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Reed

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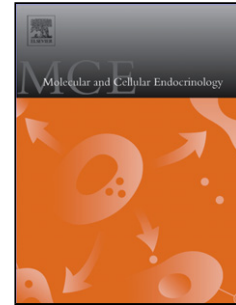


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17 β -HSD3 inhibition

Development of hormone-dependent prostate cancer models for the evaluation of inhibitors of 17 β -hydroxysteroid dehydrogenase Type 3.

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ABSTRACT

17 β -Hydroxysteroid dehydrogenases (17 β -HSDs) are responsible for the pre-receptor reduction / oxidation of steroids at the 17-position into active / inactive hormones, and the 15 known enzymes vary in their substrate specificity, localisation, and directional activity. 17 β -HSD Type 3 (17 β -HSD3) has been seen to be over-expressed in prostate cancer, and catalyses the reduction of androstenedione (Adione) to testosterone (T), which stimulates prostate tumour growth. Specific inhibitors of 17 β -HSD3 may have a role in the treatment of hormone-dependent prostate cancer and benign prostate hyperplasia, and also have potential as male anti-fertility agents.

A 293-EBNA-based cell line with stable expression of transfected human 17 β -HSD3 was created and used to develop a whole cell radiometric TLC-based assay to assess the 17 β -HSD3 inhibitory potency of a series of compounds. STX2171 & STX2624 (IC₅₀ values in the 200-450 nM range) were two of several active inhibitors identified. In similar TLC-based assays these compounds were found to be inactive against 17 β -HSD1 and 17 β -HSD2, indicating selectivity.

A novel proof of concept model was developed to study the efficacy of the compounds *in vitro* using the androgen receptor positive hormone-dependent prostate cancer cell line, LNCaPwt, and its derivative, LNCaP[17 β -HSD3], transfected and selected for stable expression of 17 β -HSD3. The proliferation of the parental cell line was most efficiently stimulated by 5 α -dihydrotestosterone (DHT), but the LNCaP[17 β -HSD3] cells were equally stimulated by Adione, indicating that 17 β -HSD3 efficiently converts Adione to T in this model. Adione-stimulated proliferation of LNCaP[17 β -HSD3] cells was inhibited in the presence of either STX2171 or STX2624. The compounds alone neither stimulated proliferation of the cells nor

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caused significant cell death, indicating that they are non-androgenic with low cytotoxicity. STX2171 inhibited Adione-stimulated growth of xenografts established from LNCaPwt cells in castrated mice *in vivo*.

In conclusion, a primary screening assay and proof of concept model have been developed to study the efficacy of 17 β -HSD3 inhibitory compounds, which may have a role in the treatment of hormone-dependent cancer. Active compounds are selective for 17 β -HSD3 over 17 β -HSD1 and 17 β -HSD2, non-androgenic with low toxicity, and efficacious in both an *in vitro* proof of concept model and in an *in vivo* tumour model.

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INTRODUCTION

The 17 β -hydroxysteroid dehydrogenases (17 β -HSDs) are responsible for the reduction or oxidation of hormones, fatty acids and bile acids *in vivo*. All require NAD(P)(H) for activity (Shi & Lin, 2004). Fifteen 17 β -HSDs have been identified to date (Luu-The, Belanger & Labrie, 2008; Lukacik *et al.*, 2006; Jansson *et al.*, 2006), and with one exception, 17 β -HSD Type 5 (17 β -HSD5), an aldo-keto reductase (AKR) (Penning *et al.*, 2000), they are all short chain dehydrogenases / reductases (SDRs) (Peltoketo *et al.*, 1999; Duax *et al.*, 2005; Vikho *et al.*, 2006). The major substrates of the 17 β -HSDs are hormones, and their reduction or oxidation by 17 β -HSDs regulates the amount of active steroid available to bind to a particular receptor. Despite having bidirectional capabilities *in vitro*, catalysing either the oxidative or reductive reaction in the presence of NAD(P)⁺ or NAD(P)H respectively, *in vivo* they appear to function unidirectionally (Khan *et al.*, 2004; Luu-The *et al.*, 1995).

5 α -Dihydrotestosterone (DHT), formed from testosterone (T) by 5 α -reductases 1 and 2, is the main intracellular androgen in the prostate and stimulates the growth of hormone-dependent prostate tumours via its interaction with the androgen receptor (AR). 17 β -HSD3, microsomally expressed almost exclusively in the testes (Geissler *et al.*, 1994; Luu-The *et al.*, 1995), specifically converts non-androgenic androstenedione (Adione) (Laplante & Poirier, 2007) to active circulating T (Figure 1) in the presence of NADPH. Another 17 β -HSD, 17 β -HSD5 (AKR1C3), can also convert Adione to T, and is expressed more ubiquitously than 17 β -HSD3, having been found in the prostate and in other tissues (Pelletier *et al.*, 1999). However, *in vitro* it has also been shown to catalyse the reduction of many other substrates, including steroids and prostaglandins, in various positions, including 3 β -, 17 β -, 20 α -

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of steroids, and 11 α - and 11 β - of prostaglandins, forming oestradiol, 20 α -progesterone, 9 α ,11 β -prostaglandin F₂, and other active compounds (Penning *et al.*, 2000; Komoto *et al.*, 2004). As yet, its major physiological function remains to be determined. The importance of 17 β -HSD3 in T production in men is exemplified by the autosomal recessive disorder, male pseudohermaphroditism (Geissler *et al.*, 1994) in which there is a defect in 17 β -HSD3 expression. Affected individuals have a high Andione to T ratio and are usually reared as females, often having been born with female external genitalia and the absence of a prostate, despite having testes and Wolffian duct-derived male internal genitalia (Andersson & Moghrabi, 1997).

