

STX2171, a 17 β -hydroxysteroid dehydrogenase type 3 inhibitor, is efficacious *in vivo* in a novel hormone-dependent prostate cancer model

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Abstract

17 β -Hydroxysteroid dehydrogenases (17 β -HSDs) catalyse the 17-position reduction/oxidation of steroids. 17 β -HSD type 3 (17 β -HSD3) catalyses the reduction of the weakly androgenic androstenedione (adione) to testosterone, suggesting that specific inhibitors of 17 β -HSD3 may have a role in the treatment of hormone-dependent prostate cancer and benign prostate hyperplasia. STX2171 is a novel selective non-steroidal 17 β -HSD3 inhibitor with an IC₅₀ of ~200 nM in a whole-cell assay. It inhibits adione-stimulated proliferation of 17 β -HSD3-expressing androgen receptor-positive LNCaP(HSD3) prostate cancer cells *in vitro*. An androgen-stimulated LNCaP(HSD3) xenograft proof-of-concept model was developed to study the efficacies of STX2171 and a more established 17 β -HSD3 inhibitor, STX1383 (SCH-451659, Schering-Plough), *in vivo*. Castrated male MF-1 mice were inoculated s.c. with 1 \times 10⁷ cells 24 h after an initial daily dose of testosterone propionate (TP) or vehicle. After 4 weeks, tumours had not developed in vehicle-dosed mice, but were present in 50% of those mice given TP. One week after switching the stimulus to adione, mice were dosed additionally with the vehicle or inhibitor for a further 4 weeks. Both TP and adione efficiently stimulated tumour growth and increased plasma testosterone levels; however, in the presence of either 17 β -HSD3 inhibitor, adione-dependent tumour growth was significantly inhibited and plasma testosterone levels reduced. Mouse body weights were unaffected. Both inhibitors also significantly lowered plasma testosterone levels in intact mice. In conclusion, STX2171 and STX1383 significantly lower plasma testosterone levels and inhibit androgen-dependent tumour growth *in vivo*, indicating that 17 β -HSD3 inhibitors may have application in the treatment of hormone-dependent prostate cancer.

Key Words

- ▶ prostate
- ▶ cancer
- ▶ androgen
- ▶ 17 β -hydroxysteroid dehydrogenase (17 β -HSD)
- ▶ inhibition

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Introduction

Treatment of hormone-dependent prostate cancer by androgen ablation is usually initially successful, reducing primary tumour burden and increasing 5-year survival rates. Currently, androgen ablation is achieved using orchidectomy, androgen receptor (AR) blockers, LH-releasing hormone agonists or 5 α -reductase inhibitors. Despite the success of these early-stage treatments, prostate cancer is the second highest cause of male cancer-related death in the UK, as tumours often present at an advanced, apparently androgen-independent, stage in older men, having gone unnoticed for several years. However, studies now indicate that almost all advanced prostate cancers are not androgen-independent, and, instead, have developed apparent independence via changes in AR signalling, such as AR up-regulation or mutation, or co-regulator modification, allowing activation by either the low levels of androgen present during ablation therapy or other ligand (Mizokami *et al.* 2004, Rau *et al.* 2005, Pienta & Bradley 2006). High levels of testosterone and 5 α -dihydrotestosterone (DHT) have been found in the prostate of patients with recurrence during ablation therapy, suggesting that surgical or chemical castration treatments may not completely inhibit androgen formation, and that *in situ* testosterone formation from adrenal androgens may continue (Titus *et al.* 2005).

17 β -Hydroxysteroid dehydrogenases (17 β -HSDs) catalyse the reduction or oxidation of hormones, fatty acids and bile acids, regulating their binding activities. Fifteen 17 β -HSDs have been identified to date (Jansson *et al.* 2006, Lukacik *et al.* 2006, Luu-The *et al.* 2008), all requiring NAD(P)(H) for activity (Shi & Lin 2004). All but one, 17 β -HSD type 5 (17 β -HSD5), an aldo-keto reductase (Penning *et al.* 2000), are short-chain dehydrogenases/reductases (Peltoketo *et al.* 1999, Duax *et al.* 2005, Vihko *et al.* 2006). *In vivo*, the 17 β -HSDs appear to function unidirectionally (Luu-The *et al.* 1995, Khan *et al.* 2004), despite having bidirectional capabilities *in vitro*, dependent on NAD(P)⁺ or NAD(P)H availability.

17 β -HSD3 specifically converts the weakly androgenic adione (Laplante & Poirier 2008) to active circulating testosterone (Fig. 1) in the presence of NADPH. The importance of 17 β -HSD3 in testosterone production in men is exemplified by the autosomal recessive disorder male pseudohermaphroditism, where there is defective 17 β -HSD3 expression (Geissler *et al.* 1994). Affected individuals have a high plasma adione-to-testosterone ratio and are usually brought up as females (Andersson & Moghrabi 1997).



Figure 1

Interconversion of adione and testosterone by 17 β -HSD3 and 17 β -HSD2.

Although 17 β -HSD3 is expressed almost exclusively in testis microsomes (Geissler *et al.* 1994, Luu-The *et al.* 1995), there have been some reports of its expression in other tissues, such as blood platelets and megakaryocytes (Gnatenko *et al.* 2005), and its up-regulation in tumours of the prostate. Expression of 17 β -HSD3 mRNA increased over 30-fold in prostate tumour biopsies in one report (Koh *et al.* 2002), with a corresponding decrease in 17 β -HSD2 mRNA expression, indicating that the reductive formation of testosterone is favoured, and was up-regulated in an AR-positive prostate cell line, LNCaP, after a 48-h treatment with dutasteride (Biancolella *et al.* 2007), a 5 α -reductase 1 and 2 inhibitor. An increased risk of prostate cancer is conferred by a polymorphism in the *HSD17B3* gene (Margiotti *et al.* 2002).

