Inhibition of steroid sulphatase activity in endometriotic implants by 667 COUMATE: a potential new therapy

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BACKGROUND: Local biosynthesis of estrogens is thought to be important for the maintenance and growth of endometriotic implants. In addition to the formation of estrogen via the aromatase pathway, steroid sulphatase (STS), which is responsible for the hydrolysis of estrogen sulphates, may be an important source of estrogens in endometriosis. METHODS: Eutopic and ectopic endometrial samples from 14 women with minimal or mild (MM) endometriosis and from 13 women with moderate to severe (MS) endometriosis were analysed for aromatase and STS activities. RESULTS: Aromatase and STS activity were detected in all samples. STS enzyme activity in both eutopic and ectopic endometrium was considerably higher and less variable than aromatase activity. Moreover, STS, but not aromatase, activity in endometriotic implants correlated with the severity of the disease (mean ± SEM: 203 ± 38 nmol/4 h/g wet weight tissue in MM disease versus 423 ± 44 nmol/4 h/g wet weight tissue in MS endometriosis, P < 0.001). The STS inhibitor 667 COUMATE almost completely blocked STS activity (>99%) in both eutopic and ectopic tissues. CONCLUSIONS: The high levels of STS activity detected in ectopic endometrium and the correlation with severity of disease suggest that STS inhibitors could be useful for the treatment of endometriosis.

Keywords: steroid sulphatase; aromatase; estrogen; endometriosis; therapy

Introduction

Endometriosis is a disease characterized by ectopic growth of endometrial tissue outside of the uterine cavity. Most lesions are located within the peritoneal cavity, involving the peritoneum, bowel, ovaries and pelvic cul-de-sac. Endometriosis is one of the most common benign gynaecological conditions, estimated to affect 5–10% of women of reproductive age (Vigano et al., 2004). It is associated with severe pelvic pain and other debilitating symptoms such as dysmenorrhea, dyspareunia and menstrual disturbances. Endometriosis is also a frequent cause of subfertility and infertility. The most likely cause of endometriosis is thought to be retrograde menstruation, a theory first proposed in the 1920s (Sampson, 1927). However, retrograde transportation of menstrual effluent occurs in most women of reproductive age, indicating that other factors, such as altered local immune responses, are likely to be involved in the development of endometriosis (Halme et al., 1998).

Endometriosis only rarely occurs in post-menopausal women, suggesting that estrogens play a crucial role in the development and maintenance of this condition. Current medical therapies for endometriosis therefore include GnRH agonists, progestins, estrogen-progestin combinations and drugs such as danazol which, by interfering with gonadotropin production, reduce estradiol synthesis by the ovaries. Although these therapies can provide some symptomatic relief, there is an urgent need for new therapies to treat this common benign condition more effectively.

In addition to ovarian estrogens, local production of estrogens in ectopic endometrial tissues is thought to be important for disease progression. Transcripts for aromatase (CYP19A1) that encode the enzyme responsible for the conversion of androstenedione to estrone have been detected in endometriotic tissue samples (Noble et al., 1996). Expression of aromatase was found to be higher in samples obtained from ovarian endometriosis than from peritoneal endometriosis with the lowest level of expression being detected in deep endometriotic (adenomyotic) nodules (Heilier et al., 2006). Aromatase activity in stromal cells cultured from ectopic tissue has also been reported to be much higher than in cells derived from eutopic endometrium from the same patient (Noble et al., 1997).

In addition to the aromatase, steroid sulphatase (STS) may also be involved in the intracrine production of estrogens in peripheral tissues (Reed et al., 2005). STS hydrolysates the
conversion of estrone sulphate (E1S) and estradiol sulphate (E2S) to their unconjugated forms (Fig. 1). High concentrations of E1S and E2S are found in blood and tissues where they are thought to act as a reservoir for the formation of active estrogens via the action of STS. The activity of this enzyme is blocked by the potent inhibitor, 667 COUMATE.