Although 17 β -HSD3 is expressed almost exclusively in the testes, there have been some reports of its up-regulation in tumours of the prostate, and of its expression in other tissues, such as blood platelets and megakaryocytes (Gnatenko *et al.*, 2005). One report indicated that expression of 17 β -HSD3 mRNA increased over 30-fold in cancerous prostate biopsies (Koh *et al.*, 2002). The authors noted a corresponding decrease in 17 β -HSD2 mRNA expression, indicating that the reductive formation of T is favoured, but found no change in the expression of 17 β -HSD5 mRNA. Expression of 17 β -HSD3 was also up-regulated in an AR-positive prostate cell line, LNCaP, after it was treated for 48 hours with dutasteride (Biancolella *et al.*, 2007), an inhibitor of 5 α -reductases 1 and 2. A polymorphism in the HSD17B3 gene, G289S, has been linked to an increased susceptibility to prostate cancer (Margiotti *et al.*, 2002).

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 various approaches which include orchidectomy, and / or the use of AR blockers such as bicalutamide (Fradet, 2004), luteinising hormone releasing hormone (LHRH) agonists such as

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goserelin (Akaza, 2004), or 5 α -reductase inhibitors, such as finasteride and dutasteride (Tindall & Rittmaster, 2008). However, the use of some of the more radical treatments, such as orchidectomy and LHRH agonists, can result in undesirable adverse effects on quality of life, including loss of sexual interest and function and bone mineral density.

Despite the success of these early stage treatments, prostate cancer is the third highest cause of male cancer-related death as tumours are often unnoticed for several years, presenting at an advanced, apparently androgen-independent, stage in older men. However, many studies now indicate that the majority of advanced prostate cancers may not be independent of androgen signalling. Instead tumours have developed apparent independence via mechanisms such as AR up-regulation, an adaptation to the low levels of androgen present during ablation therapy, or mutations to the pathways involved in the activation of the AR, such as mutations in the receptor itself, or in its co-regulators, allowing enhanced activation by the low level androgens (Mizokami *et al.*, 2004) or ligands other than androgens to activate the proliferative pathways (Rau *et al.*, 2005; Pienta & Bradley, 2006). High levels of T and DHT have been found in the prostate of patients with recurrence during ablation therapy, suggesting that surgical or chemical castration treatments may not completely remove active androgens, and that *in situ* formation of T from adrenal androgen production may continue (Titus *et al.*, 2005).

A decrease in the formation of T by the inhibition of 17 β -HSD3 should provide an effective treatment for hormone-dependent prostate cancer. Because of the enzyme's unique expression profile and substrate specificity, use of 17 β -HSD3 inhibitors may result in fewer side effects than other hormonal treatments, such as orchidectomy, or LHRH inhibitors which act upstream of the 17 β -HSD3 reaction and

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affect the systemic balance of other hormones, not just the conversion of Adione to T. The inhibitors should be similarly effective in the treatment of benign prostate hyperplasia and may also have a role as male anti-fertility agents. This report describes the identification and characterisation of two novel 17 β -HSD3 inhibitors, and the development of *in vitro* and *in vivo* models to investigate their efficacy as therapies for androgen-dependent prostate cancer.

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MATERIALS & METHODS

Candidate 17 β -HSD3 inhibitor compounds

The syntheses of the candidate 17 β -HSD3 inhibitor compounds have been reported (Vicker *et al.*, 2007). All compounds exhibited spectroscopic and analytical data in accordance with their structure. Their purity (>95%) was confirmed by high performance liquid chromatography analysis. A compound previously identified by Bristol Myers Squibb (BMS) as a potential 17 β -HSD3 inhibitor (DBT 1; Fink *et al.*, 2006), was synthesised using a modified published method (Yale *et al.*, 1972).

Cell culture

LNCaP cells (LGC Promochem, Teddington, UK) were cultured in RPMI-1640 medium (Sigma Aldrich Company Ltd., Poole, UK) supplemented by 10% foetal bovine serum (FBS), 2 mM L-glutamine, 2 mM sodium pyruvate, 1% non-essential amino acids and 0.075% sodium bicarbonate (all from Sigma Aldrich Company Ltd.). 293-EBNA cells (Invitrogen Ltd., Paisley, UK) were maintained in Dulbecco's modified Eagle's medium (Sigma Aldrich Company Ltd.) containing 10% FBS, 2 mM L-glutamine, 0.075% sodium bicarbonate, and 250 μ g/ml G418 (Sigma Aldrich Company Ltd.). All cells were maintained at 37°C in a humidified atmosphere at 5% CO₂.

Cloning

The complete 17 β -HSD3 (IMAGE) cDNA sequence was cloned into the pCEP4 expression vector (Invitrogen) to form pCEP4.17 β -HSD3 for expression in

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mammalian cells. The construct was sequenced to confirm correct insertion of the cDNA into the vector.

Expression of 17 β -HSD3 cDNA

293-EBNA and LNCaP cells were transfected with 1 μ g pCEP4.17 β -HSD3 or vector alone in 6 well plates at 50-70% confluency using a 1:3 (w/v) DNA:FuGENE 6 transfection reagent ratio (Roche Diagnostics, Burgess Hill, UK). After 48 hours cDNA expression was analysed by RT-PCR and Adione to T activity assays. Transfected cells were selected for stable expression of the plasmids over several weeks by the addition of 250 μ g/ml Hygromycin B (Invitrogen) to the growth medium.

RT-PCR analysis

mRNA was purified from T75 flasks or 6 well plates at approximately 80% confluency using RNeasy and QIAshredder kits (QIAGEN, Crawley, UK) and stored at -80°C. A 5 μ g aliquot of each mRNA sample was reverse transcribed in a final volume of 33 μ l to form cDNA using the 'First-Strand cDNA Synthesis Kit' (GE Healthcare, Amersham, UK) and stored at -20°C. RT-PCR reactions were performed in a 'Rotor Gene 2000 Real-Time Cycler' (Corbett Life Science, Cambridge, UK) with 0.5 μ l cDNA in a final volume of 10 μ l, using Taqman universal PCR master mix and Taqman expression assays containing primers and probes for 17 β -HSD3, and for the endogenous control gene, RPLO (Applied Biosystems, Warrington, UK). The conditions were as follows: 95°C for 10 minutes; followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. Relative mRNA expression was calculated using

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the comparative quantitation algorithm in the Rotor Gene 6 software (Corbett Life Science).