Decreasing the formation of testosterone by the inhibition of 17 β -HSD3 should provide an effective treatment for hormone-dependent prostate cancer. Hormonal treatments acting upstream of the 17 β -HSD3-catalysed reaction can affect the systemic balance of other hormones, causing undesirable adverse effects including loss of sexual interest, function and bone mineral density. However, because of its unique expression profile and substrate specificity, use of 17 β -HSD3 inhibitors may result in fewer side effects (Day *et al.* 2008b). Furthermore, in castrate-resistant prostate cancer (CRPC), *de novo* androgen synthesis plays a key role in LNCaP xenograft growth (Locke *et al.* 2008), and human CRPC exhibits up-regulated 17 β -HSD3 expression compared with hormone-resistant prostate cancer (Montgomery *et al.* 2008).

STX2171 is a 17 β -HSD3 inhibitor with an IC₅₀ of ~200 nM in a whole-cell assay (Day *et al.* 2009b, Vicker *et al.* 2009). *In vitro*, it has been shown to be selective for 17 β -HSD3 over 17 β -HSD2 inhibition, non-androgenic with low toxicity and to inhibit adione-stimulated proliferation of prostate cancer cells *in vitro* that overexpress 17 β -HSD3. Here, we show that it also has efficacy *in vivo*, both in the inhibition of testosterone formation in intact male mice, and in a

novel adione-stimulated castrated nude mouse prostate cancer xenograft model, inhibiting both tumour growth and plasma testosterone levels.

Materials and methods

Candidate 17 β -HSD3 inhibitor compounds

STX2171 synthesis has been reported (Vicker *et al.* 2009). Its purity (>95%) was confirmed by HPLC. STX1383, previously identified by Schering-Plough (Kenilworth, NJ, USA) as a potential 17 β -HSD3 inhibitor (SCH-451659, Guzi *et al.* 2004), was synthesised as described previously. The structures of STX2171 and STX1383 are shown in Fig. 2A.

Cell culture

LNCaP, T47D, MDA-MB-231 and MLTC-1 cells (LGC Promochem, London, UK) were all cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids and 0.075% sodium bicarbonate (all from Sigma–Aldrich Company Ltd.). PC-3 cells (LGC Promochem) were grown in DMEM/Ham's F12 medium (Sigma–Aldrich Company

Ltd.) supplemented with 10% FBS and 2 mM L-glutamine. 293-EBNA (Invitrogen Ltd) and LNCaP cells which had been previously transfected and selected for stable expression of the pCEP4.17 β -HSD3 plasmid (Day *et al.* 2009b), 293-EBNA(HSD3) and LNCaP(HSD3), respectively, were cultured in the LNCaP medium above, and in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 0.075% sodium bicarbonate and 250 μ g/ml of G418 (Promega) respectively. Both media were additionally supplemented with 250 μ g/ml of hygromycin B (Invitrogen) to maintain the selection of the transfected cells. All cells were maintained at 37 °C in a humidified atmosphere at 5% CO₂.

17 β -HSD activity assays

For the 17 β -HSD3 assay, 293-EBNA(HSD3) cells and MLTC-1 mouse Leydig tumour cells were plated at 50 000 cells/well in 24-well tissue culture plates in medium free of the antibiotics G418 or hygromycin B 48 h before the assay. The activity of the cells in the presence or absence of STX2171 and STX1383 was determined as described previously (Day *et al.* 2009b), and the inhibitory IC₅₀ values determined.

The 17 β -HSD1 and 17 β -HSD2 inhibitory activities of the two compounds were determined using T47D and MDA-MB-231 human breast cancer cells, respectively, as described previously (Day *et al.* 2009b).

Proliferation assay

The growth inhibitory IC₅₀ for STX1383 and STX2171 in LNCaPwt and PC-3 prostate cancer cells plated at a density of 3500 cells/well was determined as described previously (Stengel *et al.* 2010). A known anti-proliferative compound, STX140 (Leese *et al.* 2006, Day *et al.* 2009a), was included in the assay as a positive control. All experiments were performed in triplicate.

RT-PCR analysis

Cells in T75 flasks or six-well plates at ~80% confluency were homogenised using the QIAshredder kit (Qiagen); and excised tumour tissue samples (30 mg) were homogenised in 600 μ l RNeasy RLT buffer (Qiagen) and centrifuged for 3 min at 3000 g mRNA was purified using the RNeasy kit (Qiagen) and stored at –80 °C. cDNA was formed from a 2 μ g aliquot of each mRNA sample using the High Capacity cDNA RT kit (Applied Biosystems) and stored at –20 °C. RT-PCRs were performed in a Rotor Gene 2000 Real-Time Cycler (Corbett Life Science, Sydney,

