The novel toluidine sulphonamide EL102 shows pre-clinical in vitro and in vivo activity against prostate cancer and circumvents MDR1 resistance

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Background: Taxanes are routinely used for the treatment of prostate cancer, however the majority of patients eventually develop resistance. We investigated the potential efficacy of EL102, a novel toluidine sulphonamide, in pre-clinical models of prostate cancer.

Methods: The effect of EL102 and/or docetaxel on PC-3, DU145, 22Rv1 and CWR22 prostate cancer cells was assessed using cell viability, cell cycle analysis and PARP cleavage assays. Tubulin polymerisation and immunofluorescence assays were used to assess tubulin dynamics. CWR22 xenograft murine model was used to assess effects on tumour proliferation. Multidrug-resistant lung cancer DLKP was used to assess EL102 in a MDR1-mediated drug resistance background.

Results: EL102 has in vitro activity against prostate cancer, characterised by accumulation in G2/M, induction of apoptosis, inhibition of Hif1α, and inhibition of tubulin polymerisation and decreased microtubule stability. In vivo, a combination of EL102 and docetaxel exhibits superior tumour inhibition. The DLKP cell line and multidrug-resistant DLKP variant (which exhibits 205 to 691-fold greater resistance to docetaxel, paclitaxel, vincristine and doxorubicin) are equally sensitive to EL102.

Conclusion: EL102 shows potential as both a single agent and within combination regimens for the treatment of prostate cancer, particularly in the chemoresistance setting.
ablation therapies, eventually their cancer will become refractory and they will succumb to their illness. In the mid-2000s, introduction of taxane-based therapies improved the outcomes of patients with metastatic castrate-resistant prostate cancer, extending survival by several months. The taxane family, which includes paclitaxel, docetaxel and the newly approved cabazitaxel are natural or semi-synthetic plant derivatives that are widely used in the treatment of metastatic castrate-resistant prostate cancer (mCRPC). Their mechanisms of action have been widely reported (Rowinsky et al, 1996; Jackson et al, 2007) and have been shown to act as mitotic arresting agents (Wani et al, 1971; Douros and Saffness, 1981). The dynamic ability of a cell to assemble and disassemble the architecture of the microtubules from and to tubulin components, respectively, is curtailed greatly by the introduction of taxanes (Manfredi and Horwitz, 1984). Phase III trials demonstrated that docetaxel–estramusine combinations conferred median survival advantage of ~3 months compared with the standard mitoxantrone–prednisone combination (Petrylak et al, 2004; Berthold et al, 2008). Since 2010, an additional six drugs have been approved for use in patients with metastatic castrate-resistant prostate cancer. These include drugs targeting androgen receptor activity (abiraterone acetate and enzalutamide), drugs targeting bone metastasis and the microenvironment (denosumab and alaphradin), immunotherapeutics (sipuleucel-T) and new taxanes (cabazitaxel) (Heidegger et al, 2013).

It is postulated that combination treatments of docetaxel with alternative cytotoxic agents could prevent this late-stage resistance, with such other compounds acting in an additive or synergistic fashion. While phase II trials with various combinations of new drugs have suggested promise for emerging docetaxel combination therapies (Oh et al, 2003; Ferrero et al, 2006; Tester et al, 2006; Kikuno et al, 2007; Garcia et al, 2011), of note is the fact that no drug has yet been shown to provide survival benefit when combined with docetaxel in phase III trials (Antonarakis and Eisenberger, 2013). This suggests that there is a need to identify novel compounds for efficacy as single agents or for use in combination with taxane-based therapies.

Here, we present preliminary data on the efficacy of a novel toluidine sulphonamide, EL102, in vitro against prostate cancer cell lines and in an in vivo prostate cancer xenograft mouse model, demonstrating EL102’s ability to work in combination with docetaxel, and circumvent multiple drug resistance mediated by P-glycoprotein (Pgp). EL102 was identified by Elara Pharmaceuticals as a potential chemotherapeutic agent during a screen of novel small molecule inhibitors using the NCI-60 cell line panel assessing for growth inhibition potential. EL102 is a later generation derivative of the family of toluidine sulphonamide hypoxia-induced factor 1 (Hif1α) inhibitors described by (Wendt et al, 2011). This is the first report on the biological actions of EL102 on cancer cells, focusing on its use as an anti-prostate cancer chemotherapeutic.
addition of 50 μl of 1 M NaOH to each well. The plates were read on a dual beam plate reader at 405 nm with a reference wavelength of 620 nm. A percentage viability curve was calculated based on these values and the IC_{50} was determined. Error was presented at ± the percentage coefficient variant (%CV). All cytotoxicity assays were conducted in triplicate.