17 β -HSD3 activity assay

Cells at ~80% confluency were incubated with 2-3 nM ^3H -Adione (65.0 Ci / mmol; Perkin Elmer, Boston, MA) in 24 well tissue culture plates. For inhibitor screening assays, 293-EBNA[HSD3] cells were plated at 50,000 cells/well 48 hours pre-assay in medium free of the antibiotics G418 or Hygromycin B and at assay the substrate was added in the presence or absence of candidate 17 β -HSD3 inhibitors. Blank incubations were carried out in parallel by incubating labelled steroid in cell-free wells. After incubation of the substrate (+/- inhibitor) at 37°C for 2 hours, the steroids were isolated from the mixture by extraction with diethyl ether in the presence of ^{14}C -T (5000 dpm; Perkin Elmer) to monitor procedural losses and 25 μg of unlabelled T for product identification after thin layer chromatography (TLC). Separation of ^3H -T from ^3H -Adione was achieved by TLC using a dichloromethane : ethyl acetate (DCM:EtOAc) 4:1 v/v solvent mix, and the production of ^3H -T was calculated from the ^3H counts detected and recovery of ^{14}C -T (Beckman LS 6000SC; Beckman Coulter UK Ltd., High Wycombe, UK).

17 β -HSD1 activity assay

T47D human breast cancer cells in 24 well tissue culture plates were incubated with 2 nM ^3H -oestrone (E1; 65.0 Ci / mmol; Perkin Elmer) in the presence or absence of 10 μM candidate 17 β -HSD3 inhibitor. After 30 minutes at 37°C, the steroids were isolated from the mixture by extraction with diethyl ether, using ^{14}C -oestradiol (E2; 5000 dpm; Perkin Elmer) to monitor procedural losses. Separation of ^3H -E2 from ^3H -

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E1 was achieved using TLC in DCM:EtOAc 4:1 v/v solvent, and the production of ³H-E2 was calculated from the ³H counts detected and recovery of ¹⁴C-E2 (Beckman LS 6000SC).

17 β -HSD2 activity assay

MDA-MB-231 human breast cancer cells in T25 flasks were incubated with 2 nM ³H-E2 (41.3 Ci / mmol; Perkin Elmer) in the presence or absence of 10 μ M candidate 17 β -HSD3 inhibitors. After 3 hours at 37°C, the steroids were isolated from the mixture by extraction with diethyl ether, using ¹⁴C-E1 (5000 dpm; Perkin Elmer) to monitor procedural losses. Separation of ³H-E1 from ³H-E2 was achieved using TLC (DCM:EtOAc, 4:1 v/v) and the amount of ³H-E1 produced was calculated from the ³H counts detected and recovery of ¹⁴C-E1 (Beckman LS 6000SC).

In vitro Adione-stimulated model

LNCaPwt or LNCaP[HSD3] cells were seeded in a 24 well plate at 40,000 cells/well in 10% FBS growth medium (Day 1). On Day 4 the cells were washed with phosphate buffered saline (PBS) and the medium was replaced with 0.1% FBS growth medium. The cells were treated on Day 6 with 10 nM of either the appropriate steroid(s) or hydroxyflutamide in the presence or absence of 17 β -HSD3 inhibitor or vehicle (100% methanol) in 0.1% FBS growth medium, and cultured for a further 15 days with the medium and treatments renewed every 48-72 hours. On Day 19-21 the cells were counted using a Coulter cell counter (Beckman Coulter UK Ltd.).

In vivo inhibition of Adione-stimulated LNCaP tumour proliferation in castrated mice

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All animals received food and water *ad libitum* and were maintained in positive pressure isolators under a 12 hour light-dark cycle. The experiments were approved by the Imperial College London ethical review committee.

Castrated male MF-1 nude mice (Harlan UK Ltd., Bicester, UK) were inoculated s.c. with 1×10^7 LNCaPwt cells in 50 μ l Matrigel (BD Biosciences, Bedford, MA) into one flank of each mouse. LNCaPwt tumour growth was stimulated using 100 μ g (s.c.) Adione in 10% ethanol / 90% propylene glycol (PG) per mouse per day. When the tumours were 50-100 mm³, animals were treated additionally with 25 mg/kg/day STX2171 or vehicle (5% THF / 95% PG) in a combined s.c. injection with Adione. Every seven days tumour measurements were recorded using electronic callipers and the mice were weighed. Tumour volume was calculated using the formula: $volume = (length \times width^2)/2$. At the end of the studies the mice were culled, and final body weights and tumour sizes were determined.

Statistical analysis

Student's *t*-test was used to determine the significance of differences in *in vivo* data.

All values are represented as mean +/- s.d.

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RESULTS

Screening of candidate inhibitors

Screening assay development

RT-PCR analysis indicated that the expression of 17 β -HSD1, 2, 3 & 5 mRNA in 293-EBNA cells (Invitrogen) is negligible (results not shown). However, 48 hours after transfection with pCEP4.17 β -HSD3, there was a high level of expression of 17 β -HSD3 mRNA in the 293-EBNA cells. This expression level was maintained after Hygromycin B (Invitrogen) selection of the transfected cells, resulting in 293-EBNA[HSD3], a cell line with stable expression of 17 β -HSD3 (Figure 2a).

The 293-EBNA[HSD3] cell line was used to develop a whole cell radiometric assay for Adione to T activity and inhibition (detailed in the Materials and Methods section of this paper). The TLC-based assay uses physiologically relevant levels of ³H-Adione as substrate, at 2-3nM, and assay sensitivity is maintained by the addition of a known amount of ¹⁴C-T to monitor procedural losses. When measuring the activity of ~200,000 cells / well of a 24 well plate, the optimal duration of the assay was found to be 2 hours, as at this time point there was efficient conversion of substrate without the reaction being saturated. Assay of wild type or mock-transfected 293-EBNA cells resulted in negligible activity in this assay (Figure 2b).