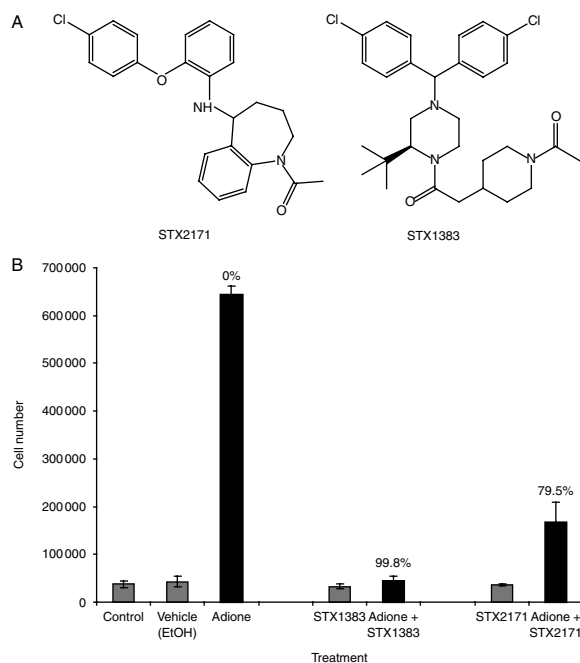


Figure 2 17 β -HSD3 inhibitors and their effects in the *in vitro* proof-of-concept model. (A) Structures of STX2171 and STX1383. (B) Effects of 500 nM STX1383 or STX2171 in the 15-day 10 nM adione-stimulated LNCaP(HSD3) *in vitro* proof-of-concept assay.

Australia) using 1 μ l cDNA in Taqman universal PCR master mix and Taqman expression assays containing primers and probes for 17 β -HSD3 or AR, and for the endogenous control gene, *RPLP0* (Applied Biosystems). The conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Relative mRNA expression was calculated using the comparative quantitation algorithm in Rotor Gene 6 Software (Corbett Life Science).

In vitro adione-stimulated model

The effect of the compounds on the adione-stimulated proliferation of LNCaP(HSD3) cells was determined as described previously (Day *et al.* 2009b).

In vivo studies

All animals received food and water *ad libitum* and were maintained in positive pressure isolators under a 12 h light:12 h darkness cycle. The experiments were approved by the Imperial College London Ethical Review Committee and comply with UK Home Office regulations.

Establishing an *in vivo* proof-of-concept model: inhibition of adione-stimulated LNCaP(HSD3) tumour proliferation in castrated mice

Castrated male MF-1 nude mice (Harlan UK Ltd., Wyton, Cambridgeshire, UK) were inoculated s.c. with 1×10^7 LNCaP(HSD3) cells in 50 μ l Matrigel (BD Biosciences, Oxford, UK) into one flank 24 h after an initial daily dose of either 100 μ g/50 μ l testosterone propionate (TP) in 4% ethanol/96% propylene glycol (PG) (s.c.) or vehicle alone. When tumours treated with TP were 50–100 mm³, the tumour-bearing TP-dosed animals were separated into four groups, and animals in group 1 continued on the daily 100 μ g/50 μ l TP dosing regimen for the remainder of the study, while TP dosing was discontinued in the other three groups. For the following week, these three groups were instead given 100 μ g adione/50 μ l vehicle (s.c. daily). After this week, group 2 continued to receive the daily 100 μ g adione/50 μ l vehicle (s.c.) dosing regimen, whereas group 3 received vehicle alone (s.c. daily), and group 4 received 100 μ g adione/50 μ l vehicle plus 20 mg/kg per day STX2171 in a combined s.c. injection ($n=6-7$ per group). A schematic of the dosing protocol is shown in Fig. 3A. Every 7 days, tumour measurements were recorded using electronic callipers and mice were weighed. Tumour volume was calculated using the formula: volume=(length \times width²)/2. After 4 further weeks, final body weights and tumour sizes were determined, blood was taken by cardiac puncture 1–2 h

after administration of the final dose; mice were culled and tumour samples were taken.

Comparative efficacy of STX2171 and STX1383 in the *in vivo* proof-of-concept model

LNCaP(HSD3) xenografts were established in castrated male MF-1 mice using TP as above. When tumours measured 50–100 mm³, mice received 100 μ g adione/50 μ l vehicle (s.c. daily) for 1 week. For the following 5 weeks, group 1 continued to receive adione, group 2 received adione plus 20 mg/kg per day STX2171 (s.c.) and group 3 received adione plus 20 mg/kg per day STX1383 (s.c.) ($n=6-7$ per group). Tumour measurements and mouse weights were recorded weekly. After 5 weeks, final body weights and tumour sizes were determined, blood was taken by cardiac puncture 1–2 h after administration of the final dose; mice were culled and tumour samples taken.

Intact mouse study In a 12-day study, intact male MF-1 nude mice (Harlan UK Ltd.) were dosed with vehicle, or with 20 mg/kg per day STX2171 or STX1383 in 4% ethanol/96% PG s.c. daily ($n=5$ per group) on days 1–5 and 8–12. On day 12, 1 h after administration of the final dose, blood samples were taken and the animals were culled.

Plasma testosterone concentration Cardiac puncture blood was collected into heparinised tubes and centrifuged at 13 000 r.p.m. for 1 min. Plasma was stored at –20 °C until assay. Testosterone levels were measured using the Active Testosterone coated-tube RIA ¹²⁵I kit (DSL-4000, Beckman Coulter, High Wycombe, Buckinghamshire, UK), according to the manufacturer's instructions (50 μ l sample/assay).

