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Inhibition of IL-6+IL-6 soluble receptor-stimulated aromatase activity by the IL-6 antagonist, Sant 7, in breast tissue-derived fibroblasts

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Interleukin 6 (IL-6) and its soluble receptor (IL-6sR) can markedly stimulate aromatase activity in cultured fibroblasts derived from normal or malignant breast tissues. IL-6 acts by binding to a low-affinity membrane-spanning receptor (IL-6R), which must associate with a high-affinity receptor (gp130) for signal transduction to occur. Sant 7 is a mutated form of IL-6 that can bind to the IL-6R, but inhibits its ability to interact with the gp130 signal transducing protein. In this study, we have used Sant 7 to examine its ability to inhibit IL-6+IL-6 soluble receptor (IL-6sR)-stimulated aromatase activity in breast tissue-derived fibroblasts. As previously observed, IL-6+IL-6sR markedly stimulated aromatase activity (7.7–20.8-fold) in fibroblasts derived from reduction mammoplasty tissue, tissue proximal to tumours and breast tumours. Sant 7 inhibited basal aromatase activity in some fibroblasts by 25–30% that had a high basal activity, but almost completely blocked the ability of IL-6+IL-6sR to stimulate aromatase activity. The IC_{50} for the inhibition of IL-6+IL-6sR-stimulated aromatase activity by Sant 7 was 60 ng ml^{-1}. A comparison of the effects of prostaglandin E_2 (PGE_2), which can also regulate aromatase activity, and IL-6+IL-6sR revealed a greater degree of aromatase stimulation by IL-6+IL-6sR. Sant 7, however, inhibited PGE_2-stimulated aromatase activity by 70% suggesting that PGE_2 acts, in part, by stimulating IL-6 production. Much of the IL-6 and IL-6sR available to stimulate breast tumour aromatase activity may originate from infiltrating macrophages and lymphocytes. The ability to block aromatase stimulation by these factors may offer a novel therapeutic strategy for reducing oestrogen synthesis in breast tumours.

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A number of potent aromatase inhibitors have now been introduced for use in postmenopausal women with hormone-dependent breast tumours (Miller, 1999). While their development represents an important advance in the therapies available for the treatment of women with breast cancer, the complete and partial response rates, when used as second-line therapy, remain relatively low (10–20%) with the time to tumour progression being relatively short (3–6 months) (Santen and Harvey, 1999). However, in a recent trial into the adjuvant use of an aromatase inhibitor *vs* tamoxifen, alone or in combination, disease-free survival was significantly longer for subjects receiving aromatase inhibitor therapy (ATAC Trialist Group, 2002). In addition, a number of adverse side effects, including gastrointestinal problems, dizziness and nausea, are associated with the use of some of the inhibitors (Buzdar *et al*, 1997). The use of aromatase inhibitors in postmenopausal women with breast cancer has also been reported to have an unfavourable effect on the serum lipid profile (Elisaf *et al*, 2001). There is, therefore, a need to develop new methods of inhibiting aromatase activity that may act specifically within the breast but spare other oestrogen-sensitive tissues.

The aromatase enzyme complex, which converts androstenedione to oestrone, has a pivotal role in controlling oestrogen synthesis in peripheral tissues in postmenopausal women. The enzyme is present not only in adipose tissue but also in normal and malignant breast tissues (James *et al*, 1987). Previous studies have revealed that the expression of the aromatase gene is regulated in a tissue-specific manner by the use of a number of different promoters (Mahendroo *et al*, 1993; Zhao *et al*, 1995a, b). In adipose tissue expression is regulated by promoter PI.4. The 5′-upstream region of this promoter contains a glucocortcoid response element and a GAS (IFNγ activating sequence) element, which can bind transcription factors of the signal transducer and activation of transcription (STAT) family (Zhao *et al*, 1995a, b). Cytokines in the presence of glucocorticoids regulate gene expression via PI.4. Promoter switching may occur in malignant breast tissues with an increase in the levels of PII and PI.3 being detected (Harada *et al*, 1993; Agarwal *et al*, 1996). Expression of the aromatase gene via PII and PI.3 is regulated by cyclic AMP (cAMP) and there is evidence that prostaglandin E_2 (PGE_2) may be the major factor regulating expression via these promoters (Zhao *et al*, 1996). Evidence has been obtained, however, to suggest that

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PGE_2 may act, at least in part, to stimulate aromatase activity by induction of IL-6 (Zhang *et al*, 1988; Hinson *et al*, 1996).