Assay of candidate 17 β -HSD3 inhibitors

A series of approximately 50 candidate 17 β -HSD3 inhibitor compounds was tested in the assay. The inhibitory IC₅₀ value, the concentration at which the reaction is 50% inhibited, was determined for those with >70% activity at 10 μ M. STX2171

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and STX2624 (Figure 3), with IC₅₀ values in the 200-450 nM range, were two of several active inhibitors identified (Vicker *et al.*, 2007). These activities were comparable to that of a dibenzothiazocine compound DBT 1 (STX2622; Figure 3), previously identified by BMS as a potential 17 β -HSD3 inhibitor (Fink *et al.*, 2006).

In similar whole cell radiometric TLC-based assays (Day *et al.*, 2006a), STX2171 and STX2624 had negligible activity against the oxidative 17 β -HSD2 enzyme which catalyses the reverse reaction, T to Adione (inhibiting 17 β -HSD2 activity at 10 μ M by 20.3 +/- 3.5% and 18.9 +/- 2.9%, respectively). STX2171 was also shown to be inactive in the inhibition of 17 β -HSD1, the enzyme that catalyses the reduction of the inactive oestrogen, oestrone, to active oestradiol (1.7 +/- 2.6% inhibition at 10 μ M).

***In vitro* proof of concept model**

A model was developed to investigate whether candidate 17 β -HSD3 inhibitors can block Adione-stimulated proliferation of 17 β -HSD3-expressing AR-positive prostate cancer cells.

Preparation of an appropriate cell line

To establish whether LNCaP cells would be suitable for use in this model, the mRNA levels of steroid metabolising enzymes in LNCaPwt cells were investigated by RT-PCR. Of those enzymes tested, which included steroid sulphatase (STS), aromatase, and 17 β -HSD Types 1, 2, 3, 5 and 12, the cells were found to have some expression of STS, high expression of 17 β -HSD12, but negligible expression of 17 β -HSD Types 1, 2, 3 and 5 (Figure 4a). Despite their lack of naturally expressed 17 β -HSD3, they were suitable for use in the proof of concept model as they could be

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transfected with 17 β -HSD3 to create a stable cell line which converted Adione to T without interference from other steroid converting enzymes such as aromatase, which would convert the Adione substrate to oestrone, or other 17 β -HSDs, such as the reductive 17 β -HSDs 1 and 5, and the oxidative 17 β -HSD2.

An LNCaPwt-derived cell line with stable expression of 17 β -HSD3, LNCaP[HSD3], was established by pCEP4.17 β -HSD3 cDNA transfection and selection with Hygromycin B over several weeks. Both 17 β -HSD3 mRNA expression (Figure 4b) and Adione to T activity levels (Figure 4c) of the LNCaP[HSD3] cell line were comparable to those of the 293-EBNA[HSD3] cells.

Development of the in vitro proof of concept model

The LNCaPwt cell line was used to establish the optimal conditions for androgen-dependent stimulation of LNCaP-derived cells. The cells were plated in 24 well plates at low density, and allowed to adhere for 3 days before the FBS content of the medium was reduced from 10% to 0.1%. After 48 hours the cells were cultured in the presence of varying concentrations of T, from 1 nM to 1 μ M, and maintained without passage, with medium and supplements replaced every 3-4 days, until the wells at highest density were approaching confluency (~13-15 days). A concentration of 10 nM T was found to be optimal for stimulation (results not shown), so this dose was used for all following experiments.

The assay was repeated using 10 nM Adione or 10 nM DHT to stimulate growth. As seen with 10 nM T, DHT also effectively stimulated proliferation of the cells, whereas 10nM Adione resulted in only 50% of the stimulation seen with DHT (Figure 4d). However, in LNCaP[HSD3] cells, 10 nM Adione, T or DHT all efficiently stimulated proliferation (Figure 4e) indicating that in this model the 17 β -

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HSD3 expressed by the cells actively converts all available Adione to T. In both cell lines the positive control, 1 μ M hydroxyflutamide, most efficiently stimulates cell proliferation to 150% that of 10 nM DHT (Figures 4d&e).

Assay of lead inhibitors in the in vitro proof of concept model

Using the LNCaP[HSD3] cell line, the two lead compounds, STX2171 and STX2624, were tested at 5 μ M in the *in vitro* proof of concept model against the BMS positive control inhibitor, STX2622 (Figure 5a). Cell proliferation was efficiently stimulated by 10 nM Adione, to 550% of the number in 0.1% FBS medium alone. In the presence of 5 μ M STX2171 and STX2624, Adione-stimulated proliferation was inhibited by 99% and 66% respectively. STX2622 did not inhibit this stimulation (98% of Adione-stimulated cell number). None of the compounds stimulated proliferation of the cells in the absence of Adione, indicating that they are non-androgenic. However, in the absence of Adione, STX2171 decreased the number of non-stimulated cells to 5%, indicating that it is cytotoxic at this dose.

The assay was repeated, using a lower 500 nM dose of STX2171, and STX2624 at both 5 μ M and 500 nM (Figure 5b). Again 10 nM Adione efficiently stimulated proliferation of the LNCaP[HSD3] cells, to 1200% of non-stimulated cell number. At 500 nM, STX2171 efficiently inhibited Adione-stimulated proliferation by 74% without causing toxicity in the non-stimulated wells. STX2624 was also efficacious in this model, inhibiting Adione-stimulation by 79% at 5 μ M, and by 52% at 500 nM, with no indication of androgenicity and little indication of toxicity in the absence of Adione.

To confirm that the effect of 500 nM STX2171 is due to the inhibition of Adione to T conversion, proliferation of LNCaPwt cells was stimulated by 10 nM

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Adione, T, or DHT, or by 1 μ M hydroxyflutamide, in the presence or absence of 500 nM STX2171 (Figure 5c). STX2171 had little effect on the cell numbers after T, DHT, or hydroxyflutamide stimulation, or after culture in 10% FBS medium, but fully inhibited stimulation by 10 nM Adione.