**Sub-G1 and cell cycle analysis by flow cytometry.** Cells were seeded at a density of 1.3 × 10^5 cells per well in a final volume of 2 ml/well in a six-well plate and left to attach overnight at 37 °C in a 5% CO₂ incubator. Cells were treated with 1 ml of medium spiked with appropriate concentrations of EL102, docetaxel or both. Following treatments, plates were returned to the incubator for 24, 48 and 72 h. The percentage coefficient of variation (%CV). All cytotoxicity assays were conducted in triplicate.

**Tubulin polymerisation assay.** The HST-tubulin polymerization assay kit (Cytoskeleton, Tebu-Bio, Peterborough, UK; #BK004P) was used as per the manufacturer’s instructions. In brief, the assay was performed using a 96-well plate. To each well, with the exception of the blank control, 4 mg/ml of tubulin was added. Each well contained a concentration of the drug of interest and G-PEM was spiked with appropriate concentrations of EL102, docetaxel or both. Following treatments, plates were returned to the incubator for 1 h, cells were then centrifuged at 1000 × g for 5 min using soft acceleration. Each pellet was washed in 500 μl dPBS and the supernatant was discarded. Cells were resuspended in 500 μl ice-cold dPBS (Sigma, #D85377) and transferred to labelled 1.5 ml tubes. Cell pellets were again recovered following centrifugation at 10000 × g and supernatant was discarded. Cells were resuspended in 150 μl dPBS. A volume of 350 μl ice-cold 100% ethanol was added dropwise to the cell suspension while vortexing, to avoid clumping. Cells were incubated on ice for 30 min. Following overnight storage at −20 °C, cells were then centrifuged at 10000 × g for 5 min using soft acceleration. Each pellet was washed in 500 μl dPBS and centrifugation was suspended at 10000 × g for 5 min using soft acceleration, after which supernatant was removed. Each cell pellet was resuspended in propidium iodide, PI/RNAse staining buffer (BD Pharmingen, BD Biosciences, Oxford, England; #550825). Sample suspensions were incubated in the dark for 15–20 min and measured by flow cytometry on BD FACSCanto II (BD Biosciences), channel PE. Logarithmic and linear regression was performed as needed for Sub-G1 and cell cycle analyses. Flow cytometric analyses were conducted using Cytlogic software (CyFlo Ltd, Turku, Finland).

**Western blot analysis.** Cells were seeded in 10-cm² dishes at a cell density of 1 × 10²⁶ per dish, and treated with the relevant doses of EL102 and docetaxel for the required time period. After treatment, cells were rinsed twice with cold PBS and lysed directly on the dish with cold RIPA buffer (Pierce, Fisher Scientific, Dublin, Ireland; #89900) supplemented with protease inhibitors (Pierce, #78410), scraped, and spun at 14 000 g for 15 min at 4 °C. Supernatant was collected and stored at −20 °C for western blot analysis of protein expression. Extracted protein was quantified using a BCA kit (Fisher Scientific, Dublin, Ireland). Both PARP and Hif1α levels were detected through use of primary anti-PARP rabbit polyclonal antibody (Cell Signaling Technology Inc., Danvers, Massachusetts, USA; #9542) and anti-Hif1α rabbit polyclonal antibody (Millipore, Temecula, California, USA; #07–628), respectively. The anti-PARP antibody was diluted 1:1000 and anti-Hif1α antibody was diluted 1:1500 in 5% skimmed milk reconstituted in 1× Tris-buffered saline (TBS) (pH 8) 0.1% Tween. These dilutions were added to the transfer membrane, and shaken overnight at 4 °C, following a 1 h RT blocking in 5% skimmed milk in TBS. Mouse monoclonal anti-β-actin antibody (Thermo-Scientific Pierce, Fisher Scientific, Dublin, Ireland; #10624754) was used to confirm even protein loading. Secondary antibodies used were IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences, Cambridge, UK; #926-32211) and IRDye 680LT goat anti-mouse IgG (LI-COR Biosciences; #926-68020) and detection was imaged on the LI-COR ODYSSEY CLx imaging system.

