STX140 and STX641 Cause Apoptosis via the Intrinsic Mitochondrial Pathway and Down-regulate Survivin and XIAP Expression in Ovarian and Prostate Cancer Cells

PAUL A. FOSTER\(^1\), YAIK T. HO\(^1\), SIMON P. NEWMAN\(^1\), MATHEW P. LEES\(^2\), BARRY V.L. POTTER\(^2\), MICHAEL J. REED\(^1\)† and ATUL PUROHIT\(^1\)

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

Abstract. Many anticancer drugs target microtubules and induce apoptosis. However, improved microtubule-targeting drugs, such as STX140 and STX641, are being developed. These compounds induce cell cycle arrest and apoptosis in a variety of tumour cells. The mechanisms that induce apoptosis and the key mediators involved are elucidated in this study. Results demonstrate that STX140 and STX641 depolarise mitochondrial bioenergetics and activate caspase 3/7 in A2780, LNCaP and MCF-7 cancer cells. Furthermore, both compounds cause a significant reduction in the expression of survivin and XIAP. This work details the temporal organisation of apoptosis induced by two microtubule disruptors and highlights the role that the down-regulation of survivin and XIAP may play in this process.

Currently the taxanes, paclitaxel and doxetaxel, are the preferred choice of therapy for advanced solid tumours. However, as resistance develops to these drugs there is a need for new treatments. Research focusing on 2-methoxyestradiol (2-MeOE\(_2\)), an endogenous estrogen metabolite with anti-cancer properties, has recently shown limited effects in prostate cancer patients due to sub-optimal plasma levels as a result of poor bioavailability (1, 2). Sulfamoylated derivatives of 2-substituted estrogens, such as 2-methoxyestradiol-3,17-O,O-bis-sulfamate (STX140) and 2-methoxy-3-O-sulfamoyl-17β-cyanomethyl-estra-1,3,5(10)-triene (STX641), possess greater in vitro and in vivo anti-tumour activity (3-10) and bioavailability (11) compared to 2-MeOE\(_2\). These compounds do not function as pro-drugs of 2-MeOE\(_2\) (6) and are believed to exert their effect in an estrogen receptor-independent manner (12) through microtubule disruption (13). Therefore, they represent a novel class of orally bioavailable anti-microtubule compounds with potential clinical application.

Sulfamoylated 2-substituted estrogen derivatives exert their antitumor effects through kinetic suppression of microtubule dynamics leading to mitotic arrest and apoptosis (3, 14). In common with the taxane family of anti-microtubule agents, these compounds modulate key apoptotic regulators through interaction with mitotic microtubules, Bcl-2 family protein phosphorylation, and up-regulation of p53 (3). Unlike the taxanes, which stabilize microtubules, sulfamoylated 2-substituted estrogens destabilize them.

The sequence of events involved in apoptosis induced by sulfamoylated 2-substituted estrogens is unknown. Our investigations focus on the molecular events leading up to apoptosis following treatment with STX140 or STX641 in A2780 (ovarian carcinoma), LNCaP (prostate carcinoma) and MCF-7 (breast carcinoma) cell lines. This study examines the cell type differences in temporal organization of the apoptotic response to these compounds. At a range of time points, immunoblotting for cyclin B1, p21, p53, survivin and XIAP was undertaken to assess the cell cycle state and apoptotic signalling mechanisms that occur in treated cells. Furthermore, we examined mitochondrial bioenergetics, cellular ATP levels, and caspase activation at various time points so as to determine the temporal organization of STX140 and STX641-induced apoptosis.

\(^{†}\)Deceased 2009.

Correspondence to: Dr. P.A. Foster, Oncology Drug Discovery and Women’s Health Group, Faculty of Medicine, Imperial College London, St. Mary’s Hospital, London, W2 1NY, UK. Tel: +44 2078861210, Fax: +44 2078861790, e-mail: paul.foster@imperial.ac.uk

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Materials and Methods

Compounds. All compounds were synthesized using previously published routes (15-17).