***In vivo* anti-tumour activity of STX2171**

Single LNCaPwt xenografts were established in one flank of castrated male MF-1 mice. Mice were dosed s.c. with 100 μ g Adione per day until the tumours became visible, when they were then dosed additionally with either vehicle (5%THF / 95% PG) or 25 mg/kg STX2171. Control tumours treated with vehicle plus Adione continued to grow, to 380% of the volume at the start of dosing, whereas the growth of those dosed with STX2171 was significantly inhibited by 81% in comparison to control ($p < 0.05$; Figure 6), the tumours growing to only 157% of the volume at the start of dosing. Mouse body weight was unaffected by either Adione or STX2171 treatment.

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DISCUSSION

Despite the success of inhibitors of steroidogenic enzymes in the clinic, such as those of aromatase and steroid sulphatase, the development of inhibitors of 17 β -HSDs is at a relatively early stage (Day *et al.*, 2008a; Poirier, 2003; Penning, 1996). At present, none of these inhibitors has reached clinical trial, and only recently has efficacy been demonstrated *in vivo* for the first of the enzymes, 17 β -HSD1, in models of breast cancer (Day *et al.*, 2006b; Husen *et al.*, 2006; Day *et al.*, 2008b).

17 β -HSD3 catalyses the reduction of Adione to form T, further metabolised by 5 α -reductases 1 & 2 to DHT which stimulates the growth of hormone-dependent prostate tumours. This report describes the identification of two novel 17 β -HSD3 inhibitors, and the development of *in vitro* and *in vivo* models to investigate their efficacy as therapies for androgen-dependent prostate cancer.

293-EBNA cells were transfected with 17 β -HSD3 and those with stable expression of the cDNA were selected for use in the development of a whole cell radiometric assay for Adione to T activity and inhibition. Using this assay, STX2171 and STX2624, with IC₅₀ values of less than 500 nM, were identified from a panel of around 50 compounds. These activities were comparable to that of a dibenzothiazocine compound, DBT 1 (STX2622), published by BMS (Fink *et al.*, 2006). The screening assay was set up as a whole cell assay, rather than using the purified enzyme, so that the resulting IC₅₀ value for each compound was a measure of both its 17 β -HSD3 inhibitory activity, as well as its ability to penetrate the cell, a property necessary for its therapeutic use. Another property that a successful 17 β -HSD3 inhibitor requires is a lack of inhibition of 17 β -HSD2, as this catalyses the opposite reaction to that of 17 β -HSD3, the oxidation of active T to inactive Adione.

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Both of these compounds were selective for 17 β -HSD3 inhibition, causing <20% inhibition of 17 β -HSD2 activity at 10 μ M. STX2171 was also tested at 10 μ M against the 17 β -HSD1 enzyme, which forms active oestradiol from oestrone, and was found to be inactive.

Having established that these two compounds were effective and selective inhibitors of 17 β -HSD3, a proof of concept model was developed to study whether this inhibitory activity would be of benefit in the treatment of hormone-dependent cancer. This model was initially established *in vitro*, with a view to transferring it into the *in vivo* setting once optimised. LNCaP prostate cancer cells were investigated for use in this model as they are androgen receptor positive, respond to androgenic stimulation, and grow as xenografts in mice (Horoszewicz *et al.*, 1983).

RT-PCR analysis of LNCaP mRNA indicated that the wild type cells (LNCaPwt) have negligible expression of the steroidogenic enzymes 17 β -HSD1, 2, 3 and 5, and aromatase, some expression of STS, and a high level of expression of 17 β -HSD12. As the expression of 17 β -HSD3 is negligible in this cell line, the cells were transfected with 17 β -HSD3 cDNA, and a cell line (LNCaP[HSD3]) with stable expression of the enzyme was derived for use in the model. The expression of 17 β -HSD12 and STS should not interfere in the use of this cell line for the 17 β -HSD3 model. 17 β -HSD12, in addition to 17 β -HSD1, has been reported to catalyse the reduction of oestrone to oestradiol (Luu-The, Tremblay & Labrie, 2006), but work from this laboratory has shown that it is unable to mediate this reaction (Day *et al.*, 2008b). Instead it is thought to be involved in the metabolism of fatty acids (Moon & Horton, 2003). STS, however, is involved in steroidogenesis, but as it acts to activate steroid sulphates by hydrolysis of their sulphate groups (Stanway *et al.*, 2006), its activity will not affect the study of the effects of 17 β -HSD3 inhibition in this model.

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Conditions for the proof of concept model were optimised using the LNCaPwt cell line. In experiments to monitor hormone-dependent cell proliferation *in vitro*, cells are often grown in medium containing charcoal-stripped FBS as this removes any steroids usually present in FBS (Macdiarmid *et al.*, 1994). However, as the LNCaP cells were not viable in this medium without supplementation for the duration required for this assay, but were viable without proliferation in medium containing 0.1% FBS, 0.1% FBS-containing medium was used as the basal medium for steroid stimulation assays.

Proliferation of both LNCaPwt and LNCaP[HSD3] cells was efficiently stimulated by the addition of 10 nM T or DHT to the basal medium. Despite being an AR antagonist, hydroxyflutamide also efficiently stimulated the growth of both cell lines. This effect is well-documented in LNCaP cells, as they express a mutated AR which is stimulated by some anti-androgens (Veldscholte *et al.*, 1992). Hydroxyflutamide was therefore used as a positive control in all LNCaPwt and LNCaP[HSD3] steroid stimulation assays. Although 10 nM Adione caused some stimulation of proliferation in the LNCaPwt cells, the expression of 17 β -HSD3 caused this to increase in the LNCaP[HSD3] cells to the same level seen for T or DHT, indicating that the enzyme was functioning efficiently in this model.