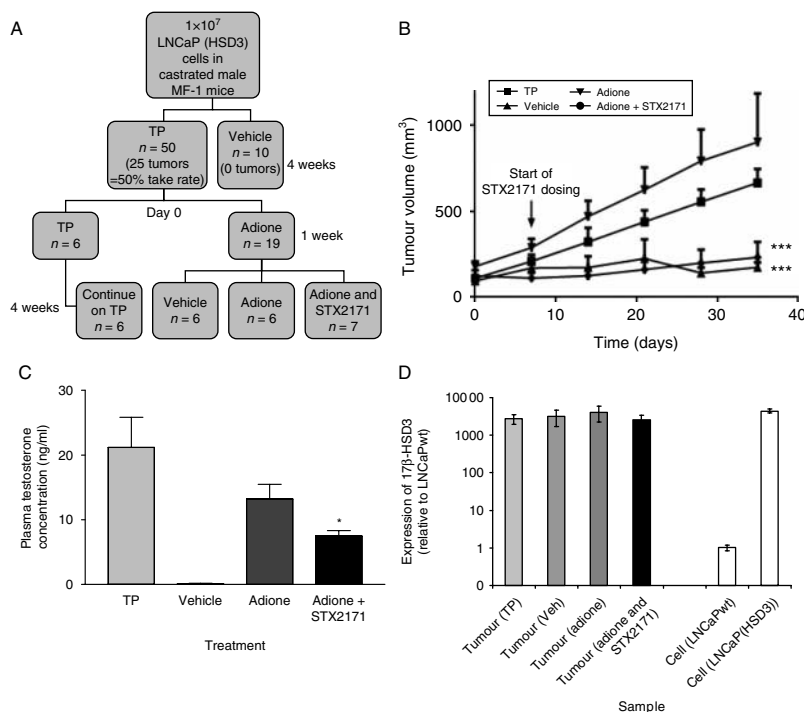
Statistical analyses

Values obtained *in vitro* are represented as mean \pm s.d., and those obtained from *in vivo* studies as mean \pm s.e.m. ANOVA, with a Bonferroni's post-test, was used to determine the significance of differences in *in vivo* tumour data, and Student's *t*-test was used to determine the significance of differences in plasma testosterone data.

Results

In vitro analysis of 17 β -HSD3 inhibitors

Inhibition of 17 β -HSD activity STX2171 had an IC₅₀ value of 208 nM in the whole-cell 293-EBNA(HSD3) radiometric TLC-based assay (Day *et al.* 2009b). In similar

**Figure 3**

Development of the *in vivo* proof-of-concept model and the effect of STX2171. (A) Dosing schedule of mice in this study. Mice were treated with either TP or vehicle for 4 weeks. After 4 weeks, 50% of those dosed with TP developed tumours, whereas none developed tumours in the vehicle-dosed group. These mice were removed from the study. TP dosing was continued for six mice for the rest of the study, whereas the other 19 were dosed with adione for 1 week, and then separated into three dosing groups and treated with vehicle, adione or adione plus STX2171 for a

further 4 weeks. (B) Effect of dosing with TP, vehicle or adione \pm STX2171 on tumour growth over the last 5 weeks of the study (mean \pm s.e.m.; $n=6-7$; *** $P<0.001$). (C) Plasma testosterone concentration (ng/ml) 1 h after administration of the final dose of TP, vehicle or adione \pm STX2171 (mean \pm s.e.m.; $n=4-7$; * $P<0.05$). (D) Expression of 17 β -HSD3 mRNA in wild-type LNCaP cells (LNCaPwt), in the LNCaP(HSD3) cell line inoculated into the animals and in the tumours of mice at the end of the study (mean \pm s.d.).

17 β -HSD1 and 17 β -HSD2 activity and inhibition assays (Table 1), STX2171 had negligible activity against the oxidative 17 β -HSD2 enzyme that catalyses the reverse reaction, testosterone to adione (inhibiting 17 β -HSD2 activity at 10 μ M by 20.3 \pm 3.5%), and was inactive against 17 β -HSD1, the enzyme that catalyses the reduction of the inactive oestrogen E1 to active E2 (1.7 \pm 2.6% inhibition at 10 μ M).

STX1383, a 17 β -HSD3 inhibitory compound identified by Schering-Plough (Guzi *et al.* 2004), had an IC₅₀ value of 2.4 nM in the 17 β -HSD3 radiometric assay, and was also selective for 17 β -HSD3 over 17 β -HSD2 and 17 β -HSD1, inhibiting these enzymes by only 19.6 \pm 8.8% at 1 μ M and 18.4 \pm 4.3% at 10 μ M respectively (Table 1).

Schering-Plough had previously indicated that STX1383 is active against the mouse 17 β -HSD3 enzyme. In a mouse Leydig tumour cell line, MLTC-1, STX2171 inhibited 17 β -HSD3 activity by 76% at 1 μ M.

Proliferation assay The AR-positive LNCaPwt prostate cancer cell line and an AR-negative prostate cancer cell line, PC-3, were treated with STX1383 and STX2171 over 96 h to establish cytotoxic effects, unrelated to their 17 β -HSD3 inhibitory activity, on prostate cancer cells. The IC₅₀ for growth inhibition by STX2171 was \sim 100-fold that for 17 β -HSD3 inhibition, at 16.6 and 23.5 μ M in LNCaP and PC-3 cells respectively. Although STX1383 was more toxic, with an IC₅₀ for growth inhibition of 5.9 μ M in LNCaP cells, and 5.8 μ M in PC-3 cells, this IC₅₀ value was more than 1000-fold that for 17 β -HSD3 inhibition by this compound (Table 1).

In vitro proof-of-concept model In an *in vitro* proof-of-concept model (Fig. 2B), 10 nM adione stimulated the proliferation of LNCaP(HSD3) cells over 15 days to \sim 1500% of the number in 0.1% FBS medium alone. In the presence of 500 nM STX2171 and STX1383, adione-stimulated proliferation was inhibited by 79.5 and 99.8%

Table 1 Properties of STX1383 and STX2171 *in vitro*.

Property/compound	STX1383	STX2171
Human 17 β -HSD3 inhibition (IC ₅₀ (nM))	2.4±0.6	208±1
17 β -HSD2 inhibition (% at 10 μ M)	18.4±4.3	20.3±3.5
17 β -HSD1 inhibition (% at 1 μ M)	19.6±8.8	1.7±2.6
Cytotoxicity (IC ₅₀ (μ M))		
LNCaP (AR+)	5.5	15.7
PC-3 (AR-)	6.8	22.0

respectively. Neither of the compounds stimulated or inhibited the proliferation of the cells in the absence of adione.