**Immunocytofluorescence.** Coverslips, pre-sterilised in 100% ethanol, were inserted to the base of each well of a six-well plate. Cells were seeded at a density of 1 × 10²⁶ per well and allowed overnight attachment at 37 °C, in a 5% CO₂ incubator. Cell treatment and fixation was carried out at the relevant time points. Cells were fixed for 10 min in ice-cold methanol. For immunocytofluorescence, primary antibodies against β-tubulin (Abcam, Cambridge, UK #AB6046), acetylated tubulin (Sigma, #T6793), diluted 1:200, and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma; #D4817) diluted 1:100 and coverslips were fixed to slides using nail varnish. Staining was imaged using Delta Vision Core Imaging System C0607 (Applied Precision, Issaquah, WA, USA). Image analysis was conducted using SoftWoRx software (Applied Precision) and FIJI software (GPL v2).

**Statistics.** Data analysis was performed using GraphPad Prism Version 5. All statistical tests were 2-sided, and an association was considered statistically significant with P-values <0.05. The Student’s t-test was used to analyse differences between treatment
groups in cell culture experiments. IC$_{50}$ values were calculated using log (inhibitor) vs normalised response curve ($Y=100/(1+10^{(X-\log IC_{50})})$). For the xenograft model, a one-way ANOVA with Tukey’s multiple comparisons test was used to determine whether there were significant differences in the tumour volumes or body weights between the treatment groups. Additionally, linear regression was used to fit a slope to the tumour growth curve to determine whether the rate of growth differed between the treatment groups.

RESULTS

**EL102 inhibits prostate cancer cell line viability in vitro.** EL102, whose chemical structure is shown in Figure 1A, is a novel toluidine sulphonamide. To determine whether EL102 could have utility as a chemotherapeutic agent in prostate cancer, we determined the effects of increasing doses of EL102 on prostate cancer cell line viability in comparison to the clinically used docetaxel. A panel of four prostate cancer cell lines were used in this study, including CWR22 (androgen receptor (AR)-positive, androgen dependent, non-metastatic), its daughter cell line 22Rv1 (AR-positive, androgen independent, non-metastatic), PC-3 (AR-negative, derived from metastatic bone lesion) and DU145 (AR-negative, derived from metastatic brain lesion). Figures 1B and C demonstrate the effects of increasing doses of EL102 and docetaxel as single agents, respectively, on prostate cancer cell viability in comparison to the clinically used docetaxel. A classic method of chemotherapeutic drug resistance involves the overexpression of drug resistance pump Pgp. We tested EL102 in a poorly differentiated squamous lung carcinoma cell line pair: DLKP and its doxorubicin-selected variant DLKPA (Glynes et al, 1992). Table 2 shows that DLKPA is cross-resistant to the taxanes, docetaxel (253-fold) and paclitaxel (258-fold). Its utility as a chemotherapeutic agent in prostate cancer, we determined the effects of increasing doses of EL102 on prostate cancer cell line viability.

| Cell line    | Docetaxel (nM) IC$_{50}$ ± s.d. | EL102 (nM) IC$_{50}$ ± s.d. |
|--------------|---------------------------------|-----------------------------|
| CWR22        | 0.4 ± 0.01                      | 24.0 ± 1.41                 |
| 22Rv1        | 0.6 ± 0.15                      | 21.7 ± 2.31                 |
| DU145        | 1.5 ± 0.18                      | 40.3 ± 7.71                 |
| PC-3         | 3.8 ± 0.76                      | 37.0 ± 2.00                 |