STX2171 caused cell cytotoxicity at 5 μ M, but both STX2171 and STX2624 inhibited Adione-stimulated proliferation of LNCaP[HSD3] cells at 500 nM without noticeable toxicity, STX2171 being most efficacious. A dibenzothiazocine compound, STX2622, previously identified by BMS as a potential 17 β -HSD3 inhibitor (DBT 1; Fink *et al.*, 2006), however, failed to inhibit the stimulation by Adione, even at 5 μ M, despite having a similar IC₅₀ for 17 β -HSD3 inhibition to STX2171 and STX2624 in the whole cell assay. It is possible that this may be due to either the stability of

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this compound in this longer term assay, or to its effects on other enzymes as its selectivity over 17 β -HSD1 and 17 β -HSD2 inhibition has not been determined. None of the three compounds tested caused any stimulation of growth in the absence of Adione, indicating that the compounds were not themselves androgenic.

Further evidence of the specificity of the inhibitory effect of STX2171 for 17 β -HSD3-dependent Adione conversion was obtained in a similar study using LNCaPwt cells, in which cells were grown in either 0.1% or 10% FBS medium, or in 0.1% FBS medium and stimulated by Adione, T, DHT or hydroxyflutamide. Only Adione-stimulated growth was inhibited by STX2171, indicating that STX2171 does not have antagonistic effects on the AR, and that it is not cytotoxic at this dose.

In a preliminary model, LNCaPwt xenografts were established in castrated mice supplemented with 100 μ g Adione (s.c.) / day. After two weeks of dosing at 25 mg/kg/day (s.c.), in comparison to vehicle-treated animals, STX2171 efficiently inhibited the growth of these tumours by 81%. Despite the fact that several groups are now working on inhibitors of 17 β -HSD3 (Bérubé & Poirier, 2007; Fink *et al.*, 2006; Lota *et al.*, 2006; Tchédam Ngatcha *et al.*, 2005; Le Lain *et al.*, 2002; Maltais, Luu-The & Poirier, 2002), this is the first published report of anti-tumour activity of a 17 β -HSD3 inhibitor in an *in vivo* model. However, this is a preliminary model and thus has limitations, despite its demonstration of STX2171 bioavailability and anti-tumour activity. In this report we have demonstrated that the wild type cells *in vitro* are stimulated to some extent by Adione, despite their low level of expression of 17 β -HSD3, and that this stimulation is inhibited by STX2171. This indicates that the inhibition of tumour growth *in vivo* may also be due to inhibition of the minimal levels of 17 β -HSD3 expressed by these tumours. To determine whether the *in vivo* activity is entirely a result of 17 β -HSD3 inhibition, whether STX2171 also has any

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cytotoxic effect on the tumours, or whether 17 β -HSD5 activity could be involved in Adione to T conversion in these tumours, the model requires further refinement. Initially, the preferred model for future studies would be the use of xenografts formed from inoculation of the mice with LNCaP[HSD3] cells, developed for use in the *in vitro* studies reported in this paper. Plasma testosterone concentrations, as well as tumour volume, should be determined in non-stimulated, Adione-stimulated, and STX2171-inhibited groups. The efficacy of STX2171 in castrated and intact mice will also be explored. These LNCaP[HSD3] xenograft studies are presently on-going.

In conclusion, a primary screening assay and proof of concept model have been developed to study the efficacy of 17 β -HSD3 inhibitory compounds. Active compounds are 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. The lead compound, STX2171, has anti-tumour activity in an *in vivo* hormone-dependent prostate cancer model. These models provide evidence that 17 β -HSD3 inhibitory compounds may have application in the treatment of hormone-dependent prostate cancer.

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FIGURES

Figure 1. The interconversion of Adione and T by 17 β -HSD3 & 17 β -HSD2.

Figure 2. Expression and activity of 17 β -HSD3 after cDNA transfection and selection of 293-EBNA cells. **a.** Expression of 17 β -HSD3 mRNA in 293-EBNA cells after transfection relative to 293-EBNAwt levels (transient = 48 hours post-transfection; [HSD3] pX = no. of passages after selection of transfected cells for stable expression). **b.** Adione to T activity of the transfected cells 48 hours after transfection (2 hour assay in 24 well plates).

Figure 3. The structures and 17 β -HSD3 inhibitory activities of STX2171, STX2624 & STX2622. (IC₅₀ = concentration at which the reaction is 50% inhibited).

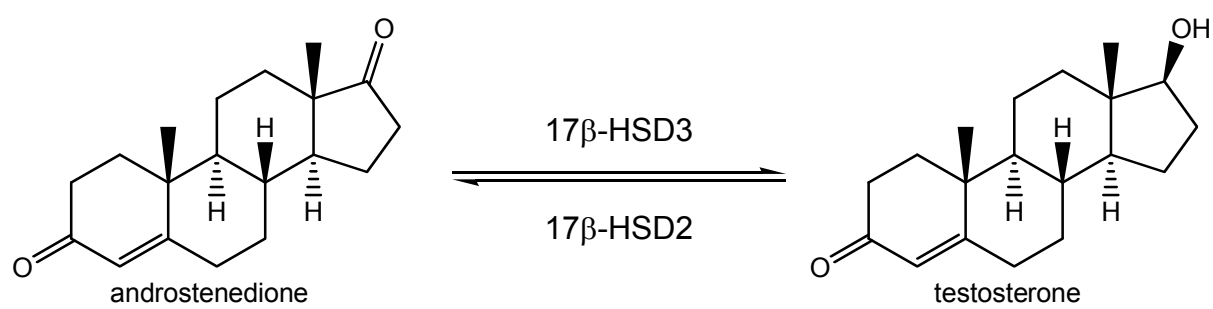
Figure 4. LNCaP model development. **a.** Expression of steroidogenic enzyme mRNA in LNCaPwt cells (relative to the expression of 17 β -HSD1). **b.** Expression of 17 β -HSD3 mRNA in LNCaP[HSD3] cells in comparison to LNCaPwt, 293-EBNAwt and 293-EBNA[HSD3] cells. **c.** Adione to T activity of LNCaPwt and LNCaP[HSD3] cells in comparison to 293-EBNAwt and 293-EBNA[HSD3] cells. **d.** Stimulation of LNCaPwt cell proliferation over 14 days (A = Adione; OH-F = hydroxyflutamide; % = % of non-stimulated growth in 0.1% FBS-containing medium). **e.** Stimulation of LNCaP[HSD3] cell proliferation over 15 days (A = Adione; OH-F = hydroxyflutamide; % = % of non-stimulated growth in 0.1% FBS-containing medium).