Cell culture. A2780, LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (LGC Promochem). Cells were grown in 5% serum supplemented RPMI medium (Sigma) and cultured at 37°C under 5% CO2 in a humidified incubator. For apoptosis studies, mitochondrial potential (ΔΨm), analysis and caspase-3/7 activation cells were seeded at 1×10^5 per T-25 flask (Triple Red Ltd.) and after 24 h the growth media was replaced with media containing compound and incubated for the times indicated. The effects of drugs on cell proliferation were determined by a microtitre plate assay (Promega).

Immunoblotting. T-25 flasks were seeded at approximately 2.5×10^5 cells per flask in 5 ml of cell-specific medium and were incubated at 37°C, 5% CO2 in a humidified incubator. After 24 h the compounds were added and the cells incubated for 8, 24, 48 or 72 h. Protein was prepared from treated cells using RIPA lysis buffer (Sigma). Equal amounts of protein were loaded in all wells. Proteins were separated by electrophoresis through a 4-12 % NuPAGE Bis-Tris gel (Invitrogen) and subsequently transferred to Hybond-P membrane (GE Healthcare). Detection was carried out using anti-human Cyclin B1 (sc-752) (Santa Cruz Biotechnology Inc.), p21 (05-655), (Upstate Biotechnologies Ltd.) and p53 (ab-28-100) (Abcam plc.), survivin (Abcam plc.), XIAP (Abcam plc.) primary antibodies, respectively.

Determination of mitochondrial membrane potential. Mitochondrial membrane potential (ΔΨm) was assessed using FACS analysis in trypsinized cells (1-2×10^6 cells/ml) washed in PBS and then re-suspended in RPMI-1640 medium containing 1 μM J-aggregate-forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Invitrogen). The carboxyamine dye JC-1 is considered a reliable and sensitive fluorescent probe for detecting differences in the ΔΨm due to its dual emission characteristics (18, 19). At low concentrations JC-1 exists mainly in a monomeric form which emits green fluorescence. At high concentrations this molecule forms aggregates, known as J-aggregates, which emit orange-red fluorescence. Thus, since the amount of cationic dye taken up by the mitochondrion depends on its transmembrane potential, at low ΔΨm the fluorescence emission will be mostly green, whereas at high ΔΨm it will shift to orange-red. Therefore, a low green fluorescence (FL-1) to high orange/red fluorescence (FL-2) indicates active mitochondria. JC-1 was allowed to equilibrate with the cells for 45 min at 37°C. Preliminary experiments demonstrated that under these conditions dye uptake was maximal and gave a fluorescence response to a fall in ΔΨm induced by the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (5 μM; data not shown; Sigma). Results are determined as the mean fluorescent index (MFI), the percent change in FL-2/FL-1 compared to control.

Caspase-3/7 activation. Determination of caspase-3/7 activation was measured using the Vybrant® FAM Caspase-3 and -7 Assay Kit (Invitrogen) as per manufacturer’s instructions. Briefly, cells were harvested at various time points after treatment and co-incubated with a fluorescent caspase inhibitor which binds irreversibly to active caspase-3 or -7. Measurement of fluorescence was performed using a FACS Calibur (BD Biosciences) flow cytometer and CellQuest Pro software (BD Biosciences).

Apoptosis analysis. Apoptosis was quantified by double-staining with annexin V-FITC and propidium iodide. Briefly, treated cells were trypsinized and washed with cold PBS before being re-suspended in 1x binding buffer (10 mM HEPES / NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at 1×10^6 cells/ml. Cells were then stained with flourescin-conjugated annexin V (BD Biosciences) antibody, which binds to phosphatidylserine (PS) on the outer leaflet of the plasma membrane, and propidium iodide (5 μg/ml) (Sigma) before analysis using a FACS Calibur (BD Biosciences) and CellQuest Pro software (BD Biosciences). Cells undergoing apoptosis are defined as cells positive for annexin V and negative for propidium iodide staining.