Table 1. Prostate cancer cell line inhibition by docetaxel and EL102

| Protein       | DLKP IC$_{50}$ ± s.d. | DLKPA IC$_{50}$ ± s.d. | Fold change |
|---------------|-----------------------|------------------------|-------------|
| Adriamycin    | 24 ± 2                | 4900 ± 300             | 204         |
| Docetaxel     | 0.15 ± 0.04           | 38 ± 3.0               | 253         |
| Paclitaxel    | 1.2 ± 0.5             | 310 ± 25               | 258         |
| EL102         | 14.4 ± 0.8            | 16.3 ± 1.2             | 1.1         |
| Vincristine   | 0.91 ± 0.1            | 629 ± 160              | 691         |

Table 2. Cross-resistance profile of DLKP and DLKPA

Figures 1B and C demonstrate the effects of increasing doses of EL102 and docetaxel as single agents, respectively, on prostate cancer cell line viability over a 3-day drug exposure. This demonstrates that while docetaxel is more potent than EL102, both EL102 and docetaxel decrease prostate cancer cell viability in a dose-dependent manner. Table 1 shows that CWR22 and 22Rv1 are equally sensitive to docetaxel (IC$_{50}$ 0.4–0.6 nM), while bone metastatic cell line, PC-3, is 2.5–10 fold more resistant to docetaxel than the other cell lines (IC$_{50}$ 3.8 nM). EL102 inhibited cell proliferation with an IC$_{50}$ of ~21–40 nM. By comparison, bone metastatic PC-3 cells were twofold more resistant than CWR22 and 22Rv1 to EL102, and were equally as sensitive as brain metastatic cell line DU145.

Cell lines with MDR1-mediated drug resistance are sensitive to EL102. A classic method of chemotherapeutic drug resistance involves the overexpression of drug resistance pump Pgp. We tested EL102 in a poorly differentiated squamous lung carcinoma cell line pair: DLKP and its doxorubicin-selected variant DLKPA (Glynes et al, 1992). Table 2 shows that DLKPA is cross-resistant to the taxanes, docetaxel (253-fold) and paclitaxel (258-fold). Its utility as a chemotherapeutic agent in prostate cancer, we determined the effects of increasing doses of EL102 on prostate cancer cell line viability.

![Figure 1. Impact of EL102 and docetaxel on prostate cancer cell line viability in vitro.](image-url)

(A) Chemical structure of EL102. (B) Dose response effects of EL102 on prostate cancer cell line viability over 72-h exposure. (C) Dose response effects of docetaxel on prostate cancer cell line viability over 72-h exposure. (D) Effect of EL102 on doxorubicin and docetaxel-resistant DLKPA lung cancer cell line viability vs DLKP parental lung cancer cell line. (E) Comparison of docetaxel sensitivity in the doxorubicin and docetaxel-resistant DLKPA lung cancer cell line viability vs DLKP parental lung cancer cell line.

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EL102 pre-clinical anti-prostate cancer activity

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Figure 2. Impact of EL102 and docetaxel alone and in combination on CWR22 xenograft tumour volume. (A) Effect of vehicle vs 12 mg kg⁻¹ docetaxel, vs 12 mg kg⁻¹ EL102, vs 15 mg kg⁻¹ EL102, vs 12 mg kg⁻¹ docetaxel plus 12 mg kg⁻¹ EL102, vs 12 mg kg⁻¹ docetaxel plus 15 mg kg⁻¹ EL102, on CWR22 tumour volume using a 5-day on/2-day off schedule (tumour volume (cm³) ± s.e.m.). (See Supplementary Table 1 for one-way ANOVA comparing tumour volume at each time point. (B) Effect of vehicle vs 12 mg kg⁻¹ docetaxel, vs 12 mg kg⁻¹ EL102, vs 15 mg kg⁻¹ EL102, vs 12 mg kg⁻¹ docetaxel plus 12 mg kg⁻¹ EL102, vs 12 mg kg⁻¹ docetaxel plus 15 mg kg⁻¹ EL102, on mouse body weight (Note: vehicle group killed on day 24 due to tumour size). (See Supplementary Table 2 for one-way ANOVA comparing body weight at each time point.)

