Reversible control of oestradiol-stimulated growth of MCF-7 tumours by tamoxifen in the athymic mouse

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Summary We investigated the ability of high concentrations of oestradiol to reverse the growth inhibitory action of tamoxifen on MCF-7 breast cancer cells in vivo. Tamoxifen inhibits the oestradiol-stimulated growth of MCF-7 cells in athymic mice. Using a sustained release preparation of tamoxifen we consistently achieved serum concentrations of the drug in the 40 to 50 ng ml⁻¹ range and much higher levels in tissues. These serum levels are sufficient to inhibit the oestrogen stimulated growth of MCF-7 tumours exposed to physiologic (i.e. 300–600 pg ml⁻¹) serum oestradiol concentrations. However, by administering dosages that increase serum oestradiol concentrations to 900–2000 pg ml⁻¹, mimicking the increase often observed clinically in premenopausal women taking tamoxifen, we show that the growth inhibitory action of tamoxifen can be partially reversed. Serum tamoxifen levels were elevated to nearly 400 ng ml⁻¹ by injecting 1 mg day⁻¹ tamoxifen (IP 3 × weekly); this dosage was more effective at inhibiting oestradiol stimulated tumour growth than subcutaneous tamoxifen capsules alone. Our data suggest that tamoxifen may not act optimally. There may be a need to monitor tamoxifen levels in premenopausal patients to ensure that they are high enough not to be overcome by a tamoxifen induced increase in ovarian steroidogenesis.

Materials and methods

Tumours

MCF-7 breast tumours were maintained as serially passaged solid tumours in ovariectomised athymic nude mice (Harlan-Sprague Dawley, Madison, WI), bearing 1.0 cm oestradiol capsules (described below). Tumours were routinely passaged by removing a >1.0 cm diameter tumour from an oestradiol treated animal, trimming away all fat, skin, and necrotic tissue, and mincing the remaining viable tissue into pieces of approximately 1 mm³ in a bath of cold CMF-HBSS. Tumour pieces were then implanted into the thoracic mammary fat pads (1/side) of 4 to 5 week old athymic mice by using a 13 gauge trocar. At the time of tumour transplantation, all animals were also implanted subcutaneously with a 1.0 cm Silastic capsule containing 17β-oestradiol (described below).

Tumour measurements were performed weekly using calipers. Tumour cross sectional areas were calculated using the formula:

\[(\text{length}/2 \times \text{width}/2) \times \pi\]

After 5 weeks of oestrogen treatment, tumours had reached an average size of 0.5 cm³. Animals were then randomised into six groups, and the oestradiol capsules were removed. All of the animals in each group then received one of the following treatments: a 1.0 cm oestradiol capsule, a 2.0 cm tamoxifen capsule, or a 2.0 cm oestradiol pellet, or a 2.0 cm, 1.0 cm or 0.5 cm oestradiol capsule. Tumour cross sectional areas were recorded for each animal, means for each time point were calculated and then standardised to be expressed as a percentage of the tumour area at the outset of treatment. Standard errors were calculated from these standardised tumour areas.

In another experiment athymic mice (18) bitransplanted with MCF-7 tumours were treated with oestradiol (1 cm silastic capsule) until the tumour areas were approximately 0.6 cm³. Animals were divided into three groups of six mice: oestradiol alone, oestradiol plus a 2 cm tamoxifen implant or oestradiol, tamoxifen (2 cm capsule) and tamoxifen 1 mg IP 3 × per week (MWF).

Tumours were measured for 4 weeks, after which animals were sacrificed and tissues were taken for determination of tamoxifen. Oestrogen and progesterone receptors were determined by immunoassay using ER-EIA and PR-EIA kits.

Tamoxifen, a non-steroidal antioestrogen, is the first line antihormonal therapy for breast cancer. Tamoxifen was originally introduced to treat advanced breast cancer in postmenopausal patients (Cole et al., 1971), however the drug has proved to be effective in premenopausal patients as well (Buchanan et al., 1986; Ingle et al., 1986; Manni & Pearson, 1980; Sawka et al., 1986). Recently tamoxifen has been evaluated as an adjuvant therapy in premenopausal women with either node positive (CRC Adjuvant Breast Trial Working Party, 1988; Novladez Adjuvant Trial Organization, 1988) or node negative disease (Breast Cancer Trials Committee, Scottish Cancer Trials Office, 1987; CRC Adjuvant Breast Trial Working Party, 1988; Fisher et al., 1989). An early analysis of the clinical trials data demonstrates an increase in disease free survival in those women receiving the antioestrogen. Indeed the use of tamoxifen is being extended to treat normal premenopausal women to evaluate whether an antioestrogen can prevent breast cancer (Powles et al., 1990).