Results

Proliferation assay. Initial cytotoxicity assays, using a microtitre plate assay, revealed that the IC50 values for STX140 and STX641 were 280 nM and 90 nM in A2780 ovarian carcinoma cells; 260 nM and 60 nM in LNCaP prostate carcinoma cells; and 250 nM and 150 nM in MCF-7 breast carcinoma cells respectively (Figure 1).

Cell cycle proteins. To determine whether the cells were arrested during mitosis, treated cells were harvested at various time points, protein prepared and immunoblotting for cyclin B1 was performed. Cyclin B1 is a phasic cell cycle protein maximally expressed during the G2-M phase and its degradation is requisite for metaphase to anaphase transition during mitosis (20). Sustained cyclin B1 expression would therefore provide evidence that the cells have not progressed through the mitotic spindle checkpoint. In A2780 cells, STX140 and STX641 treatment induced cyclin B1 expression at 8 h (Figure 2A) in conjunction with G2-M arrest. However, total degradation of cyclin B1 occurred by 24 h in both STX140- and STX641-treated A2780 cells. In LNCaP cells, cyclin B1 expression was sustained 24-48 h post-treatment (Figure 2A). These findings correlate with previously published findings (3, 14) and are consistent with prolonged G2-M arrest of cells by anti-microtubule agents (20). In MCF-7 cells
immunodetection of cyclin B1 revealed that it is degraded after 8 h in both STX140- and STX641-treated cells. Fluorescent microscopy revealed that these cells feature fragmented nuclei and possess disrupted cytoskeleton as visualized by β-tubulin staining (data not shown).

Treatment of A2780 cells with STX140, but not with STX641, caused p53 induction by 24 h compared to untreated cells (Figure 2A), along with a concomitant induction of p21. STX641 treatment, while not appearing to stabilize p53, resulted in an accumulation of p21 at 48 and 72 h in treated cells.

Figure 2. Analysis of phasic cell cycle proteins in STX140- and STX641-treated cells. Expression of cell cycle proteins cyclin B1, p21, p53 in A2780, LNCaP and MCF-7 cells treated with 1 μM STX140 or STX641 for 8, 24, 48 and 72 h (A). Panel B shows the expression of anti-apoptosis proteins survivin and XIAP in A2780, LNCaP and MCF-7 cells treated with 1 μM STX140 or STX641 for 24, 48 and 72 h.
A2780 cells. In LNCaP cells low endogeneous levels of p53 were not induced by treatment with STX140 or STX641. However, possibly through a p53-independent mechanism, p21 induction still occurred in STX140- and STX641-treated cells (Figure 2A). MCF-7 cells showed induction of p53 24 h after treatment with STX140 and STX641, along with a concurrent increase of p21. It was also noticed that in all three cell lines, p21 was initially present in untreated cells but was down-regulated after 24-48 h, possibly due to the confluence of cells after seeding.

Apoptosis proteins. All cell lines examined were shown to express the anti-apoptotic protein survivin (Figure 2B). This expression was down-regulated upon treatment with either STX140 or STX641 in A2780 and LNCaP cell lines by 72 h. However, STX140 and STX641 had no effect on survivin expression in MCF-7 cells. This pattern is similar for another anti-apoptotic protein, XIAP. Both STX140 and STX641 down-regulated this protein by 48 h and 72 h in A2780 cells and by 72 h in LNCaP. Again, both treatments had no effect on XIAP expression in MCF-7 cells.
Mitochondrial depolarization. The induction of cyclin B1 and apoptosis by STX140 and STX641 suggest that these compounds act via disruption of the mitotic process and induction of the intrinsic apoptotic pathway. Activation of the intrinsic apoptotic pathway results in mitochondrial depolarization and release of pro-apoptotic factors, such as cytochrome c, into the cytoplasm. Therefore, mitochondrial depolarization of treated cells was investigated using JC-1, a cationic dye which alters its fluorescence emission spectrum in response to changes in Δψm.