EL102 potentiates the effects of docetaxel in vivo. To determine whether EL102 could be used in combination with docetaxel in vivo, we examined the ability of the combination of docetaxel with EL102 to inhibit tumour growth in a CWR22 xenograft mouse model (Figure 2A). While administration of 12 mg kg⁻¹ EL102 using a 5-day on/2-day off regimen did not significantly inhibit rate of tumour growth compared with vehicle (slope (R²): vehicle 0.1414 ± 0.01438 (0.9603) vs EL102 12 mg kg⁻¹, 0.1210 ± 0.01179 (0.9462), F-test: P = 0.3385), increasing the dosage to 15 mg kg⁻¹ EL102 did inhibit the rate of tumour growth compared with vehicle (slope (R²): vehicle 0.1414 ± 0.01438 (0.9603) vs EL102 15 mg kg⁻¹, 0.08451 ± 0.006934 (0.9612), F-test: P = 0.003).

Administration of 12 mg kg⁻¹ docetaxel decreased the rate of tumour growth more efficiently than EL102 (slope (R²): vehicle 0.1414 ± 0.01438 (0.9603) vs docetaxel 12 mg kg⁻¹, 0.04230 ± 0.002531 (0.9688), F-test: P < 0.0001), while the combination of both drugs had the largest effect on inhibition of tumour growth, suggesting that these drugs work well together in combination in vivo (slope (R²): vehicle 0.1414 ± 0.01438 (0.9603) vs docetaxel 12 mg kg⁻¹ and EL102 12 mg kg⁻¹, 0.01533 ± 0.0008838 (0.9709), F-test: P < 0.0001 or vehicle, 0.1414 ± 0.01438 (0.9603) vs docetaxel 12 mg kg⁻¹ and EL102 15 mg kg⁻¹, 0.01537 ± 0.001704 (0.9003), F-test: P < 0.0001). Comparison of the docetaxel arm vs the combination arms showed a significant difference in the rate of tumour growth indicating that the addition of EL102 to docetaxel improves anti-tumour activity (F-test, P < 0.0001). Supplementary Table 1 describes the results of a one-way ANOVA test on this model, using a Tukey’s post-hoc test to assess statistical difference in tumour volume between the treatment arms at different time points. Additionally to determine if combining EL102 and docetaxel was well tolerated by the mice with minimal adverse effects, we compared changes in mean body weight between the treatment arms and found no significant differences between the groups compared with vehicle or between different treatment arms (Figure 2B). Supplementary Table 2 describes the results of a one-way ANOVA test on this model, using a Tukey’s post-hoc test to assess statistical difference in body weights between the treatment arms at different time points.

EL102 is cytotoxic to prostate cancer cell lines and induces cellular apoptosis. As demonstrated in Figure 1B and Table 1, EL102 is a potent inhibitor of prostate cancer cell viability, and when combined with docetaxel in vivo inhibits tumour growth to a greater extent than either alone (Figure 2A). In an attempt to further address the mechanisms driving the combination of the two agents we performed an in vitro combination assay looking at the impact of the combining EL102 and docetaxel on cell viability (Figure 3). Results show that in vitro, combining EL102 and docetaxel does not have an additive effect on inhibition of cell viability. To determine whether these effects were cytostatic or cytotoxic, we quantified the number of cells in subG1 phase indicating loss of cellular DNA and entry into late apoptosis using logarithmic scale propidium iodide flow cytometry (Figure 4). Cells were exposed to increasing doses of EL102 and docetaxel. EL102 was an equally strong inducer of apoptosis at 100 nM in all four prostate cancer cell lines, while it failed to induce apoptosis at 10 nM of EL102 (Figure 4A–D), despite inhibiting cell viability by approximately 25–30% at 10 nM (Figures 3A–D). Apoptosis was detectable at 24 h and steadily increased over the next 48 h (72 h total), indicating that EL102-dependent inhibition of cell viability is partially due to cytotoxic effects, namely induction of apoptosis. Similarly, docetaxel induced apoptosis in all 4 cell lines in a dose-dependent and temporal manner. When EL102 and docetaxel were administered in combination in vitro, no additive effects were seen on the levels of apoptosis in these cell lines (Figure 4), similar to the cell viability assays (Figure 3). Of note though is that while 10 nM of EL102 failed to induce increased apoptosis (Figure 4), it did lead to significantly decreased % cell viability compared to control (Figure 3) in each cell line, indicating non-apoptosis effects at low concentrations. Figures 5A–D shows representative histograms from these experiments in the DU145 prostate cancer cell line. In addition to demonstrating an increase in subG1 accumulation, the histograms indicated that combining the agents altered the cell cycle dynamics. These effects in DU145 are quantified at 24 (Figure 5E), 48 (Figure 5F) and 72 (Figure 5G) h, and demonstrate that combining EL102 and docetaxel causes greater loss of cells from G1 and accumulation in G2/M than either alone by 24 h at low doses. Also of interest in the combination assays cell profile images is a peak beyond the G2/M peak which
represents a subset of cells with increased DNA content (8X). Apoptosis induction upon in vitro EL102 and docetaxel administration was further evidenced by detection of PARP cleavage in protein extracted from DU145 cell lysate, 24 and 48 h post-treatment. PARP cleavage increases in a dose-dependent manner and over time with the strongest detection seen in lysates of cells cultured with dual treatments at 48 h (Figure 4E).