Inhibition of adione-stimulated LNCaP(HSD3) tumour growth in castrated nude mice

A schematic diagram showing the treatment regimens for mice is shown in Fig. 3A and further explained in the Materials and methods section.

Although there was little tumour growth in mice dosed with vehicle alone (171.3±29.0 mm³, most growth occurring during the initial week of TP dosing), the tumours stimulated with either TP or adione grew to 665.3±81.3 mm³ by day 35 (TP, 665.3±81.3 mm³; adione, 903.3±284.0 mm³; $P<0.001$; Fig. 3B). This indicates that, as seen *in vitro*, 17 β -HSD3 expressed in the LNCaP(HSD3) cells efficiently converted adione to testosterone in mice, stimulating tumour growth. However, in animals given a combined dose of adione plus STX2171, tumour growth was significantly inhibited (229.4±91.2 mm³ at day 35; $P<0.001$) to the level of those dosed with vehicle alone, indicating that STX2171 inhibited androgen stimulation of tumour growth completely at this dose.

Blood was obtained from four of the six animals in each of the vehicle-, TP- and adione-dosed groups, and from all seven of the adione plus STX2171-dosed group 1 h after final dose administration, and plasma testosterone concentration was measured by RIA (Fig. 3C). Castrated mice dosed with vehicle alone demonstrated a negligible measurable plasma testosterone (0.1±0.1 ng/ml), but mice given TP or adione had plasma concentrations of 21.2±4.6 and 13.2±2.3 ng/ml respectively. In the adione plus STX2171-dosed group, serum concentration of testosterone was significantly decreased to 7.5±0.8 ng/ml ($P<0.05$) compared with those dosed with adione alone.

RT-PCR analysis indicated that 17 β -HSD3 mRNA expression in the established LNCaP(HSD3) tumours was unaffected by any treatment, remaining at the level seen in the LNCaP(HSD3) cell line *in vitro* (Fig. 3D). Xenograft tumour 17 β -HSD3 expression was maintained throughout

the study period. Additionally, AR expression was unchanged in tumour samples and mouse body weight was unaffected by treatment (data not shown).

Comparison of the efficacy of STX2171 and STX1383 in the *in vivo* proof-of-concept tumour model

This novel *in vivo* proof-of-concept model was used to compare the efficacy of STX2171 and STX1383 in adione-dependent tumours. In mice dosed with adione alone, tumours grew to 592.5±161.7 mm³ by day 35 (the start of 17 β -HSD3 inhibitor dosing), whereas in those dosed with adione plus STX2171 or STX1383, the final tumour volumes were significantly lower ($P<0.05$) at 283.3±53.2 and 326.2±73.0 mm³ by day 35 respectively (Fig. 4A).

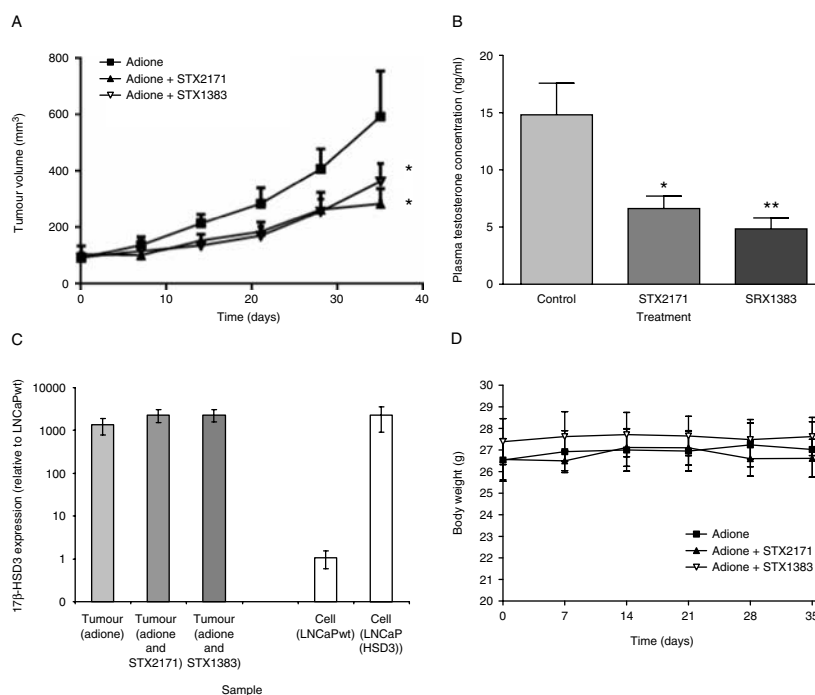
Blood hormone concentrations were determined from all mouse groups at the end of the study (Fig. 4B). Plasma testosterone concentration was 14.8±2.7 ng/ml in mice dosed with adione alone, but was significantly decreased in both the 17 β -HSD3 inhibitor-dosed groups: at 6.6±1.0 ng/ml in those dosed with adione plus STX2171 ($P<0.05$) and at 4.8±1.0 ng/ml in animals dosed with adione plus STX1383 ($P<0.01$).

As in the previous study, RT-PCR analysis indicated that 17 β -HSD3 (Fig. 4C) and AR mRNA expression (results not shown) in the LNCaP(HSD3) tumours were unaffected by any treatment, remaining at the levels seen in the LNCaP(HSD3) cell line *in vitro*. Additionally, mouse body weight was unaffected by any of the treatments (Fig. 4D), demonstrating that STX2171 and STX1383 do not have significant toxicological problems when dosed at 20 mg/kg per day.