Fibroblasts derived from normal or malignant breast tissues have been used as a model to investigate aromatase activity as these cells have a much higher level of activity than epithelial cells (Singh *et al*, 1999). Using such fibroblasts a number of cytokines including interleukin 6 (IL-6) and tumour necrosis factor α (TNFα) were identified as important regulators of fibroblast aromatase activity (Reed *et al*, 1992; Macdiarmid *et al*, 1994). Peripheral aromatase activity is increased in elderly and obese subjects (Grodin *et al*, 1973; Hemsell *et al*, 1974) and production of IL-6 is also increased in these conditions (Wei *et al*, 1992; Mohamed-Ali *et al*, 1997).

The ability of IL-6 to stimulate aromatase activity in cultured fibroblasts is markedly potentiated (up to 21-fold) by its soluble receptor, IL-6sR (Singh *et al*, 1995; Zhao *et al*, 1995a, b). IL-6 is produced by fibroblasts derived from normal and malignant breast tissues, whereas IL-6sR was only detected in conditioned medium collected from malignant fibroblasts (Singh *et al*, 1995). Many breast tumours are infiltrated by macrophages and lymphocytes and there is evidence that these cells may be a major source of factors that are able to stimulate aromatase activity within the breast (Purohit *et al*, 1995; Reed and Purohit, 1997).

Like other cytokines IL-6 acts by binding to a membrane-spanning receptor. The IL-6R complex consists of an 80 kDa (gp80) ligand-binding subunit and a 130 kDa (gp130) signal-transducing protein (Taga *et al*, 1989; Kishimoto *et al*, 1995). The gp80 subunit, which can also exist in a soluble form (Rose-John and Heinrich, 1994), binds IL-6 with low affinity and must associate with the larger gp130 for high affinity binding and signal transduction to occur. While IL-6 monoclonal antibodies (Mabs) have been used to abrogate the effects of IL-6, they are only partially effective (Klein *et al*, 1991). The Mabs form a complex with IL-6, which results in reduced clearance of the cytokine.

As an alternative approach to block the actions of IL-6 Ciliberto and colleagues generated a number of IL-6 receptor antagonists, which were mutated forms of IL-6, that can bind to the IL-6R but block it in an inactive configuration (Demartis *et al*, 1996). This inhibits its ability to interact with the gp130 signal-transducing protein. The IL-6 variant Sant 7 was identified as the most potent superantagonist and it was able to inhibit the IL-6-stimulated growth of several different malignant cell types. In view of the important role that IL-6+IL-6sR have in regulating aromatase activity, the ability of Sant 7 to block cytokine-stimulated aromatase activity has been investigated. Sant 7 was also employed to obtain further information about the process by which PGE_2 regulates aromatase activity in breast tissue-derived fibroblasts.

MATERIALS AND METHODS

Culture of fibroblasts

Samples of breast adipose tissue were collected from women undergoing reduction mammoplasty. In addition, samples of breast adipose tissue proximal to breast tumours (i.e. 'non-involved' tissue) and breast tumours were collected from women undergoing lumpectomy or mastectomy. Tissue samples were collected after obtaining subjects' informed consent to the study, which was approved by the hospital Ethics Committee.