**EL102 has both cytostatic and cytotoxic effects.** Figure 5 indicated that EL102 may cause accumulation of cells in G2/M. To further quantify the accumulation of cells in the various phases of the cell cycle after exposure to EL102 we performed linear scale propidium iodide flow cytometry. Figure 5 shows that EL102 causes loss of cells in G1, and accumulation of cells in G2/M within 24 h. This is accompanied by an increase in the number of cells in subG1, indicating that EL102 has both cytostatic and cytotoxic effects. By 72 h, the majority of cells have entered apoptosis as indicated by accumulation in subG1, and the decrease in the number of cells in G2/M. Additionally, we again observed a peak beyond G2/M which may represent a subset of cells which advanced through the cell cycle with incomplete cell division.

**EL102 inhibits tubulin polymerisation and microtubule formation.** The cytotoxic activity of taxanes is exerted by promoting and stabilizing microtubule assembly, while preventing physiological microtubule depolymerisation. To determine the effects of EL102 on taxane induced microtubule assembly, we examined the effects of docetaxel and EL102 on the rate of tubulin polymerisation (Figure 6). As expected docetaxel increased the rate of tubulin polymerisation compared with control untreated tubulin. In contrast, EL102 exhibited a decreased rate of polymerisation compared with control, indicating that EL102 may be an inhibitor of tubulin polymerisation. We also examined the effect of combining EL102 with docetaxel on tubulin polymerisation rates, which resulted in inhibition of docetaxel induced tubulin polymerisation to levels of inhibition similar to EL102 alone, suggesting that these drugs may antagonise each other with respect to their effects on tubulin polymerisation. To connect tubulin polymerisation in a cell-free system to effects on mitosis, we have performed immunofluorescence assays of β-tubulin and acetylated tubulin in DU145 to visualise the microtubules and examine the effects of EL102, docetaxel and combination of both (Figure 7). The data shows an increase in the expression of β-tubulin and acetylated tubulin in response to docetaxel, while EL102 causes a reduction in acetylated tubulin. The combination of EL102 and docetaxel caused a marked change in the distribution of acetylated tubulin becoming increasingly disorganised consistent with a destabilising effect. This coupled with the cell cycle analysis showing loss of cells from G1 and accumulation in G2/M at 24 h post treatment (Figure 8) and induction of apoptosis does suggest that microtubule destabilisation is responsible in part for the cytotoxic effects of EL102.

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**Figure 3. Impact of EL102 and docetaxel combination treatment on prostate cancer cell line viability in vitro.** Effect of EL102 and docetaxel in combination of in vitro cell viability after 72 h in (A) CWR22, (B) 22Rv1, (C) PC-3 and (D) DU145 prostate cancer cell. (See Supplementary Table 3 for results of one-way ANOVA comparing cell viability between each treatment.)
EL102 inhibits Hif1α protein expression. EL102 is a later generation derivative of the family of toluidine sulphonamide designed to inhibit Hif1α described by (Wendt et al., 2011). We therefore examined the ability of EL102 to inhibit Hif1α in prostate cancer cells (Figure 9). In normoxia, EL102 modestly inhibited Hif1α expression at 50 and 100 nM, but had no effect at 10 nM. We then used cobalt chloride to artificially induce hypoxia-increasing Hif1α expression, and found that EL102 decreased Hif1α at as little as 10 nM.