STS can generate either estrone (E1) from E1S or the biologically active estrogen, estradiol (E2) from E2S. Aromatase however leads directly to the formation of E1. This estrogen requires reduction by 17β-hydroxysteroid dehydrogenase Type 1 (17β-HSD1) to form E2. RT–PCR and Northern analyses have confirmed that 17β-HSD1 transcripts are present in endometriotic tissue (Zeitoun et al., 1998). The ability of E2 to exert estrogenic effects in endometriotic tissues is further enhanced by the lack of expression of 17β-HSD2, one of the enzymes capable of inactivating E2 (Zeitoun et al., 1998). The activities of all the enzymes required for estrogen synthesis, i.e. aromatase, STS and 17β-HSD1, can be stimulated by cytokines (Purohit et al., 1996; Reed and Purohit, 1997). These include IL-6 and TNFα, which are present in peritoneal fluid (Keenan et al., 1994, 1995). Thus, the local inflammatory response associated with endometriosis may contribute to the production of E2 in ectopic lesions.

The realization that local production of estrogens has a role in supporting the growth of endometriotic tissues has led to the use of aromatase inhibitors for the treatment of this condition (Bulun et al., 2000). Clinical pilot studies have shown that treatment with an aromatase inhibitor, in combination with a progestin, may relieve symptoms in patients resistant to standard therapy (Takayama et al., 1998). Although aromatase inhibitors are in widespread use for the treatment of hormone-dependent breast cancer, the development of STS inhibitors is at a much earlier stage (Reed et al., 1996).

A number of potent irreversible STS inhibitors, including estrone sulphamate and 667 coumarin sulphamate (667 COUMATE; Fig. 1), have now been identified (Woo et al., 2000). These compounds are active in vivo and 667 COUMATE recently completed the first-ever phase I trial of any drug in this class in post-menopausal women with endometriosis.

Figure 1: Pathways for the local formation of estrogens in ectopic endometrial implants
Androstenedione and testosterone can be converted to estrone and estradiol, respectively, by the aromatase. Estrone and estradiol can be converted to their sulphated forms by estrogen- and phenol-sulphotransferases. The sulphated estrogens act as a reservoir for the formation of active estrogens via the action of STS.
metastatic breast cancer (Stanway et al., 2006). In this study, treatment with 667 COUMATE resulted in almost complete inhibition of STS activity in breast tumour and peripheral tissues and significant reductions in serum E1 and E2 concentrations. The availability of potent STS inhibitors, such as 667 COUMATE, suggests that this new type of drug might also have therapeutic potential for the treatment of endometriosis. The present study was designed to measure aromatase and STS activities in matched endometriotic and eutopic endometrial samples and to determine the ability of 667 COUMATE to block STS activity in these tissues.

Materials and Methods
Patients and tissue sampling
Samples of eutopic and ectopic endometrial tissue were collected from 27 patients undergoing laparoscopy for either pelvic pain or subfertility. None of the patients had received hormonal treatment for at least 3 months prior to surgery.

The severity of the disease was scored according to the Revised American Fertility Society classification of endometriosis (1985). Fourteen women were deemed to have MM endometriosis, and 13 MS endometriosis. The mean age (± SD) of patients with MM disease was 37.8 (± 8.0) years and of patients with MS endometriosis 34.9 (± 5.0) years (NS). A comparable number of women were studied during the proliferative (n = 13) and secretory (n = 14) phase of the cycle. Biopsies were taken from peritoneal implants in each patient and the presence of endometriosis was confirmed by histology. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until assayed.