Resected tissues were minced with scalpels and incubated in Eagles' modified minimum essential medium (EMEM) for 18–24 h at 37° with collagenase (200 μg ml^{-1}). The dispersed cells were harvested by centrifugation and washed twice with medium to remove collagenase. Dispersed cells were seeded into 25 cm^2 culture flasks and allowed to attach. Cells were grown to confluence in EMEM containing HEPES buffer (20 mmol l^{-1}), 10% fetal calf serum (FCS) and supplements (Reed *et al*, 1992). Cells were routinely passaged two to three times after which replicate 25 cm^2 culture flasks were seeded and grown to 70–80% confluency. The medium was replaced with 2%c. charcoal-stripped FCS, phenol red-free EMEM and treatments were added in this medium for 48 h in the presence of dexamethasone (100 nM) and included: IL-6+IL-6sR (50 and 100 ng ml^{-1}, R&D Systems Ltd, Abingdon, Oxon, UK) and PGE_2 (10 μM, Sigma, Poole, Dorset, UK).

Sant 7

The IL-6R superantagonist Sant 7 was obtained from Sigma-Tau (Rome, Italy) and synthesised as previously described (Salvati *et al*, 1995). Sant 7 was dissolved in culture medium before adding to cells.

Aromatase assay

Aromatase activity was measured in intact fibroblast monolayers using (1β-^{3}H) androstenedione (15–30 Ci mmol^{-1}, NEN- Du Pont, Stevenage, Herts, UK) over a 3–20 h period (Reed *et al*, 1992). Briefly, fibroblast monolayers were washed once with Earle's balanced salt solution EM (2.5 ml) unless stated otherwise. To each flask ^{3}H androstenedione (0.25 μCi) was added to give a final substrate concentration of 3–4 nM. Fibroblast monolayers were incubated with substrate for 3–20 h at 37°C depending on the basal aromatase activity in the cells. Flasks containing no cells were also incubated with substrate and serum-free medium as assay blanks. After incubation, an aliquot of medium (2 ml) was removed from each flask and aliquots were extracted twice with diethylether (5 ml), which was discarded. The remaining aqueous phase was treated with an equal volume of a solution containing charcoal (5.0%) and dextran (0.5%), centrifuged and an aliquot of the supernatant (1 ml) was taken to determine its radioactive content by liquid scintillation spectrometry. It has previously been established that aromatase activity, as measured in fibroblasts, is linear with respect to time for up to 24 h (Macdiarmid *et al*, 1994).

Statistics

The significance of differences in aromatase activity in treated and control cells was assessed using Student's *t*-test. Representative example of results are shown for experiments that were repeated 2–3 times.

RESULTS

The ability of Sant 7 to inhibit cytokine-stimulated aromatase activity was initially examined in fibroblasts derived from breast adipose tissue of a subject undergoing reduction mammoplasty (Figure 1). In these fibroblasts, IL-6 alone, (at 50 ng ml^{-1}) increased aromatase activity by 27%. The addition of IL-6sR in combination with IL-6, however, markedly potentiated its ability to stimulate aromatase activity (7.7-fold compared with controls). Sant 7 caused a significant ($P<0.05$) decrease in basal aromatase activity and IL-6-stimulated activity ($P<0.001$). Sant 7 was able to almost completely block the ability of IL-6+IL-6sR to stimulate aromatase activity. In a further series of experiments, the ability of Sant 7 to block cytokine-stimulated aromatase activity in fibroblasts derived from tissue proximal to a tumour (proximal fibroblasts) and also the tumour (tumour fibroblasts) from the same subject was examined (Figures 2A and B). In the presence of dexamethasone, basal aromatase activity was 10 times higher in proximal fibroblasts than in tumour fibroblasts. The extent to which IL-6+IL-6sR-stimulated aromatase was also greater in proximal fibroblasts (20.8-fold) than in tumour fibroblasts (7.9-fold). No apparent differences were detected in the cellular homogeneity or viability of fibroblasts derived from tumour or