However, the question can be asked whether antioestrogen therapy in premenopausal women is an optimal strategy. Tamoxifen is known to cause an elevation in ovarian steroidogenesis whether given in a short course (Groom & Griffiths, 1976; Senior et al., 1978) or as continuous therapy (Jordan et al., 1987; Jordan et al., 1991; Manni et al., 1979; Sherman et al., 1979). Clearly, if tamoxifen is a competitive inhibitor of oestrogen action through the oestrogen receptor, then an increase in oestrogen levels might reverse the antitumour action of tamoxifen. We have addressed this question in a laboratory model. Tamoxifen inhibits the oestrogen-stimulated growth of tumours derived from the MCF-7 breast cancer cell line that have been inoculated into athymic mice (Gottardis et al., 1988). We have now evaluated the relative ability of oestradiol and tamoxifen to control the growth of MCF-7 tumour cells in vivo. The significance of our findings is discussed in its clinical context.

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(Abbott Laboratories, Chicago, IL). Assays were performed following the standard kit protocol, except that tumours were homogenised in buffer containing 0.4 M KCl during cytosol preparation.

Drug administration

Oestradiol was administered by subcutaneous implantation of either Silastic capsules or sustained release cholesterol pellets containing 1.7 mg oestradiol (Innovative Research of America, Toledo, OH). Tamoxifen was administered by either subcutaneous implantation of a Silastic capsule or IP injection.

Silastic capsules were prepared by plugging one end of various length pieces of medical grade Silastic tubing (0.078 in ID by 0.125 in OD; Dow corning, Midland, MI) with Silastic silicone type A medical adhesive (Dow Corning) and then filling with either crystalline tamoxifen – free base (Sigma Chemical Co., St Louis, MO) or 17β-oestradiol (Sigma) mixed 1:3 (w/w) with silastic 382 medical grade elastomer (Dow Corning) without catalyst. Capsules were completed by filling the open end with adhesive and sterilising with radiation (>200 Gy).

Tamoxifen injections (IP) were prepared with 1 mg in 0.1 ml peanut oil. The tamoxifen was added to the required volume of peanut oil and mixed with ethanol to aid solution. The ethanol was evaporated under N2, with gentle heating (50–60°C) on a mantle.

Oestradiol and tamoxifen measurements

Circulating 17β-oestradiol and tamoxifen levels were measured as previously described (Jordan et al., 1987; Langan Fahey et al., 1990) in serum samples taken from tumour bearing mice. Blood samples were obtained by bleeding from the eye orbit under light ether anaesthesia or at autopsy. After clotting overnight, samples were centrifuged at 2,000 g; serum was removed and stored at −20°C until analysis.

Tamoxifen measurements in tissues were made using normal phase HPLC with fluorescent detection of the parent compound and metabolites as previously described (Robinson et al., 1991).

Statistical analyses

All statistical calculations were performed using Minitab version 6.1 (Minitab, Inc., State College, PA) on an IBM PS/2 model 50Z or Tandy 3000 HD personal computer.

Results

Serum and tissue levels of tamoxifen in athymic mice during treatment with a sustained release silastic capsule

In order to assess the efficacy of our sustained release preparation of tamoxifen, non-tumour bearing athymic mice were implanted with 2 cm tamoxifen capsules. At 2 week intervals beginning 2 weeks after capsule implantation serum samples were obtained by drawing blood from the eye orbit of a random sample of mice; a subset of the mice were sacrificed and liver, uterus and muscle samples were collected for analysis of tamoxifen concentration.

As Figure 1a shows, serum tamoxifen levels did not change significantly, remaining around 30–40 ng ml−1 (0.08–0.11 μM) for the duration of treatment. Although a slight downward trend was evident towards the end of the experiment, neither the values were significantly different from each other.

Figure 1b shows the concentrations of tamoxifen in muscle, liver and uterine tissue samples taken during the course of the experiment. Tamoxifen levels in uterus and muscle remained fairly constant, showing slight decreases over time, possibly as drug levels in the capsules were gradually depleted. Mean tamoxifen levels in liver tissue, although fairly constant over time, showed much greater variability at each time point than any of the other tissues examined. This may be due to inter-animal variation in hepatic metabolic ability, or variability in the efficiency of extraction of drug from the lipid rich hepatic tissue. However, the internal enclomiphene control used in the tamoxifen assay makes the latter possibility relatively unlikely.