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Figure 5. Inhibition of Adione-stimulated proliferation of LNCaP[HSD3] and LNCaPwt cells *in vitro*. **a.** The effect of 5 μ M of the 17 β -HSD3 inhibitors, STX2171, STX2624 and STX2622, on proliferation of LNCaP[HSD3] cells stimulated by 10 nM Adione (A). (OH-F = hydroxyflutamide) **b.** The effect of STX2171 at 500nM and STX2624 at both 500 nM and 5 μ M on proliferation of LNCaP[HSD3] cells stimulated by 10 nM Adione. **c.** The effect of 500 nM STX2171 on the stimulation of LNCaPwt cells by 10 nM Adione (A), T or DHT, or 1 μ M hydroxyflutamide (OH-F).

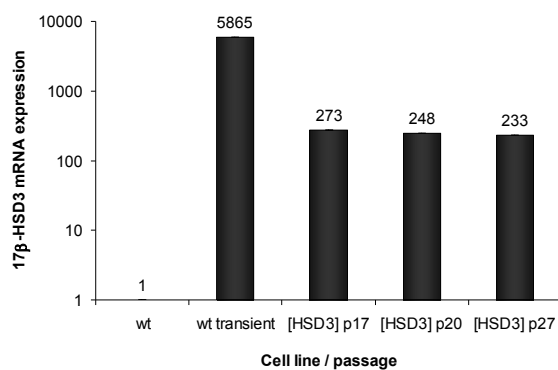
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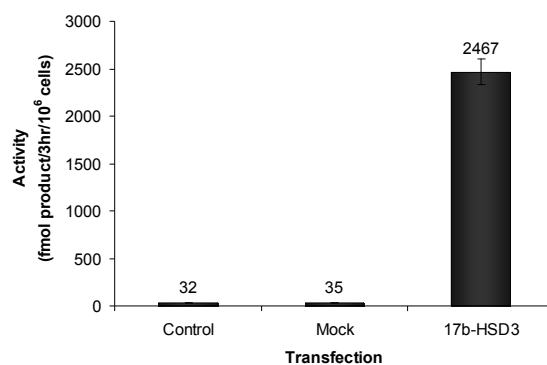