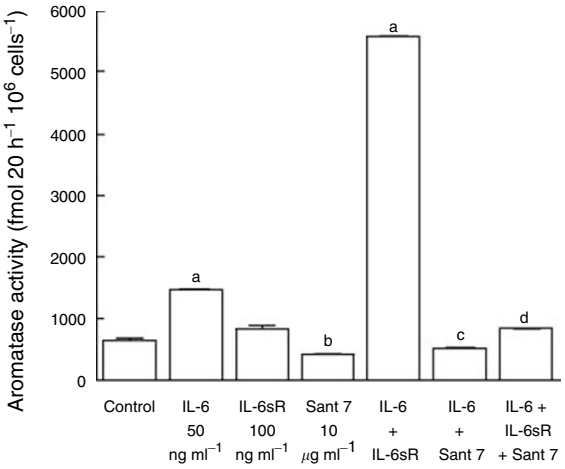

Figure 1 Effect of the IL-6 receptor superantagonist, Sant 7, on IL-6, IL-6sR or IL-6+IL-6sR-stimulated aromatase activity in fibroblasts derived from reduction mammoplasty breast adipose tissue. Fibroblasts were cultured in 2% stripped FCS, phenol red-free EMEM and treatments were added in this medium for 48 h in the presence of dexamethasone (100 nM). Aromatase activity was measured in intact fibroblast monolayers using [^{3}H-1β]androstenedione as the substrate. The significance of differences in treated and control cells was assessed using Student's *t*-test (a, $P<0.001$ *vs* controls; b, $P<0.05$ *vs* controls; c, $P<0.001$ *vs* IL-6; d, $P<0.001$ *vs* IL-6+IL-6sR, means $\pm$ s.d., $n=3$).

proximal breast tissues that might explain the marked differences in basal and IL-6+IL-6sR-stimulated aromatase activity in these different fibroblasts. In both types of fibroblasts Sant 7 completely blocked IL-6+IL-6sR stimulation of aromatase activity. Preincubation of proximal fibroblasts with Sant 7, prior to the addition of IL-6+IL-6sR, did not increase its ability to block aromatase stimulation (Figure 2A). A dose–response study was carried out using proximal fibroblasts. The IC_{50} was calculated as the concentration of Sant 7 that inhibited IL-6+IL-6sR-stimulated aromatase activity by 50% and was 60 ng ml^{-1} (Figure 3).

To examine if dexamethasone was an absolute requirement for the ability of Sant 7 to block IL-6+IL-6sR-induced aromatase activity, an experiment was carried out in the absence or presence of this glucocorticoid (Figure 4). As shown, a similar pattern of responses to IL-6, IL-6sR or Sant 7, alone or in combination was seen in the absence or presence of dexamethasone. However, the extent of simulation by IL-6+IL-6sR was considerably greater (772%) in its presence than in its absence (252%). Sant 7 did inhibit the IL-6+IL-6sR-induced aromatase activity in the absence of dexamethasone.

The regulation of aromatase gene expression is complex and controlled by factors such as PGE_2 and IL-6 (Mahendroo *et al*, 1993). There is evidence, however, that PGE_2 may act, in part, to stimulate aromatase activity by the induction of IL-6 (Singh *et al*, 1999). As Sant 7 effectively blocks stimulation of aromatase activity by IL-6+IL-6sR, it was used to obtain further insight into its regulation by IL-6 or PGE_2. In proximal and tumour fibroblasts, derived from the same subject, PGE_2 stimulated aromatase activity by 521 and 103%, respectively (Figures 5A and B). The combination of IL-6+IL-6sR was considerably more potent at simulating activity in proximal and tumour fibroblasts than PGE_2, by 9.6– and 7.1-fold, respectively. In these fibroblasts Sant 7 itself inhibited basal aromatase activity by 30%. Sant 7 reduced the ability of PGE_2 to stimulate aromatase activity in proximal and tumour fibroblasts by 69 and 75%, respectively. In these experiments the ability of Sant 7 to act in a reversible or irreversible manner was also examined (Figures 4A and B). Fibroblasts were preincubated for 12 h with Sant 7, after which it was removed from the cells by washing with phosphate-buffered