Partial reversal of the growth inhibitory action of tamoxifen on breast tumours and uterine tissue in vivo by increasing serum concentrations of oestradiol

MCF-7 breast cancer cells, when injected into the mammary fat pads of oestrogen treated ovariectomised athymic mice, form tumours at high frequency. These tumours can be serially transplanted into other oestradiol treated athymic mice. However, if the oestradiol was removed from animals bearing these tumours and replaced with tamoxifen, or if the tumours were left in an oestrogenised environment and tamoxifen was added, tumour growth rate was reduced to nearly zero (Figure 2). Moreover, if the amount of oestrogen administered to tumour bearing animals was increased, resulting in a corresponding increase in serum oestradiol concentrations, the growth inhibitory effect of tamoxifen was reversed, although even extremely large doses of oestrogen did not return tumour growth rate to that seen in a tumour exposed to oestradiol alone in the absence of tamoxifen (Figure 2). Two-sample t-tests performed on mean relative tumour areas from the final week of this experiment for every possible pairing of groups show that the mean relative tumour area for each group was significantly different from every other group, with one exception. There was no statistically significant difference between the mean relative
tumour areas for the groups treated with tamoxifen plus either a 2.0 cm or a 1.0 cm oestradiol capsule. Uterine wet weights were also measured in all test animals at the end of the experiment. Only the highest dose of oestradiol administered could partially reverse the inhibition of uterine growth by tamoxifen (Figure 3). The anti-oestrogenic action of sustained release preparations of tamoxifen in the mouse uterus has previously been noted (Jordan et al., 1990).

Oestradiol and tamoxifen concentrations in the serum and various tissues of experimental animals

Table I shows the circulating levels of 17β-oestradiol and tamoxifen detected in the serum of the animals in each group in the variable oestrogen dose experiment. Significant differences in serum oestradiol concentrations between groups are addressed individually in Table I. No significant differences were detected among the serum tamoxifen levels in any of the tamoxifen treated groups.

Mean tamoxifen levels in the serum as well as in several tissues of animals in each experimental group receiving tamoxifen are shown in Figure 4. No significant differences were detected among any of the experimental groups for any of the tissues with one exception. Tamoxifen levels in the uteri of animals receiving tamoxifen in conjunction with either an oestradiol pellet or a 2.0 or 1.0 cm oestradiol capsule were

| Table I | Serum oestradiol and serum tamoxifen levels in each experimental group |
| --- | --- |
| **Group** | Serum oestradiol (pg ml^-1)*** | Serum tamoxifen (ng ml^-1)*** |
| 1.0 cm capsule | 755.8±152.3 | N/A |
| TAM++ + E2 pellet | 1949.4±537.7 | 47.14±8.19† |
| TAM++ + E2 2.0 cm capsule | 927.7±76.5 | 54.00±6.36 |
| TAM++ + E2 1.0 cm capsule | 543.3±91.8 | 42.12±6.69 |
| TAM++ + E2 0.5 cm capsule | 365.2±87.6 | 47.60±6.21 |
| TAM++ alone | 9.1±3.6 | 52.07±8.27 |

*All animals were treated with E2 1.0 cm capsules for the first 5 weeks and then were divided into six groups. **Circulating 17β-oestradiol was measured by RIA 6 weeks after animals were divided into six groups. ***Circulating tamoxifen was measured by HPLC fluorescence assay (Langan-Fahey et al., 1990; Robinson et al., 1991) 6 weeks after animals were divided into six groups. †Mean ± s.e. ††TAM: 2.0 cm tamoxifen capsule. *E2 alone vs tamoxifen + E2 pellet, *P = 0.07. ‡Significantly different from tamoxifen alone, *P < 0.01. §Significantly different from tamoxifen + 0.5 cm E2 capsule, *P < 0.05. ¶Significantly different from tamoxifen + 1.0 cm E2 capsule, *P < 0.05. #Significantly different from tamoxifen + 2.0 cm E2 capsule, *P < 0.005. **Significantly different from tamoxifen + E2 pellet, *P < 0.05. ***Significantly different from E2 alone, *P < 0.05.
significantly lower than those in animals receiving only tamoxifen. It should also be noted that of all tissues measured, tamoxifen levels were consistently highest in tumour tissue of each experimental group.

Table II shows concentrations of tamoxifen and its principal metabolites in serum, muscle and tumour and oestradiol concentrations in serum for the experiment in which animals received either a 2 cm capsule as their only source of tamoxifen, or were also administered 1 mg IP injections of tamoxifen three times weekly. This dose of tamoxifen brought about a 10 fold increase in serum levels, and was coupled with greater than 20-fold increases in tamoxifen levels in tumour tissue and nearly a 30 fold increase in tamoxifen concentration in the non-oestradiol target muscle tissue.

