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PROGESTERONE RECEPTOR INDUCTION BY DANAZOL IN CULTURED CANCER CELLS AND THE RAT UTERUS

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Summary—We have previously reported that clinical trials relating to the use of danazol in the management of benign breast disease show a positive correlation between favourable clinical response and an induction of progesterone receptors in the affected tissue which is maintained for a period of at least 6 months subsequent to the cessation of treatment.

Further studies designed at elucidating more clearly the actions of danazol at the cellular and molecular levels have confirmed that progesterone receptors are down-regulated by short-term progestin action at the level of the mRNA transcript, but that danazol is subsequently able to produce an enhanced cellular response, inducing progesterone receptors in the presence of oestrogenic agents. Uteri from danazol-treated rats showed a doubling of progesterone receptor concentrations compared with the control uteri. In the mammary cancer cell line T-47D, cells treated with danazol had increased progesterone receptor concentrations of 558.4 ± 32.0 compared with 152.6 ± 7.0 fmol/mg protein in the control cells. In both cases, these inductions were observed following a period of progesterone receptor suppression. Short-term molecular studies on T-47D cells indicated that progesterone and danazol initially inhibit mRNA transcription, but that 24 h after treatment an induction is observed. This is especially marked in the danazol-treated cells.

INTRODUCTION

Mastalgia, with or without nodularity, is symptomatic of a heterogenic condition which comes within the classification of benign breast disease. So far, no clearly defined aetiological factor has been determined. It has been suggested that a hormonal abnormality may be involved and this is reflected in current treatment strategies [1, 2].

Danazol is a synthetic 2,3-isoxazol derivative of 17-α-ethinyltestosterone [3] which has been used extensively in the treatments of endometriosis and benign breast disease [4–7]. It has long been regarded as possessing progestin-like actions, particularly on the human endometrium [8]. Previous studies have shown that danazol can bind to the progesterone receptor (PgR), conferring growth inhibition in a human endometrial cell line [9]. However, its well documented success in the management of mastalgia (currently the most effective agent available with 70% of cyclical cases responding favourably) would not appear to arise from direct progestin action since clinical trials report progestins as not being particularly efficacious [2]. In addition, a clinical trial carried out by our group [10, 11] showed that danazol induced PgR in those patients who responded favourably to treatment. These raised PgR levels were maintained for a period of at least 6 months after the cessation of treatment in those patients who also maintained a favourable clinical response. Such a PgR induction is not typically associated with progestin action [12].

Danazol has a complex endocrine profile, and much of its clinical effectiveness may lie with its anti-gonadotropin effects [13]. It has been suggested that danazol inhibits hypothalamic release of LH [14] and that this action is mediated by androgen receptors (ARs) [15]. The androgenic properties of danazol have been thought to be responsible for the atrophy of endometriotic lesions [16] and also to contribute to side effects such as hirsutism and acne. Such androgenic properties may arise not only through its direct action on the AR but also as a result of danazol suppression of sex hormone binding globulin (SHBG) biosynthesis [17] and of its competition with testosterone for binding

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with SHBG, both leading to an increase in free testosterone levels [18]. Danazol is also known to inhibit several enzymes involved in steroidogenesis [19] and, in addition, has been shown to bind to PgRs but not at all with oestrogen receptors (ERs) [20–22]. Its first use in mastalgia was reported in 1971 [5] and its efficacy has since been verified by numerous clinical trials [23, 24]. It has also been shown to induce an objective response in human breast cancer and with DMBA-induced tumours in the rat [25, 26].

Danazol thus possesses a complex mechanism of action. It has many known effects on hormonal environment and regulation and is observed to generate many metabolic products, including ethisterone [27, 28] which together have compounded to hinder progress aimed at elucidating its precise mode of action. The present studies were aimed at defining more closely the effects of danazol treatment on steroid receptor concentrations. Neither the mammary cancer cell lines, T-47D and MCF-7, nor the rat uterus are representative of the benign breast condition but do, nonetheless, possess functional steroid hormone receptors, and as such are a suitable model for determining the influence of danazol on their mechanism of action. In this paper, the short-term actions of danazol on cellular PgRs and mRNA transcript levels, together with the effects on PgR concentrations following longer treatments, are reported.