Intact mouse study

As an initial simple *in vivo* screen, an intact mouse model of 17 β -HSD3 inhibition was established. However, this model relies on the inhibition of the mouse 17 β -HSD3 enzyme, rather than that of the human enzyme as in the tumour model.

At the end of a 12-day study, in which intact male MF-1 nude mice were given daily doses of either 20 mg/kg per day STX2171 or STX1383 (s.c.) on days 1–5 and 8–12, plasma testosterone concentration was significantly decreased in both dosing groups ($P<0.05$; $n=5$ per group; Fig. 5). In mice dosed with STX2171, plasma testosterone concentration decreased to 0.41±0.09 ng/ml, 11.2% of that of the control (3.65±2.45 ng/ml), and in the STX1383-dosed animals, the concentration was 0.63±0.20 ng/ml, 17.4% of that measured in control vehicle-dosed mice.

**Figure 4**

Effects of the 17 β -HSD3 inhibitors in the *in vivo* proof-of-concept model. (A) The effect of dosing with adione \pm STX2171 or STX1383 on tumour growth (mean \pm s.e.m.; $n=6-8$; $*P<0.05$). (B) Plasma testosterone concentration (ng/ml) 1 h after administration of the final dose of adione \pm STX2171 or STX1383 (mean \pm s.e.m.; $n=6-7$; $*P<0.05$ and $**P<0.01$).

(C) Expression of 17 β -HSD3 mRNA in wild-type LNCaP cells (LNCaPwt), in the LNCaP(HSD3) cell line inoculated into the animals, and in the tumours of mice at the end of the study (mean \pm s.d.). (D) Effect of the dosing on mouse body weight (mean \pm s.e.m.; $n=6-8$; NS, $P>0.05$).

Discussion

Inhibitors of several steroidogenic enzymes are successfully used to treat hormone-dependent cancers in the clinic. These include, for use in early-stage prostate cancer and in benign prostate hyperplasia (BPH), 5 α -reductase inhibitors such as finasteride and dutasteride, and in ER-positive breast cancer, aromatase and steroid sulfatase inhibitors (Stanway *et al.* 2007). However, contrastingly, the development of 17 β -HSD inhibitors is at a relatively early stage (Day *et al.* 2010). At present, none of these inhibitors has reached clinical trial, and only recently has efficacy been demonstrated *in vivo* for inhibitors of the first of the enzymes, 17 β -HSD1, in models of breast cancer (Day *et al.* 2006, 2008a, Husen *et al.* 2006) and endometrial hyperplasia (Saloniemi *et al.* 2010). 17 β -HSD3 catalyses the reduction of adione to form testosterone, further metabolised by 5 α -reductases to DHT, which stimulates the growth of hormone-dependent prostate tumours. This report describes the development of *in vivo* models to investigate the efficacy of 17 β -HSD3 inhibitors as therapies for androgen-dependent prostate cancer.

Previously, we described the development of *in vitro* assays and models to investigate the use of compounds as inhibitors of 17 β -HSD3 (Day *et al.* 2009b). As the expression of 17 β -HSD3 and 17 β -HSD2 was found to be negligible in AR-positive LNCaPwt prostate cancer cells, a stable 17 β -HSD3-expressing cell line was selected for after transfection with pCEP4.17 β -HSD3. This cell line, LNCaP(HSD3), provided a good model in which to study the effects of 17 β -HSD3 inhibition in prostate cancer cells, especially as LNCaP cells are known to be responsive to androgenic stimuli, both *in vitro* and *in vivo* (Horoszewicz *et al.* 1983). In the present study, we have taken this adione-stimulated model from the *in vitro* to the *in vivo* setting, stimulating growth of the cells as androgen-stimulated LNCaP(HSD3) xenografts in castrated nude mice. Additionally, we have set up a simpler, faster *in vivo* assay as an initial *in vivo* screen for candidate 17 β -HSD3 inhibitors that relies on the endogenous 17 β -HSD3 present in intact mice.

STX2171 was previously identified as the most active of a panel of 50 compounds designed as 17 β -HSD3

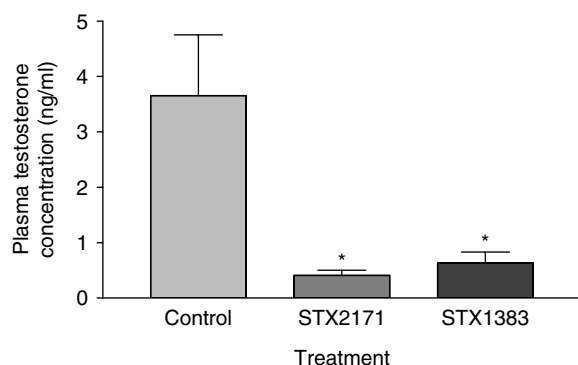


Figure 5

Inhibition of testosterone formation by 17 β -HSD3 inhibitors in intact male mice *in vivo*. Plasma testosterone concentrations (ng/ml) in intact male MF-1 nude mice 1 h after administration of the final dose of STX2171 or STX1383 in a 12-day study (mean \pm S.E.M.; $n=5$; * $P<0.05$).

inhibitors (Vicker *et al.* 2009), with an IC₅₀ of 208 nM in our whole-cell assay. STX1383, which in our assay inhibits 17 β -HSD3 with an IC₅₀ of 2.4 nM, was originally synthesised by Schering-Plough as SCH-451659 (Guzi *et al.* 2004). In this study, we have used STX2171 to develop the *in vivo* models, and have compared its efficacy with that of STX1383 *in vivo*.