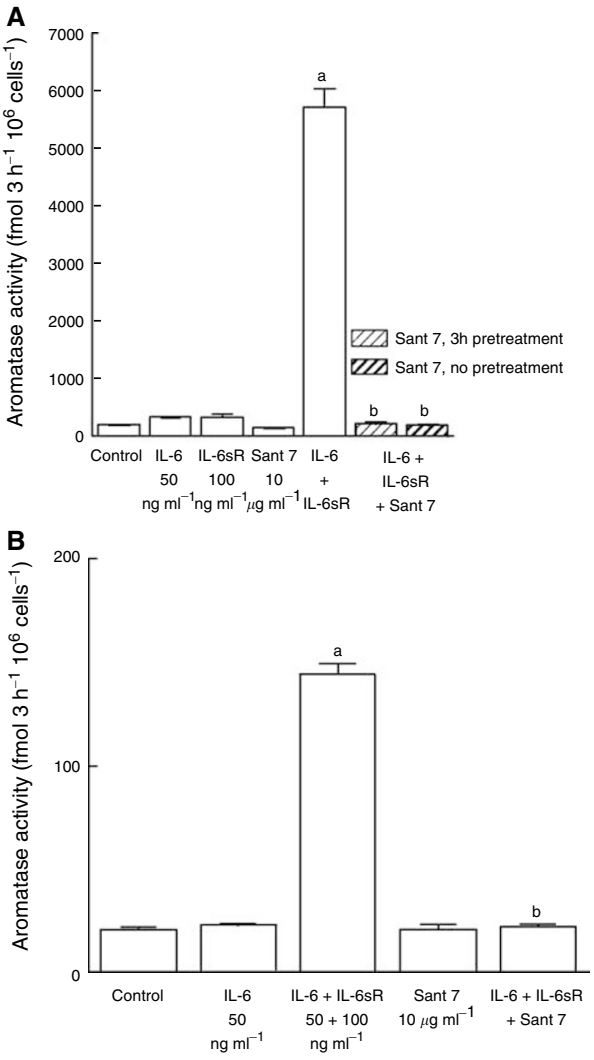

Figure 2 (**A**) Effect of Sant 7 on IL-6, IL-6sR or IL-6+IL-6sR-stimulated aromatase activity in fibroblasts derived from tissue proximal to a breast tumour. Fibroblasts were cultured in 2% stripped FCS, phenol red-free EMEM and treatments were added in this medium for 48 h in the presence of dexamethasone (100 nM). Aromatase activity was measured in intact monolayers using [^{3}H-1β]androstenedione as the substrate. One set of cells (pretreatment) was preincubated with Sant 7 for 3 h before the addition of IL-6+IL-6sR, while for another set (no pretreatment) Sant 7 was added at the same time as IL-6+IL-6sR (a, $P<0.001$ *vs* controls; b, $P<0.001$ *vs* IL-6+IL-6sR, means $\pm$ s.d., $n=3$). (**B**) Effect of Sant 7 on IL-6 or IL-6+IL-6sR-stimulated aromatase activity in fibroblasts derived from a breast tumour. Culture conditions were as described above (a, $P<0.001$ *vs* controls; b, $P<0.001$ *vs* IL-6+IL-6sR, means $\pm$ s.d., $n=3$).

saline. Subsequent addition of IL-6+IL-6sR showed that they were able to stimulate aromatase activity indicating that Sant 7 did not bind to the IL-6R in an irreversible manner.

DISCUSSION

The results obtained from these studies have confirmed previous findings that IL-6+IL-6sR can markedly stimulate aromatase activity in breast tissue-derived fibroblasts (Singh *et al*, 1995; Zhao *et al*, 1995a, b). The potentiation by IL-6sR of the IL-6 stimulation of aromatase activity presumably results from an increase in the interaction of the IL-6–IL-6sR complex with the gp130 signal-transduction protein. In some cell systems the