Figure 3A shows the quantification of Δψm for all three cell lines. In A2780 cells, STX140 and STX641 resulted in a reduction of Δψm after 24 h, and complete collapse of mitochondrial potential after 48 h. LNCaP cells exposed to STX140 and STX641 exhibited a gradual decline of Δψm starting from 24 h (75% and 50% of control respectively), leading to eventual collapse after 72 h of treatment. MCF-7 cells also exhibited a similar collapse of Δψm in response to exposure to both STX140 and STX641.

Caspase-3/7 activation. Having determined the duration of STX140- and STX641-induced cyclin B1 down-regulation and subsequent passage of arrested cells through the mitotic checkpoint, the time course for the activation of caspase-3/7 was determined using flow cytometry. Figure 3B shows the quantified results from three independent experiments for A2780, LNCaP and MCF-7 cells respectively. It is thought that mitochondrial collapse closely precedes caspase-3/7 activation, and in these investigations, activation of caspase-3/7 was found to occur at 48 h in A2780 and LNCaP cells. In MCF-7 cells, caspase-3/7 activation was observed after 96 h treatment. All three cell lines exhibited different kinetics of caspase-3/7 activation. In STX140- and STX641-treated A2780 cells, caspase-3/7 activation peaked at 72 h (Figure 3B). There was no significant difference in the time course of caspase-3/7 activation between STX140- and STX641-treated A2780 cells. In LNCaP cells, treatment with STX140 or STX641 induced a gradual increase of caspase-3/7 activity that peaks at 72 h.

Although MCF-7 cells are caspase-3 deficient (21), this has been shown to be insufficient to block apoptosis induced by certain antimitotic agents due to compensatory activation of caspase-7 (22). Treatment of MCF-7 cells with 1μM of STX140 or STX641 resulted in significant caspase-7 activation after 48 h, when compared to untreated cells. This elevated level of caspase-7 activity gradually peaks at 120 h, which coincides with the onset of apoptosis (Figure 3C). Although these results do not exclude the possibility of caspase-independent cell death, they implicate its involvement in STX140- and STX641-induced apoptosis, as caspase-3/7 activity correlates with PS exposure on the outer bilipid membrane, both events of classical apoptosis.

STX140 and STX641 induce apoptosis in cancer cells. Apoptosis was measured by detecting PS flip-out onto the outer cell membrane through binding of Annexin V - FITC as determined using flow cytometry. Exposure to 1 μM STX140 and STX641 caused all three cell lines to undergo apoptosis; however each cell line exhibited different kinetics of cell death. The quantification of the (±S.D.) mean of three experiments at 8, 24, 48 and 72 h are represented in Figure 3C. A2780 cells had maximal apoptosis at 48 h, LNCaP cells at 72 h, and MCF-7 cells at 168 h.

Discussion

Previously the efficacy of sulfamoylated estrogen derivatives as anti-angiogenic/microtubule-disrupting agents has been demonstrated in vitro (3, 14). However, the downstream effectors of microtubule disruption are poorly defined and there is a paucity of information on the time course of apoptosis induced by these compounds. In this study, we investigated the differential effects of STX140 and a D-ring C-17 substituted analogue, STX641, on the induction of molecular events that lead up to apoptosis in three separate cell lines. A major finding in this work is that there are surprising differences in the organization and time course of apoptosis in these cell lines. Furthermore, we show novel data demonstrating that this class of compound down-regulates survivin and XIAP expression in ovarian and prostate cancer cells.

Our group has demonstrated that STX140 (2-MeOE2bisMATE) caused irreversible G2-M arrest and Bcl-2 phosphorylation followed by apoptosis in MDA-MB-231 breast cancer cells (14). In the current study, the duration of the G2-M block was measured by immunoblotting for cyclin B1, a regulator of the mitotic checkpoint. Under physiological conditions, cyclin B1 degradation occurs at the metaphase–anaphase transition during mitosis. Microtubule-interacting agents that induce G2-M arrest and prevent cytokinesis have been shown to cause a persistent expression of cyclin B1, which delays passage through the mitotic checkpoint and triggers apoptosis (20). This study shows that A2780 and MCF-7 cells exhibit an altered G2-M arrest profile compared to LNCaP cells in response to treatment with STX140 and STX641. The induction of cyclin B1 protein in A2780 and MCF-7 cells is brief; expression is induced by 8 h and is completely lost by 24 h, whereas LNCaP cells show persistent expression until after 24-48 h. In conjunction with findings that A2780 cells exhibit caspase-3/7 activation and apoptosis before LNCaP and MCF-7 cells, these data could suggest that A2780 cells undergo mitotic slippage, endoreduplication and subsequent DNA damage-stimulated apoptosis that is characteristic of multinuclei formation (23).