**DISCUSSION**

We have established the potential of a novel toluidine sulphonamide EL102 as a potential broad spectrum anti-prostate cancer therapeutic agent. We found that prostate cancer cell lines were sensitive to EL102 at an IC50 range of 20–40 nM. Our metastatic prostate cancer cell lines PC-3 and DU145, which are both AR negative and represent castrate-resistant metastatic disease, are equally responsive to EL102. The AR-positive cell lines CWR22 and 22Rv1 are twofold more sensitive to EL102 than the metastatic DU145 and PC-3 cell lines.

EL102 is a next-generation derivative of the prototype toluidine sulphonamide compound 1 Hif1α inhibitor (Wendt et al., 2011). EL102 was identified as a potential chemotherapeutic agent during a screen of compound 1-derived novel small molecule inhibitors using the NCI-60 cell line panel assessing for growth inhibition potential (not shown). Therefore, we assessed its efficacy for use in the treatment of prostate cancer as a single agent and in combination with the clinically available docetaxel. Docetaxel is a member of the taxane family and is approved for use in prostate cancer patients with castrate-resistant metastatic disease, having been found to provide a modest increase in median survival time.
Figure 5. Cell cycle analysis of DU145 cell accumulation in G1, S, G2/M and subG1 after EL102, docetaxel or combination treatment. Cell cycle profiles of DU145 cells, with markers indicating the G1, S, G2/M and subG1 cells, after exposure to (A) 10 nM EL102 or 1 nM docetaxel, or 100 nM EL102 and 1 nM docetaxel, (B) 100 nM EL102 or 10 nm docetaxel, or 10 nm EL102 and 10 nm docetaxel, (C) 100 nM EL102 or 1 nm docetaxel, or 100 nm EL102 and 1 nm docetaxel, (D) 100 nm EL102 or 10 nm docetaxel, or 100 nm EL102 and 10 nm docetaxel for 24, 48 and 72 h. Graphs of cell cycle analysis of accumulation of DU145 in the G1, S, G2/M and sub-G1 phase in response to 0, 10 and 100 nm EL102, 1 and 10 nm docetaxel and combinations of each at (E) 24, (F) 48, (G) 72 h as measured by propidium iodide flow cytometry.

When used in combination with prednisone, compared with mitoxantrone and prednisone (19.2 months vs 16.3 months median survival) in the TAX327 trial (Berthold et al, 2008), and when in combination with extramustine compared with mitoxantrone and prednisone (17.5 months vs 15.6 months median survival) in the SWOG9912 trial (Petrylak et al, 2004). Until the approval of six new agents in the last 3 years, docetaxel had been the standard of care in the castrate-resistant metastatic setting. Attempts to combine docetaxel with other agents have been largely unsuccessful in terms of efficacy and side-effects (Antonarakis and Eisenberger, 2013).

We observed that EL102 is a cytotoxic agent and also displays cytostatic properties, through flow cytometric analysis of PI-stained cells cultured for 24, 48 and 72 h, following treatment. This was evidenced by the increased number of cells seen in subG1 and G2/M phase of cell cycle, demonstrating that EL102 induces apoptosis and causes G2/M arrest, preventing the cell from entering into mitosis. Further investigation showed that EL102 inhibited tubulin polymerisation and caused destabilisation of the microtubules in DU145 prostate cancer cells. Induction of apoptosis, following 24 and 48 h EL102 treatment was confirmed through western blot analysis of PARP cleavage. Additionally, we found that EL102 decreased Hif1α expression in normoxia and hypoxia in vitro, indicating an additional mechanism of action to microtubule destabilisation. Future studies will explore in depth the ability of EL102 to inhibit cell migration and invasion in vitro and inhibit a PC-3 xenograft model of bone metastasis, given the role of microtubules in cell polarisation and cell invasion. We will also explore the impact of Hif1α inhibition by EL102 in PC-3 xenograft mouse models and its subsequent effects on the expression of hypoxia-inducible genes, which regulate several key biological processes, including cell proliferation, angiogenesis, metabolism, apoptosis, immortalisation and migration, essential for tumour progression (Harris, 2002).