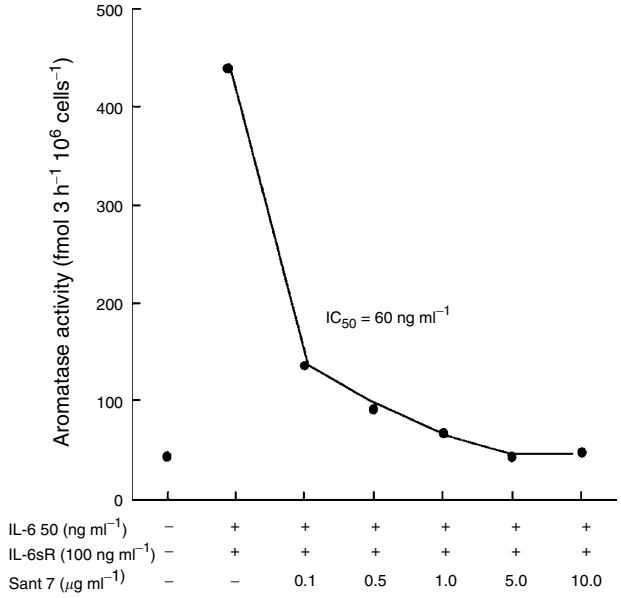

Figure 3 Dose response for the ability of Sant 7 to inhibit IL-6+IL-6sR-stimulated aromatase in fibroblasts derived from tissue proximal to a breast tumour. Sant 7 inhibited the stimulated aromatase activity with an IC_{50} of 60 ng ml^{-1} (means of triplicate measurements for which the coefficients of variation were $<10\%$).

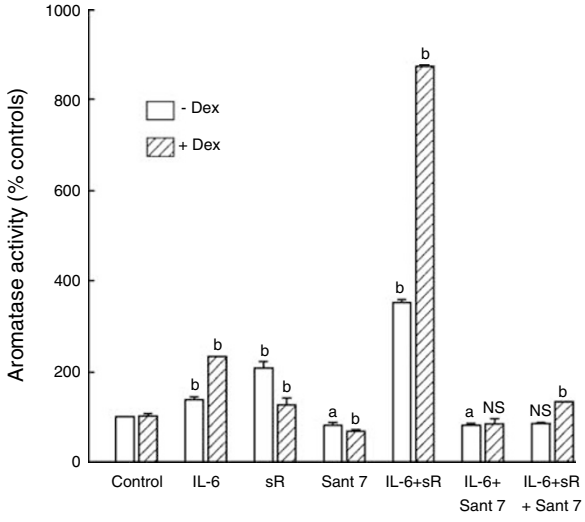

Figure 4 Ability of Sant 7 to inhibit IL-6+IL-6 sR stimulation of aromatase activity in fibroblasts derived from reduction mammoplasty tissue. Cells were treated for 48 h with IL-6 (50 ng ml^{-1}), IL-6sR (100 ng ml^{-1}) or Sant 7 (10 μg ml^{-1}), or in combinations as shown, in the absence or presence of dexamethasone (Dex, 100 nM). Aromatase activity in control cells was 1370 ± 15 fmol 20 h^{-1} 10^6 cells^{-1} and 639 ± 35 fmol 20 h^{-1} 10^6 cells^{-1} for fibroblasts cultured in the absence or presence of dexamethasone (means $\pm$ s.d., $n = 3$; a, $P<0.05$ *vs* controls; b, $P<0.001$ *vs* controls; NS, not significant).