Initially, compounds were investigated *in vitro*. Although the IC₅₀ of STX1383 was ~100-fold lower than that of STX2171, both compounds were similarly inactive against 17 β -HSD1. STX2171 had negligible activity (20.3% at 10 μ M) against the inactivating enzyme 17 β -HSD2, whereas STX1383 had slightly more activity (19.6% at 1 μ M). However, as it has 100-fold higher 17 β -HSD3 inhibitory activity *in vitro* than STX2171, its selectivity for 17 β -HSD3 over 17 β -HSD2 is comparable with that of STX2171.

In both AR-positive and AR-negative prostate cancer cells, the IC₅₀ value for each compound was similar, indicating that neither compound has any pro- or anti-androgenic effects. Although the anti-proliferative IC₅₀ values for STX1383 were around 3.5-fold lower than those of STX2171, indicating STX1383 as slightly more cytotoxic, the values for STX2171 were ~100-fold higher than its IC₅₀ for 17 β -HSD3 inhibition, and those for STX1383 were >2000-fold, suggesting that both compounds have negligible toxicity at the doses required for 17 β -HSD3 inhibition. In the *in vitro* proof-of-concept model, at 500 nM, both compounds inhibited adione stimulation of LNCaP(HSD3) cells, with STX1383 demonstrating better activity (99.8% inhibition), as would be expected from its lower IC₅₀ value for 17 β -HSD3 inhibition. The

79.5% inhibition seen for STX2171 is comparable with that obtained in a previous similar study (74%; Day *et al.* 2009b).

Having previously successfully developed an *in vivo* breast cancer model of 17 β -HSD1 inhibition (Day *et al.* 2008a), we used similar principles to develop the *in vivo* androgen-stimulated human 17 β -HSD3-dependent prostate tumour model. In the 17 β -HSD1 ovariectomised breast cancer model, wild-type human T47D breast cancer cells stimulated with E1 were used, as they are ER-positive and have a high 17 β -HSD1:17 β -HSD2 ratio. Because there is no established AR-positive human prostate cancer cell line expressing a similar ratio of 17 β -HSD3:17 β -HSD2, we developed the LNCaP(HSD3) cell line for the prostate model, as LNCaP cells are AR-dependent and have low expression of 17 β -HSD2. Castrated male animals were used so that, when stimulated with adione, the growth of the cells would be dependent on the 17 β -HSD3 activity of the human enzyme expressed by the inoculated LNCaP(HSD3) cells.

Initially, TP was used to establish the growth of the androgen-dependent LNCaP(HSD3) xenografts. Testosterone was administered as its propionate ester due to the short half-life of free testosterone (Mallidis *et al.* 2012). After 4 weeks, 25 of the 50 mice dosed with TP had developed tumours of 50–100 mm³, but tumours had not developed in any of the ten mice given vehicle alone. This indicated that castration was effective and that tumour growth was androgen-dependent. The animals that had not developed tumours by 4 weeks were removed from the study. The 50% take rate suggests that this dose of TP is just enough to stimulate the growth of these cells in castrated mice, although similarly low take rates have been documented in other studies in which xenografts have been formed from inoculated transfected LNCaP cells (Russell *et al.* 1999, Perryman *et al.* 2006).

Once the tumours had reached 50–100 mm³, the stimulus was switched to adione, and the tumours continued to grow as efficiently as those dosed with TP for the remainder of the study, indicating that 17 β -HSD3 in the tumours efficiently converted the dosed adione to testosterone. Growth was significantly inhibited in the presence of 20 mg/kg STX2171, to the level of those mice dosed with vehicle alone.

Testosterone concentration in the plasma was significantly lowered in STX2171-treated mice 1 h after administration of the final dose, to 56.7 \pm 6.3% of that in the animals dosed with adione alone. The concentration of testosterone in the plasma of those given TP was, however, unexpectedly significantly higher than those

dosed with adione, given the similar growth rate of the tumours in the two groups. This may be due to the scheduling of the final dose administration of each steroid 1–2 h before the blood sample was obtained by cardiac puncture. This short duration may not allow for the maximal conversion of the administered adione to testosterone, as would have happened during each 24 h period throughout the study. Furthermore, it is noted that STX2171 failed to completely inhibit plasma testosterone concentrations despite inhibiting tumour growth. As plasma was collected 1 h after the final dose of adione and STX2171, testosterone measured may reflect the slower delivery of the compound to the tumour compared with the hormone. Consequently, some adione would be converted by LNCaP 17 β -HSD3 activity. However, we suggest that 17 β -HSD3 inhibition did occur more completely after 1 h, blocking the conversion of remaining adione and hence inhibiting tumour growth.

These results indicated the development of a successful model in which to test clinical candidate 17 β -HSD3 inhibitors *in vivo*; we therefore used this model to compare the efficacy of STX2171 and STX1383, the Schering-Plough compound which has a more favourable 17 β -HSD3 inhibitory IC₅₀ *in vitro*. STX2171 was again tested at 20 mg/kg per day, to study the reproducibility of the model, and STX1383 was also given at this dose. Again, administration of adione resulted in the steady growth of the LNCaP(HSD3) tumours, and the growth was significantly inhibited by co-administration of either of the two 17 β -HSD3 inhibitors tested. In the last 2 weeks of study, although STX2171 maintained its inhibition of tumour growth, the effect of STX1383 appeared to decline; however, the difference between the effects of the two compounds was non-significant over this time duration. Inhibition by STX2171, to 39% of the adione-treated tumour volume, is highly comparable with the results from the first study, in which STX2171-treated tumours inhibited tumour growth to 35% of that of the adione-dosed animals, indicating that this is a robust, reproducible model. In both studies, RT-PCR analysis indicated that neither the androgens nor the inhibitors affected the expression of the transfected 17 β -HSD3 or the wild-type AR in the xenografts.

