor also overcomes potential user bias in assessing immunochemistry. For this reason, only non-necrotic tissues excised from the tumors were studied.

The mRNA was quantified for *VEGF, GLUT1, ABCB1, PGK1* and *CAIX*, all of which are reported to be regulated by HIF1 (45). STX140 and STX243 did not inhibit the expression of the HIF1-regulated genes examined *in vitro* and *in vivo*, except CAIX, suggesting that they do not down-regulate HIF1 transcriptional factor activity. The fact that they do not down-regulate *VEGF*, one of the most important biomarkers for tumor angiogenesis, corroborates these results. Although no strictly HIF2α-regulated genes were studied, HIF1α/HIF2α-common targets, such as *GLUT1*, were not affected by any treatment, suggesting that they do not alter HIF2α activity. STX140 and STX243 might affect HIF1α and HIF2α stability or translocation without altering their activities. To assess the *in vivo* anti-tumor efficacy of STX140 and STX243, paclitaxel was included in the xenograft studies as a clinically proven competitor compound. Therefore, analysis of the effects of paclitaxel on HIF1-regulated genes *in vivo* was undertaken, despite the fact that paclitaxel did not form part of the *in vitro* study. This showed that expression of HIF1-regulated genes are not significantly altered by paclitaxel. HIF1 down-regulation would appear to have little mechanistic relevance to explain the link between microtubule disruption and anti-angiogenic effects that can be induced by these compounds. Moreover, it has also been shown that STX140 and STX243 are potent tumor growth inhibitors in non-VEGF-stimulated tumor models (46).

STX140 and STX243 down-regulated *CAIX* mRNA and protein expression both *in vitro* and *in vivo*. No correlation was seen between tumor size and *CAIX* mRNA expression, suggesting that the down-regulation observed is not tumor size-related, but is due to the effects of STX140 and STX243. The CAIX enzyme is overexpressed in many tumors, where it is involved in crucial processes related to cancer progression, such as pH regulation and cell adhesion, and it is associated with a poor prognosis (47). *CAIX* transcription can be strongly activated by hypoxia *via* HIF1 transcription (20). However, as HIF1 activity is not down-regulated by STX140 and STX243, the decrease in *CAIX* transcription is most likely to be HIF1-independent, and may be activated *via* the PI-3K pathway (48). However, *in vitro* PI-3K protein expression was not altered by treatment with STX140 or STX243. Another potential pathway implicated in regulating CAIX expression is through the anti-apoptotic oncogene B-cell lymphoma 2 (*BCL2*). Decreased expression of BCL2 directly leads to *CAIX* down-regulation (49), and it is known that STX140 can down-regulate *BCL2* expression in breast cancer cells (50). Despite this, how STX140 and STX243 down-regulate *CAIX* mRNA expression remains to be fully resolved. Ho *et al.* showed that STX140 and STX243 directly inhibit CAII (12), another enzyme of the carbonic anhydrase family, whereas the parent 2-methoxyestradiol does not. This is due to the presence of a sulfamate group in STX140 and STX243 that directly coordinates with the zinc atom of CAII. Indeed, both STX140 and STX243 were previously co-crystallized with CAII and their binding modes were elucidated (37). It is, therefore, very likely that STX140 and STX243 will also directly inhibit the CAIX enzyme, which is structurally similar to CAII. This direct enzyme inhibition could explain, at least in part, the ability of these compounds to inhibit angiogenesis and tumor growth.

We proposed earlier that such sulfamate esters might inhibit not only CAII, but also CAIX and CAXII (36, 38). Since this work, the X-ray crystal structure of CAIX has been reported (34). We, therefore, used computational docking to explore possible binding modes for STX140, STX243 and the recently reported potent sulfamate-based CAIX inhibitor S4 (IV, (33)). All successfully docked into the CAIX active site. The sulfamate and its interaction with the zinc ion is a major determinant of the binding pose, as the crystal structure of CAII with 17-*O*-sulfamoylated 2-methoxy-3-hydroxyestratriene, *i.e.* STX140 with the 3-*O*-sulfamoyl moiety replaced with a hydroxyl group, binds with the 17-*O*-sulfamoyl moiety interacting with the zinc, but if the 17-*O*-sulfamoyl moiety of STX140 is removed then the 3-*O*-sulfamoyl moiety interacts with the zinc (37). In the crystal structure of CAII with STX243, with sulfamates in both the 3- and 17-positions, the 17-*O*-sulfamate interacts with the zinc ion in the binding site, at least in those crystals examined. This is presumed to be because of local steric hindrance exerted by the neighboring 2-methoxy group on the phenolic sulfamate, but this may of course simply represent the only binding mode that could be crystallized. However, the docking of STX243 (and STX140) into CAIX has the 3-*O*-sulfamate making this interaction.

Although there are some amino acid and, therefore, structural differences between the binding sites of CAII and CAIX, we can see no obvious structural explanation for the difference in binding pose and possibly both are operative *in vivo*.

S4 Has a K_i of 7 nM and a docking score of 45.45. If docking score is correlated with K_i then the docking scores of STX140 (14.35) and STX243 (16.24) suggest that the STX compounds may be weaker inhibitors of CAIX than S4. This may be related to the greater rigidity of the STX compounds compared to S4 and the consequent reduced ability to adapt to the shape of the binding site. Carbonic anhydrase inhibitors are known anticancer agents both *in vitro* and *in vivo*. In binding to the related CAII, STX140 and STX243 had IC_{50} values of 379 and 290 nM respectively in direct comparison to acetazolamide, that had a value of 25 nM. S4 has a marked anti-metastatic effect (33). If this effect is a consequence of CAIX inhibition, then this may explain the anti-proliferative effect of the STX compounds. Moreover, in a recent *in vivo* study (9), in addition to observed marked growth inhibition, tumors treated with STX140 were shown to exhibit no metastasis, in stark contrast to those treated with paclitaxel. This additional positive effect of STX140 may be due to a combination of its direct inhibition and down-regulation of *CAIX*.

In conclusion, we showed that STX140 and STX243, two potent anti-proliferative, antitumor growth and anti-angiogenic microtubule disruptors down-regulate HIF1α accumulation in nuclei *in vitro* but do not inhibit HIF1 transcriptional activity *in vitro* and *in vivo*. We also showed that these compounds have no effect on nuclear HIF2α accumulation. Furthermore, we demonstrated that STX140 and STX243 down-regulate *CAIX* mRNA transcription and expression without affecting PI-3K protein expression. Therefore, the anti-angiogenic activity observed for these compounds and other microtubule disruptors, such as paclitaxel and 2-methoxyestradiol, is unlikely to be mediated by the inhibition of HIF1α-mediated gene transcription. The excellent *in vivo* efficacy of STX140 and STX243 may, at least in part, be due to a combination of the inhibition of transcription of *CAIX*, a marker of poor prognosis in breast cancer (51), and to direct inhibition of CAIX in hypoxic environments.

Competing Interests

Acknowledgements

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