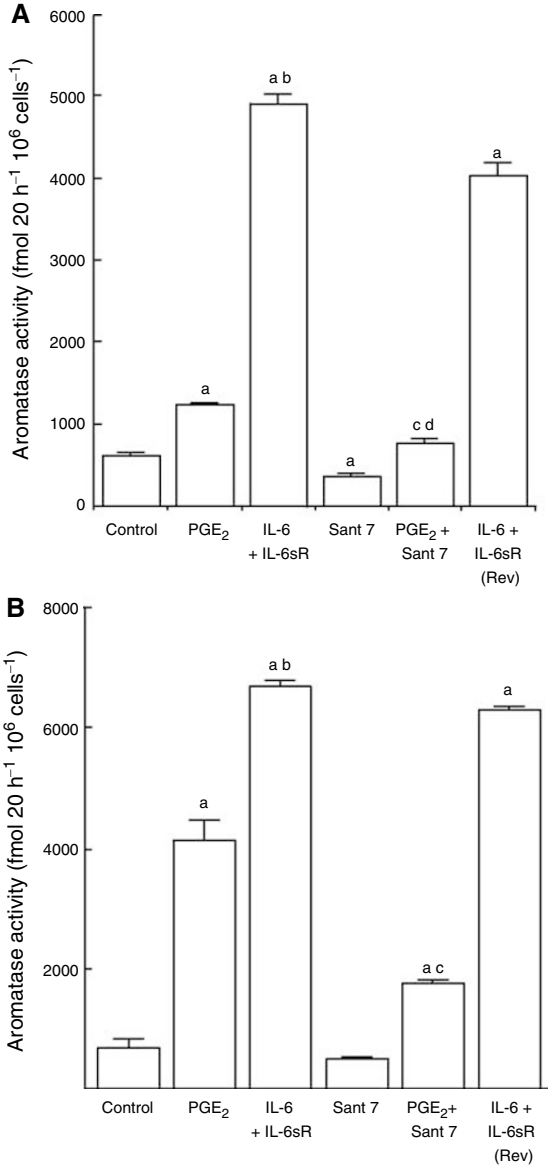

Figure 5 (**A**) Effect of Sant 7 on PGE_2-stimulated aromatase in proximal fibroblasts. IL-6 (50 ng ml^{-1})+IL-6sR (100 ng ml^{-1}) stimulated aromatase activity to a greater extent than did PGE_2 (10 μM). Sant 7, which blocks IL-6+IL-6sR-stimulated aromatase activity, significantly reduced the ability of PEG_2 to stimulate aromatase activity in these fibroblasts. To examine if the effects of Sant 7 were reversible (Rev), one set of fibroblasts (IL-6+IL-6sR (Rev)) was preincubated with Sant 7 for 12 h and then Sant 7 was removed by washing the cells with phosphate-buffered saline. IL-6+IL-6sR stimulated aromatase activity in these cells after washing to a similar extent to fibroblasts not exposed to Sant 7, showing that Sant 7 does not act in an irreversible manner. (a, $P<0.001$ *vs* controls; b, $P<0.0001$ *vs* PGE_2; c, $P<0.001$ PGE_2+Sant 7 *vs* PGE_2). (**B**) As for the legend to Figure 4A but carried out using breast tumour-derived fibroblasts (a, $P<0.001$ *vs* controls; b, $P<0.001$ *vs* PGE_2; c, $P<0.05$ *vs* controls; d, $P<0.001$ *vs* PGE_2, means $\pm$ s.d., $n = 3$).

combination of IL-6+dexamethasone can markedly upregulate the expression of gp130 mRNA (Schooltink *et al*, 1992). In a previous study, the ability of Sant 7 to inhibit the proliferation of multiple myeloma cells was found to be dependent upon the presence of dexamethasone and retinonic acid (Honemann *et al*, 2001). This was not the case in the present study where Sant 7 was able to inhibit IL-6+IL-6sR-induced aromatase activity in the absence of glucocorticoid. The ability of IL-6+IL-6sR to stimulate aromatase activity was almost completely blocked by Sant 7 in all the fibroblasts examined. Sant 7 is a mutated form of IL-6 that binds to the IL-6R with an increased affinity that results in an inactive configuration of the receptor (Demartis *et al*, 1996). In addition to blocking cytokine-stimulated aromatase activity, Sant 7 also reduced the basal activity of this enzyme in some fibroblasts that had a relatively high basal activity, by up to 30%. It has previously been shown that breast tissue-derived fibroblasts can secrete IL-6

Experimental Therapeutics

(Purohit *et al*, 1995). The finding that Sant 7 can reduce basal aromatase activity in these cells suggests that the IL-6 they produce is able to act in an autocrine/paracine manner to increase aromatase activity.