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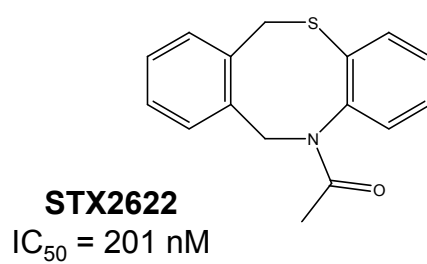
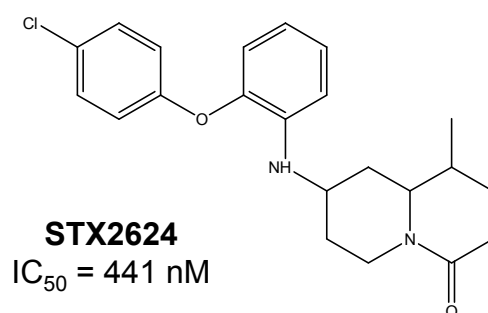
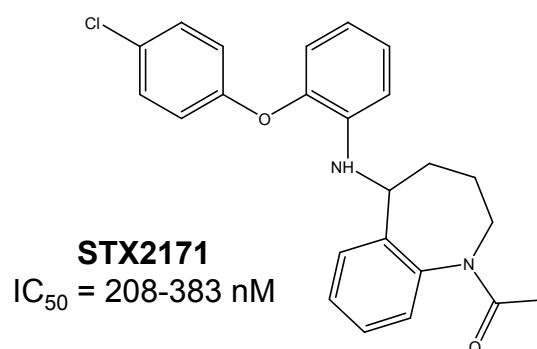
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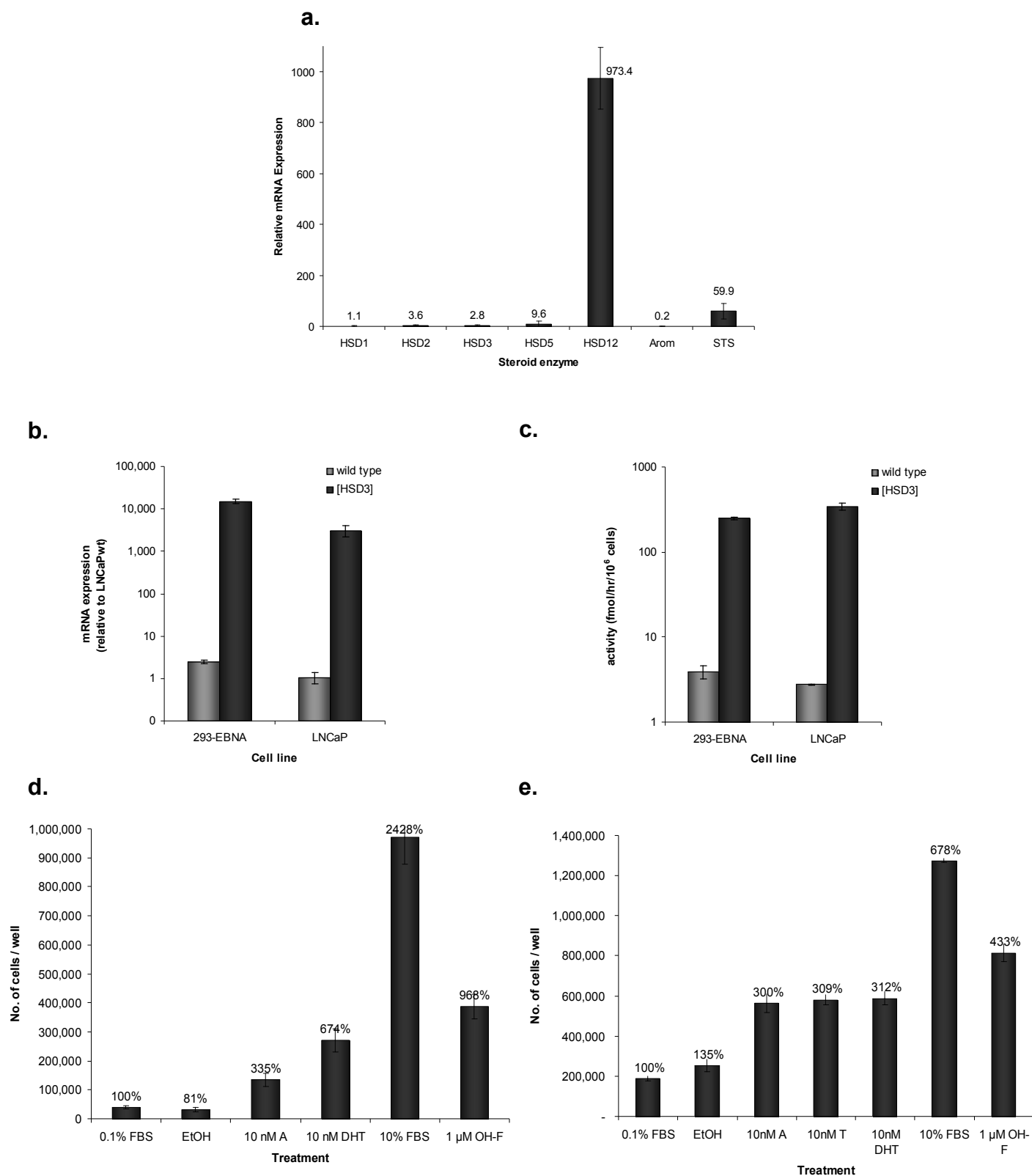
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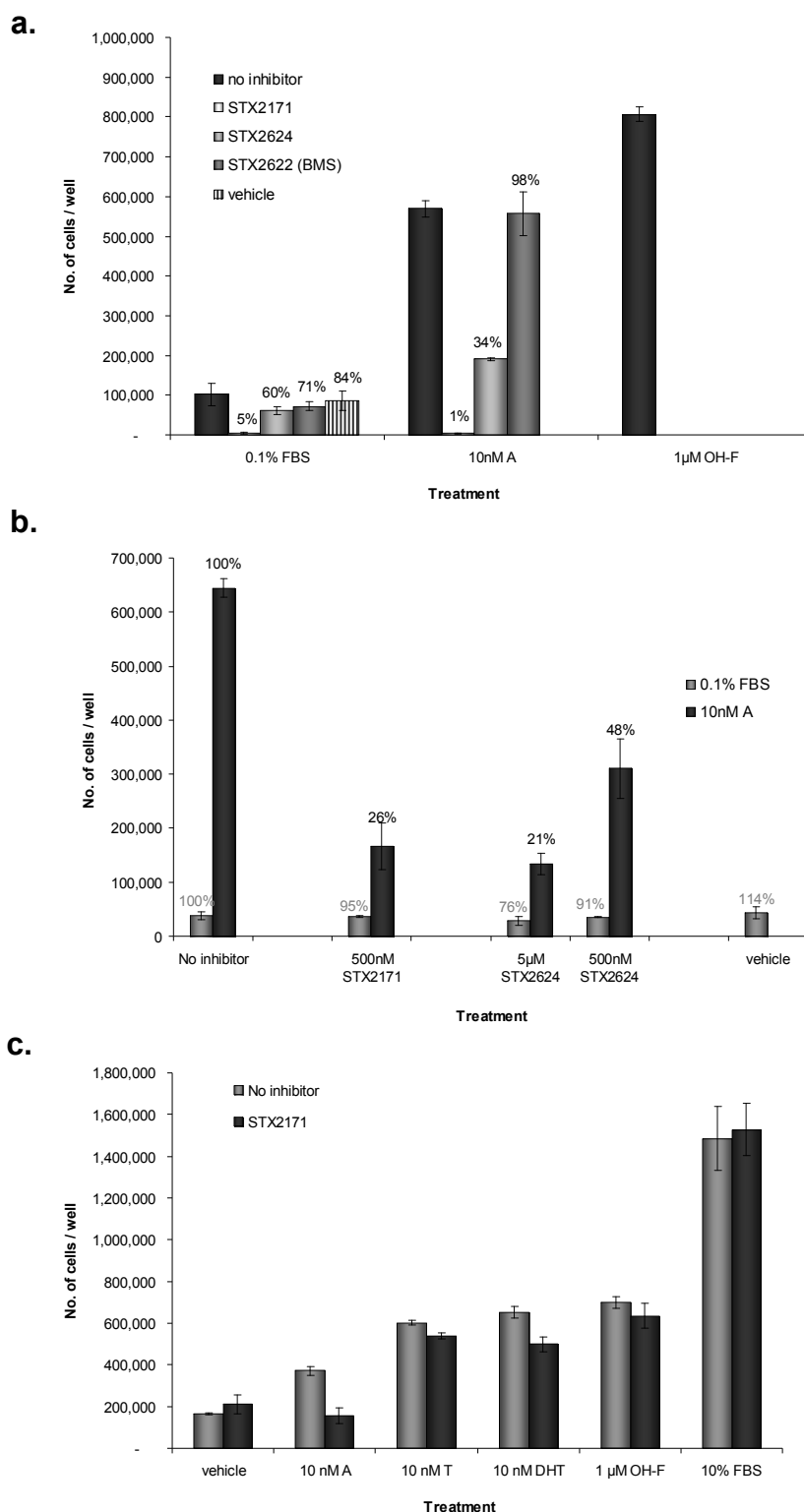
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