Several clinical trials have been conducted recently exploring the potential of neoadjuvant chemotherapy in patients with high-risk localised prostate cancer (Womble et al, 2011; Narita et al, 2012; Ross et al, 2012). The results of these trials suggest a benefit to patients in terms of reductions in tumour volume and PSA levels (Womble et al, 2011; Ross et al, 2012). Given the equal sensitivity...
of AR-positive CWR22 and 22Rv1 to EL102 despite their different sensitivity to androgen, this suggests that EL102 could potentially be used in a castrate sensitive setting before the development of hormone resistance. To further investigate this, we postulated that CWR22 cells would respond to EL102 as single agent. This was confirmed in the CWR22 prostate xenograft model.

As mentioned previously attempts to combine docetaxel with other agents have been largely unsuccessful (Antonarakis and Eisenberger, 2013). In this study, our in vivo investigations found that the combination of EL102 and docetaxel decreased tumour proliferation of CWR22 xenograft to a great extent than either drug alone. The combination of docetaxel and EL102 significantly decreased tumour growth to a greater extent than either alone in a xenograft model of CWR22. While combining the drugs in vitro doesn’t have an additive effect on induction of apoptosis, it appears to increase the loss of cells from G1 and accumulation in G2/M than either drug alone suggesting the combination may increase cytostatic effects. Additionally combining EL102 and docetaxel, one essentially a tubulin polymerisation stabiliser and the other a tubulin polymerisation stabiliser had an antagonistic effect resulting initially in a slower rate of initial polymerisation followed by inhibition of further polymerisation. Future studies will examine whether EL102’s ability to inhibit Hif1α in hypoxic tumours contributes to the observed effects of the combination. Possible downstream effects of Hif1α inhibition include inhibition of angiogenesis and metastasis.

There is no current cure for castrate-resistant metastatic prostate cancer. Novel adjuvant chemotherapies are continually being developed to address this, with the approval of six new agents since 2010. Recently, clinical trials involving next-generation taxane, cabazitaxel in combination with abiraterone acetate, have begun recruiting patients with preliminary findings expected in 2015. Cabazitaxel is a microtubule-stabilising agent, and is effective in treating patients that have become resistant to docetaxel treatment through overexpression of Pgp (O’Neill et al, 2011; Zhang et al, 2012), as cabazitaxel is not a substrate for Pgp (Mita et al, 2009). Abiraterone functions through disruption of critical steps of androgen formation by direct inhibition of CYP17 activity. This results in reduced levels of circulating androgen and slower progression of prostate cancer in castrate-resistant patients (O’Donnell et al, 2004; Agarwal et al, 2010). Thus, combining

![Figure 6. Impact of EL102 and docetaxel alone and in combination on tubulin polymerisation activity.](image)

![Figure 7. Effect of EL102 on microtubule destabilisation.](image)
cabazitaxel and abiraterone acetate, allows us to target multiple pathways in mCRPC, while also eliminating Pgp mediated drug resistance. This lends further credence to the argument for introducing novel compounds, such as EL102 which has mechanisms distinct from the mainstay therapies that may work synergistically.

Interestingly, we also found that EL102 overcame Pgp-mediated resistance in the Pgp overexpressing lung cancer cell lines DLKPA, which is cross-resistant to doxorubicin, paclitaxel, docetaxel and vincristine (Clynes et al., 1992). While Pgp is an important mechanism of drug resistance in prostate cancer, it is not the only one. Other mechanisms of resistance include altered growth factor receptor pathway activation (e.g., IGFR, VEGFR, EGFR), hypoxia-related resistance, tubulin mutation and altered tubulin isoform expression, and upregulation of other drug pumps in addition to MDR1 (e.g. BCRP, MRP1, MDR2) and NFκB activation (O’Neill et al., 2011; Seruga et al., 2011; Zhang et al., 2012).

In summary, we present data on the efficacy of EL102 as a novel chemotherapeutic agent with potential for the treatment of prostate cancer. We show that EL102 is active in both castration-sensitive and castration-resistant prostate cancer cell lines. EL102 enhances the potency of docetaxel in a xenograft model of the CWR22 prostate cancer. Finally, EL102 is not a substrate for Pgp-mediated drug resistance, indicating that it may be of use in a chemotherapy refractory setting.

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