In animals receiving high dose injections, detectable levels of the metabolites, 4-hydroxytamoxifen and N-desmethyl tamoxifen (N-des) were detectable (Table II) but at lower concentrations than the parent compound.

Effect of a dose increase of tamoxifen on oestradiol-stimulated MCF-7 tumour growth and steroid receptor content

Tumour cross sectional areas from MCF-7 bearing mice treated with oestradiol alone, oestradiol plus a tamoxifen capsule, or oestradiol with a tamoxifen capsule plus IP tamoxifen injections are shown in Figure 5. Although administration of tamoxifen capsules alone was sufficient to block further increases in tumour size, even greater inhibition, i.e., a reduction in tumour area, was achieved by the administration of a high dose of tamoxifen.

Figure 6 shows the amounts of oestrogen and progesterone receptors measured in the tumours in this experiment. It is apparent that although tamoxifen blocked the oestradiol induced increase of progesterone receptor in tumour cells in a dose dependent fashion, it also induced a dose dependent increase in oestrogen receptor levels in these tumours.

Discussion

The aim of these experiments was to determine the effectiveness of tamoxifen to control the growth of hormone responsive MCF-7 tumours grown in athymic mice under high and low oestrogen environments.

We used a sustained release method (Robinson & Jordan, 1989) to treat tumour bearing athymic mice with tamoxifen. The level of oestradiol we selected was targeted to be within the range normally observed in premenopausal patients during tamoxifen therapy (500–900 pg ml⁻¹). Tamoxifen controlled oestradiol stimulated growth; a result that parallels clinical experience (Breast Cancer Trials Committee, Scottish Cancer Trials Office, 1989; Buchanan et al., 1986; CRC Adjuvant Breast Trial Working Party, 1988; Fisher et al., 1989; Ingle et al., 1986; Manni & Pearson, 1980; Nolvadex Adjuvant Trial Organization, 1988; Sawka et al., 1986). Although the action of tamoxifen was reversed with increasing circulating concentrations of oestradiol, the levels required appeared to be at the top of the range observed clinically in premenopausal women during tamoxifen therapy (Groom & Griffiths, 1976; Jordan et al., 1987; Jordan et al., 1991; Manni et al., 1979; Senior et al., 1976; Sherman et al., 1979). Nevertheless the efficacy of tamoxifen seemed to be optimal in a low oestrogen environment. Indeed it has been suggested (Sawka et al., 1986) that ovarian steroids can reverse the action of tamoxifen since some patients who respond and then fail tamoxifen treatment can subsequently

| Table II Serum and tissue levels of tamoxifen (TAM), metabolites (N-desmethyltamoxifen (N-des), 4-hydroxytamoxifen (40HT)) and serum oestradiol (E₂) in animals receiving high doses of tamoxifen. |
|-----------------------------------------------|
| **Tissue** | **E₂ capsules** | **Treatment** | **E₂ capsules + TAM capsules** | **E₂ and TAM capsules IP TAM injection** |
| Serum TAM | 491 ± 130 pg ml⁻¹ | 304 ± 23 pg ml⁻¹ | 228 ± 21 pg ml⁻¹ |
| Serum 40HT | 38 ± 3 ng ml⁻¹ | 4 ± 2 ng ml⁻¹ | 9 ± 2 ng ml⁻¹ |
| Serum N-des | 0.1 µM | 0.01 µM | 6.5 ± 0.5 µM |
| Muscle TAM | 4.2 ± 0.1 ng ml⁻¹ | 0.001 µM | 6.5 ± 0.5 ng ml⁻¹ |
| Muscle N-des | 0.65 µM | 0.65 µM | 6.5 ± 0.5 ng ml⁻¹ |
| Tumour TAM | 1.2 ± 0.2 ng ml⁻¹ | 1.00 ± 0.05 ng ml⁻¹ | 6.5 ± 0.5 ng ml⁻¹ |
| Tumour 40HT | 5.0 ± 0.5 ng ml⁻¹ | 5.0 ± 0.5 ng ml⁻¹ | 6.5 ± 0.5 ng ml⁻¹ |
| Tumour N-des | 22.6 ± 2.6 ng ml⁻¹ | 22.6 ± 2.6 ng ml⁻¹ | 6.5 ± 0.5 ng ml⁻¹ |

*Metabolite concentrations below the limit of assay detection.
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