The cyclin-dependent kinase inhibitor p21 is induced by multiple stimuli, including DNA damage which stimulates p53-dependent and -independent p21 expression (24). Loss
of p21 expression has been shown to sensitize MCF-7 cells to paclitaxel (25), through interference with paclitaxel-mediated G2-M arrest, allowing inappropriate checkpoint traversal, formation of multi-nucleated cells and subsequent apoptosis. Immunoblotting of p53 and p21 in STX140- and STX641- treated cells also revealed that induction of these proteins is cell line dependent. LNCaP cells possess low levels of functional p53 which were not induced by treatment with STX140 or STX641, although levels of p21 increased after 24 h of exposure in contrast to stabilization of p53 and concomitant up-regulation of p21 in STX140- and STX641-treated MCF-7 cells.

However, STX140 up-regulated both p53 and p21 expression in A2780 cells, but STX641 resulted in p21 induction without p53 stabilization at the 24 h time-point. These findings suggest that apoptosis induced by sulfamolyated 2-methoxyestrogens, at least in A2780 cells, is not dependent on p53 stabilization, as caspase-3/7 activation and annexin V exposure occur without accumulation of intracellular p53. Indeed, in MDA-MB-231 cells which express a mutant p53 variant, STX140 is still efficient at causing apoptosis (6, 14).

Further investigation into the effect of STX140 and STX641 on the expression of anti-apoptotic proteins demonstrated that these compounds down-regulate survivin and XIAP expression in ovarian and prostate, but not breast, cancer cell lines. As both these factors have been shown to be up-regulated in patient tumour samples (26, 27) this finding is of significant importance. The time course of survivin and XIAP down-regulation, at 48 h post-treatment, suggests that as levels of these anti-apoptotic proteins are reduced, cell death ensues. This implies that STX140 and STX641 have a direct effect on the apoptotic machinery of cancer cells. It is interesting that MCF-7 cells do not down-regulate survivin and XIAP expression in response to treatment but still undergo apoptosis. This may be the reason why these cells take over 96 h to die and suggests that survivin and XIAP are not the primary factor in cell death pathways in this cell line. However, further work in this area still needs to be done. The effects of STX140 and STX641 on cancer cells with silenced (siRNA) XIAP and survivin would demonstrate whether these proteins are critical for apoptosis from this treatment.

The time course of STX140- and STX641-induced apoptosis in MCF-7 cells is longer than that of LNCaP and A2780 cells, despite the similarities in their relative efficacies in standard cell proliferation assays (Figure 1). These differences in onset of apoptosis may be attributed to two factors; deregulation of pro- and anti-apoptotic factors in the different cell lines and compromised integrity of the intrinsic apoptotic pathway. In the case of caspase-3-deficient MCF-7 cells (24), caspase-7 undergoes cleavage activation in response to certain antimitotic agents (28). This compensatory activation of caspase-7 does not occur immediately, and may account for the significantly longer time course of STX140- and STX641-induced apoptosis in MCF-7 cells (120 h) compared to A2780 (48 h) and LNCaP (72 h) cells.

The findings presented here are surprising and significant as they demonstrate that the orally bioavailable anti-microtubule compounds STX140 and STX641 are effective at inducing apoptosis via the mitotic checkpoint in cancer cell lines and that the rate of cell death is cell-type dependent. These findings strongly underscore their potential as anticancer therapeutic agents against prostate, ovarian and breast tumours. STX140 is set to enter clinical trials in 2010.

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Disclosure of Conflict of Interest

BVLP, MJR and AP are all consultants for Ipsen Ltd.

References


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