Steroid sulphatase activity
Samples of eutopic or ectopic endometrium (0.5–1.0 g) were homogenized in phosphate buffered saline (PBS, pH 7.4 containing 250 mM sucrose, 10 mM NaCl). This buffer, at pH 7.4, was used as previous studies have shown that although this pH is optimal for the measurement of STS activity, other aryl sulphatases remain inactive (Purohit et al., 1995c). Duplicate aliquots of the homogenized tissue were incubated for 4 h with [3H]-E1S (53 Ci/ml, 2–3 mM, Sigma, Poole, Dorset, UK). [4,14C] Estrone (1 x 10^6 dpm, Perkin Elmer, Boston, MA, USA) adjusted to a final concentration of 20 μM with unlabelled substrate (Sigma, Poole, Dorset, UK). The reaction mixture to monitor procedural losses. Samples were incubated in the absence or presence (1 μM) of the STS inhibitor 667 COUMATE, synthesized at the University of Bath (Fig. 1) (Woo et al., 1996, 1998, 2000). At the end of the incubation period, the product estrene was isolated from the reaction mixture by toluene partition (Duncan et al., 1993). An aliquot of the toluene was removed and the 3H and 14C radioactivity measured by liquid scintillation spectrometry. The mass of estrone sulphate hydrolyzed was calculated from the 3H counts detected, corrected for procedural losses. Because of the blood content of the ectopic endometrial samples, it was not possible to estimate their protein content by colorimetric assay. Results are therefore expressed as mmol product formed/4 h/g wet weight tissue.

Aromatase activity
Aromatase activity was measured in endometrial samples using a tritiated-water release assay similar to that developed to measure activity in breast tumours (Purohit et al., 1995a). For this, tissue was homogenized in phosphate buffer (pH 7.4, 0.05 M containing 0.25 M sucrose and 1 mM NADPH; 5 ml/g tissue). Aromatase activity was measured using [1β-3H] androstenedione (2–3 nM, 25 Ci/mmol, Perkin Elmer) over a 20 h incubation period. The ability of the aromatase inhibitor letrozole (1 μM) to block aromatase was also examined. Results for aromatase activity are expressed as nmol product formed/20 h/g wet weight tissue.

Results
Aromatase activity
Aromatase activity was measured in 27 matched pairs of eutopic and ectopic endometrial tissue samples, with 13 obtained during the proliferative phase and 14 during the secretory phase of the cycle. No significant difference in aromatase activity was detected in eutopic samples collected during the proliferative (median and range: 0.46, 0.06–24.5 fmol/20 h/g wet tissue) or secretory phase (median and range: 0.32, 0.06–6.28 fmol/20 h/g wet weight tissue) (Fig. 2A). However, aromatase activity in endometriotic lesions (median and range: 2.58, 0.06–616 fmol/20 h/g wet weight tissue) was significantly higher (P < 0.05) than the activity in eutopic tissues (median and range: 0.35, 0.06–24.5 fmol/20 h/g wet weight tissue) (Fig. 2B). Notably, the level of aromatase activity differed widely in both eutopic and ectopic endometrium, with the highest level (616 fmol/20 h/g wet weight tissue) detected in an endometriotic lesion obtained during the proliferative phase.

STS activity
STS activity was first measured in eutopic endometrial samples. As shown in Fig. 3A, there was a trend towards higher levels of STS activity in proliferative endometrium (mean ± SEM: 569 ± 65 nmol/4 h/g wet weight tissue) as compared with secretory samples (mean ± SEM: 482 ± 52 nmol/4 h/g wet weight tissue), although this difference was not significant (P > 0.05). In contrast to aromatase, STS activity in ectopic samples was significantly lower (mean ± SEM: 313 ± 36 nmol/4 h/g wet weight tissue) than in eutopic samples (mean ± SEM: 523 ± 41 nmol/4 h/g wet weight tissue) (P < 0.001; Fig. 3B).

Severity of endometriosis and enzyme activity
To examine if the severity of endometriosis impacts on the activities of these enzymes, the samples were divided into those from patients with MM disease and those from women with MS endometriosis (Fig. 4). For aromatase activity, the highest activity was detected in an ectopic tissue sample collected from a patient with mild endometriosis. Overall, the levels of aromatase activity in ectopic lesions did not differ between women with MM or MS endometriosis (P > 0.05; Fig. 4A). However, it is notable that the four samples with the highest activity were all obtained from patients with MM disease. This contrasted to STS activity, which was...
Figure 3: STS activity in eutopic and ectopic endometrial tissues
(A) Activity was similar in eutopic tissues collected during the proliferative and secretory phases of the menstrual cycle; (B) Overall, STS activity was significantly lower ($P < 0.001$, Student’s $t$-test) in ectopic than in eutopic endometrial tissues.