In this study, in plasma samples taken 1–2 h after administration of the final dose, although both compounds significantly lowered plasma testosterone levels, STX1383 more efficiently reduced plasma testosterone concentrations, to 32% of those dosed with adione alone, compared with a decrease to 45% of control in the STX2171-dosed animals; this is a little lower than the 57% in the first study.

These studies demonstrate the development of a novel, robust *in vivo* proof-of-concept model for the evaluation of 17 β -HSD3 inhibitors against androgen-dependent prostate cancer. However, this model has limitations for high-throughput usage as it is time-consuming and expensive, requiring more animals than are used in the final dosing phase due to the relatively low take rate of the LNCaP(HSD3) tumours, and additionally requiring castrated animals. Therefore, as an initial screen for *in vivo* efficacy and inhibitor properties, a simple 12-day intact mouse model was set up. In this model, mice were treated with the 17 β -HSD3 inhibitors on days 1–5 and 8–12, again at 20 mg/kg per day, and blood samples were taken 1 h after the final dose, so that the plasma testosterone result scheduling was directly comparable with that of the proof-of-concept tumour model. Both inhibitors significantly inhibited plasma testosterone concentration in this model, to <20% of that measured in control mice.

It is important to remember that in this intact mouse model, it is the mouse 17 β -HSD3 enzyme, and not the human homologue, which is being inhibited (contrary to the proof-of-concept model). The efficacy shown by both inhibitors in the intact mouse model supports the results in mouse Leydig cells *in vitro* which demonstrated that STX2171 is active against the mouse enzyme. Schering-Plough has previously indicated that STX1383 is active against the mouse enzyme, albeit with an IC₅₀ ~1000-fold higher than that for the human enzyme (Pachter *et al.* 2005). Although the clinical relevancy of mouse 17 β -HSD3 inhibition in this model is questionable, the intact status of mice results in less controlled steroid conditions *in vivo*, and this may thus be more similar to the steroidogenic state in prostate cancer and BPH patients. Although 17 β -HSD3 expression has been seen to be up-regulated in prostate cancer (Koh *et al.* 2002), the majority of the 17 β -HSD3 activity in humans is gonadal (Geissler *et al.* 1994). The mice in this study were dosed for 12 days so that, in addition to the effect on plasma testosterone levels, the effect of the inhibitors on prostate size could be studied. Unfortunately, due to the small size of the mouse prostate, prostate weight could not be measured accurately in these young animals.

Use of these *in vivo* models indicates that our lead 17 β -HSD3 inhibitor, STX2171, is at least as efficient as the Schering-Plough compound *in vivo*, both at lowering plasma testosterone levels and at inhibiting the proliferation of androgen-dependent prostate tumours when stimulated by adione. Although several groups are now working on inhibitors of 17 β -HSD3 (Le Lain *et al.* 2002,

Maltais *et al.* 2002, Tchédam Ngatcha *et al.* 2005, Fink *et al.* 2006, Lota *et al.* 2006, Berube & Poirier 2007), this is the first report on anti-tumour activity of a 17 β -HSD3 inhibitor *in vivo*. Having developed the primary screening assays and an *in vitro* proof-of-concept model, and selected a lead compound with good activity selective for 17 β -HSD3 over other 17 β -HSD enzymes, non-androgenic with low toxicity, and efficacious in the *in vitro* proof-of-concept model (Day *et al.* 2009b), we have now developed novel robust models to study the efficacy of this and future candidate compounds *in vivo*.

Further work on this model and STX2171 is required. Our *in vivo* studies imply that the majority of adione was converted to testosterone by LNCaP 17 β -HSD3 activity. However, it has recently been suggested that the dominant pathway of adione metabolism in CRPR is through adione conversion to 5 α -adione via SRD5A (Chang *et al.* 2011). 5 α -Adione is then the substrate for 17 β -HSD3 to synthesis DHT. Our studies did not investigate adione-induced DHT plasma concentrations. However, as 17 β -HSD3 is involved in this alternative pathway for adione metabolism, STX2171 and STX1383 should also inhibit this pathway. This fact makes these compounds significantly more interesting with regard to the treatment of CRPR.

These models provide evidence that 17 β -HSD3 inhibitory compounds may have application in the treatment of hormone-dependent prostate cancer. STX2171 represents an attractive compound class suitable for further optimisation towards preclinical development, inhibiting the growth of adione-dependent tumours in the *in vivo* prostate cancer xenograft model and significantly decreasing plasma testosterone concentrations both in this model and in intact mice.

Declaration of interest

F Schmidlin is an employee of Ipsen. A Purohit is a consultant for Ipsen. BVL Potter & MJ Reed were consultants for Ipsen.

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Author contribution statement

A Purohit, B V L Potter and M J Reed conceived and coordinated the project and designed experiments with F Schmidlin. J M Day designed the *in vitro* experiments, performed both the *in vitro* proof-of-concept model and the *in vivo* intact male mouse model, and statistical analysis. P A Foster designed and performed all *in vivo* experiments, collecting data and statistical analysis. H J Tutill helped design the *in vitro* modelling, ascertained the IC₅₀ values and ran the *in vitro* proof-of-concept assay.

B V L Potter and N Vicker designed STX2171. C M Sharland and J D Hargrave synthesised STX2171 and STX1383. P A Foster, J M Day & A Purohit wrote the manuscript with input from all authors.

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