In related studies into the control of aromatase activity, the ability of a number of 10–16 amino-acid peptides to inhibit IL-6+IL-6sR-stimulated aromatase activity was previously examined (Parish *et al*, 2001). The 16 amino-acid peptide, AROHIB, at 10 μM inhibited the ability of these cytokines to stimulate aromatase activity by 65%. AROHIB is therefore a less potent inhibitor of IL-6+IL-6sR-stimulated aromatase activity than Sant 7. Furthermore, to be effective it was necessary to preincubate cells with AROHIB prior to the addition of IL-6+IL-6sR. For Sant 7 no preincubation period was found to be necessary. Sant 7, however, does not bind to the IL-6R in an irreversible manner as preincubation of fibroblasts followed by washing with phosphate-buffered saline restored the ability of IL-6+IL-6sR to stimulate aromatase activity.

There is now good evidence that malignant fibroblasts produce IL-6 and IL-6sR and that tumour infiltrating macrophages and lymphocytes may also be an important source of factors that can stimulate oestrogen synthesis in breast tumours (Purohit *et al*, 1995; Singh *et al*, 1997). If IL-6 and IL-6sR, derived from these cells, are important regulators of aromatase activity, then the use of Sant 7 may offer a means of selectively blocking aromatase stimulation within the breast. Although small molecule-based aromatase inhibitors are being used for breast cancer therapy they can only be used in postmenopausal women. Their use in premenopausal women results in increased gonadotrophin production that overcomes the aromatase blockage. Thus, the ability to inhibit cytokine-stimulated aromatase activity in breast tissues of premenopausal women, either in the preventive or therapeutic setting, could be an important option for the use of Sant 7.

In addition to cytokines stimulating aromatase activity, PGE_2 has also been implicated in the control of this enzyme (Zhao *et al*, 1996). However, determining the extent of regulation of aromatase activity by PGE_2 in fibroblasts is complicated by the finding that PGE_2, or factors that can increase intracellular cAMP levels, can stimulate IL-6 secretion by cells (Zhang *et al*, 1988; Hinson *et al*, 1996). Sant 7 was therefore employed in an attempt to determine whether PGE_2 acts to stimulate aromatase activity by induction of IL-6. It was reasoned that if PGE_2 is acting by the induction of IL-6, then Sant 7 should block, or reduce, its ability to stimulate aromatase activity. It has previously been shown that the ability of PGE_2 to stimulate aromatase activity in breast tissue-derived fibroblasts is associated with a significant increase in IL-6 production by these cells (Singh *et al*, 1997). As consistently observed in previous studies, the ability of PGE_2 to stimulate aromatase activity in proximal and tumour fibroblasts (520 and 100%, respectively) was considerably lower than that achieved with IL-6+IL-6sR (960 and 710%, respectively). Sant 7 reduced the PGE_2 stimulation of aromatase activity by 69 and 75% in proximal and tumour fibroblasts, respectively. As Sant 7 only blocks IL-6-stimulated activity, this important finding indicates that a major part of the ability of PGE_2 to stimulate aromatase activity results from its effect on IL-6 production.

IL-6 is a pleiotropic cytokine that has a number of important physiological functions (Van Snick, 1990). Excess production is associated with a number of pathological conditions including multiple myeloma (Ludwig *et al*, 1991). IL-6 is also known to have a role in regulating androgen receptor expression in an androgen-independent manner (Chen *et al*, 2000). As a result it may be involved in making prostate tumours resistant to endocrine therapy (Lin *et al*, 2001). By blocking the action of IL-6, Sant 7 has been shown to potentiate the sensitivity of the hormone-dependent prostate carcinoma cell line PC-3 to the cytotoxic effects of etoposide and cisplatin (Borsellino *et al*, 1999). The development of IL-6R superantagonists should allow the role of IL-6 in hormone-dependent and -independent conditions, such as breast and prostate cancer, to be elucidated and may lead to their use as novel therapeutic options for their treatment.

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