Figure 2: Aromatase activity in eutopic and ectopic endometrial tissues
(A) Levels of activity in eutopic endometrium collected from patients during their proliferative or secretory phases of their menstrual cycle; note the logarithmic scale; (B) Activity in ectopic endometrial tissues (median 2.58 fmol/20 h/g wet weight tissue) was significantly higher (Mann–Whitney $U$-test, $P < 0.05$) than in eutopic endometrial tissues (median 0.35 fmol/20 h/g wet weight tissue).
significantly higher (mean ± SEM: 423 ± 44 nmol/4 h/g wet weight tissue) in samples collected from patients with MS endometriosis than in samples from patients with MM endometriosis (mean ± SEM: 203 ± 38 nmol/4 h/g wet weight tissue) (P < 0.001; Fig. 4B).

In view of the higher STS activity detected in ectopic tissues collected from patients with more advanced disease, STS activities in ectopic tissue samples were compared with their corresponding eutopic samples. STS activity in ectopic samples from patients with MM endometriosis was indeed significantly lower than in the corresponding eutopic samples (mean ± SEM: 557 ± 62 versus 203 ± 38, respectively; P < 0.001). In the presence of advanced (MS) disease, STS activity in ectopic lesions was similar to that in eutopic tissues (mean ± SEM: 423 ± 44 nmol/4 h/g wet weight tissue versus 517 ± 52 nmol/4 h/g wet weight tissue, respectively; P > 0.05). Thus, the lower overall STS activity in ectopic versus eutopic endometrium was attributable to the lower level of enzyme activity in implants from patients with minimal or mild disease.

**Inhibition of STS and aromatase of enzyme activities**

The development of potent STS inhibitors raises the possibility that these compounds may be useful for the treatment of endometriosis. To this end, we examined the ability of 667 COUMATE to inhibit in vitro STS activity in eutopic and ectopic endometrial samples. 667 COUMATE proved to be very effective at inhibiting STS activity in these tissues, reducing enzyme activity by >99% in both eutopic and ectopic endometrial tissue samples (Fig. 5A and B). This level of inhibition was comparable to the inhibition of aromatase activity by letrozole of >99% (data not shown) in both eutopic and ectopic samples.

**Discussion**

To the best of our knowledge, this is the first study to measure aromatase activity in eutopic endometrium and ectopic implants from women with endometriosis and to compare this activity with that of STS. The main findings to emerge from this study are that there are significant levels of STS activity in endometriotic implants and, importantly, that the level of activity correlates with the severity of the disease. Furthermore, the potent STS inhibitor 667 COUMATE was found to abolish STS activity in endometriotic tissues.

The limited success of therapies, such as GnRH analogs, which aim to inhibit ovarian estrogen production (Waller and Shaw, 1993), has focused attention on targeting extra-ovarian sources of estrogen production in endometriosis. Bulun and his colleagues were the first to report that transcripts for aromatase could be detected in eutopic as well as ectopic endometrium from women with endometriosis (Noble et al., 1996), an observation confirmed by others (Heilier et al., 2006; Smuc et al., 2007). Brosens et al. (2004) used real-time quantitative PCR and demonstrated that the abundance of aromatase transcripts in eutopic endometrium varied considerably between patients, independently of the phase of the cycle. In agreement, we found that endometrial aromatase activity was comparable in proliferative
and secretory endometrium. Moreover, most eutopic and ectopic endometrial samples had relatively low levels of enzyme activity, although activity ranged from as little as 0.06 to 616 fmol/20 h/g wet weight tissue in an endometriotic lesion from a patient with mild disease. Overall, the level of aromatase activity detected in most eutopic and ectopic samples was comparable to the reported basal levels (2–5 fmol/20 h/mg protein) in cultured endometriotic stromal cells (Velasco et al., 2006).

Previous studies have measured STS activity in normal or malignant endometrial tissues (Adessi et al., 1984; Naitoh et al., 1989) and demonstrated STS expression at the mRNA or protein level in endometriotic tissues (Ezaki et al., 2001; Smuc et al., 2007). In the present study, we found that STS activity is much higher than aromatase activity in eutopic as well as ectopic endometrium. The difference in the activity of both enzymes in our endometrial samples was similar to that reported for breast adipose tissue and cancer. In these tissues, aromatase activity has been shown to range from 10 to 15 fmol/3 h/mg protein, whereas STS activity is several magnitudes higher, ranging between 2 and 3 nmol/3 h/mg protein (James et al., 1987). In breast tumours, estrogen production via the STS pathway has been shown to be at least 10-fold higher than that originating via the aromatase route (Santner et al., 1984).

At a cellular level, endometriotic implants are heterogeneous lesions, consisting of not only endometrial glands and stroma but also smooth muscle, vascular and endothelial cells. Previous IHC studies have shown that STS expression is confined to endometrial epithelial cells in both eutopic and ectopic endometrium, whereas aromatase expression is confined to stromal cells (Ezaki et al., 2001; Acien et al., 2007). Thus, the relative difference in total aromatase and STS activities in eutopic versus ectopic samples may reflect, at least partially, differences in the proportion of stromal and epithelial cells.

Like aromatase, STS activity in eutopic endometrium did not vary significantly according to the phase of the cycle. However, STS activity in endometriotic implants was found to be significantly higher in patients with moderate to severe disease. This contrasted to aromatase activity, which tended to be higher, though not significantly, in MM endometriosis. Although our initial analysis indicated that STS activity is overall lower in ectopic than eutopic endometrium, further analysis revealed that this was due to the relative lower enzyme activity levels in endometriotic lesions from patients with MM disease. This may indicate that women with severe endometriosis may be particularly amenable to STS inhibitor therapy. The findings of a recent study, in which STS mRNA expression was shown to be markedly elevated in ovarian endometriosis, provides further support for the therapeutic potential of targeting STS activity in endometriosis (Smuc et al., 2007).

Danazol, a compound that was widely used for the treatment of endometriosis is known to possess weak STS inhibitory properties (Carlstrom et al., 1984a,b). However, danazol is only a weak reversible STS inhibitor and much less potent than 667 COUMATE which was initially developed for the treatment of patients with hormone-dependent breast cancer (Reed et al., 2005). Use of this drug in a phase I clinical trial resulted in almost complete inhibition of STS activity in peripheral and breast tumour tissues and a significant reduction in serum E1 and E2 levels (Stanway et al., 2006). Unexpectedly, serum concentrations of androstenedione and testosterone, the substrates for the aromatase enzyme, also decreased.

Figure 5: Inhibition of STS activity by 667 COUMATE
At 1 μM 667 COUMATE was able to almost completely block STS activity in: (A) eutopic (>99%) and (B) ectopic (>99%) endometrial tissues.
This finding suggests that, at least in post-menopausal women, most androstenedione originates from the conversion of dehydroepiandrosterone rather than from direct secretion by the adrenal cortex. The hydrolysis of dehydroepiandrosterone sulphate to dehydroepiandrosterone is also blocked by this class of inhibitor (Purohit et al., 1995b). 667 COUMATE was well tolerated in the phase I trial in post-menopausal women with breast cancer with only a few grade 1 or 2 adverse events recorded that were drug related (Stanway et al., 2006). This drug has good bioavailability and was administered orally in the phase I trial.

In summary, this study demonstrated that, in addition to aromatase, STS may also have an important role in local estrogen production in endometriosis. High levels of STS activity were detected in both eutopic and ectopic tissues. Importantly, STS activity in ectopic endometrium correlated with the severity of endometriosis, suggesting a role for this enzyme in disease progression. Furthermore, the STS inhibitor 667 COUMATE was shown to almost completely abrogate enzyme activity in endometriotic tissues, raising the possibility that this compound could be effective in the treatment of this debilitating disease.

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**References**


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