EXPERIMENTAL

Cell culture

T-47D cells (obtained from European Collection of Animal Cell Cultures, ECACC) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin and foetal bovine serum (FBS) (Flow Laboratories, Herts., England). MCF-7 cells (Michigan Cancer Foundation, U.S.A.) were maintained in Minimum Essential Medium Eagle Modified (MEMEM), supplemented as previously. Where phenol red free medium was utilized (DMEM, Flow Labs) the serum was first charcoal-stripped to remove endogenous steroids using the method of Butler *et al.* [29].

Subcellular fractionation of cell lines

Cells were harvested from the flasks with 10 ml Trypsin-EDTA solution (Flow Labs) and sonicated on ice in glycerol-phosphate buffer [30% glycerol, 50 mM sodium phosphate, 1.5 mM EDTA, 10 mM monothioglycerol (MTG), pH 7.4]. The sonicate was centrifuged for 1 h at 100,000 *g*, at 4°C to obtain cytosolic fractions (2–3 mg protein/ml cytosol).

Steroid treatment

Cells were grown in continuous monolayer culture in the presence or absence of danazol over a period of weeks. Prior to harvesting the cells, the monolayers were washed and left for 24 h, or 1 week, in danazol-free medium. The effects of progesterone and ethisterone on PgR concentrations were also investigated.

Animal studies

Progesterone, ethisterone and danazol were administered to female Wistar rats, aged 12 weeks, by daily subcutaneous injection at the following concentrations for periods of 24 h, 7 and 14 days: controls, 200 μl dimethylsulphoxide (DMSO)/rat; progesterone-treated, 1.0 mg in 200 μl DMSO; ethisterone-treated, 1.5 mg in 200 μl DMSO; danazol-treated, 1.5 mg in 200 μl DMSO.

Twenty-four hours following the final administration of these steroids the rats were killed by cervical dislocation and vaginal smears were taken to determine the stage of the oestrous cycle in each animal. The uteri were individually removed, defatted and homogenized in 4–5 ml glycerol-phosphate buffer at 4°C. Following centrifugation for 1 h, 100,000 *g* at 4°C, the supernatant was removed for steroid hormone receptor analysis.

Assay for PgRs and ERs

Single saturating dose (SSD) method. Duplicate aliquots of cytosol (100 μl) were incubated with a single saturating dose of 33 nM [^{3}H]-progesterone (Amersham) in the presence or absence of 6.6 μM norethisterone for 18–20 h, at 4°C [30]. Following the incubation period unbound steroid was extracted by dextran-coated charcoal (DCC), and radioactivity was assayed from 500 μl aliquots of each sample in 4 ml scintillation fluid, counted in a Beckman LS 7500 scintillation counter. ER analysis was carried out in a similar manner using 16 nM [^{3}H]oestradiol (Amersham) and 3.3 μM diethylstilboestrol (DES) as competitor. All non-radioactive steroids were obtained from Sigma Chemical Company (England).

Enzyme-linked immunoassay (EIA). PgRs were additionally assayed by the EIA. This was performed in accordance with instructions provided with the Abbott PgR-EIA kit (Abbott Labs, England).

mRNA determination

T-47D cells were grown to semi-confluence in DMEM, 5% FBS. The cells were then placed for 2 days in phenol red free medium, 1% FBS (charcoal stripped). Incubation was continued in this medium in the presence and absence of progesterone (10^{-6} M) or danazol (10^{-6} M). At intervals of 3, 6, 24, 30 and 48 h, four flasks of cells were harvested and RNA was prepared by the method of Cathala *et al.* [31]. 20 μg of RNA from each sample were denatured at 65°C and loaded and electrophoresed on a 1% agarose gel containing 5% formaldehyde [32] at 60 V for 2–3 h. The RNA was transferred to a nylon filter by the Northern blotting technique and fixed by u.v. transillumination. The presence of ethidium bromide in the loading buffer and running buffer allowed the integrity of the RNA to be assessed by u.v. transillumination. This method was also used to confirm that equal amounts of mRNA had been loaded in each lane.

A 1.2 kb clone (A16) of PgR cDNA covering the major part of the A/B and C domains of PgR was used as a probe to detect PgR mRNA. The probe was labelled by the random primer method [33] using ^{32}dCTP (Amersham) to a specific activity of 5×10^8 cpm/μg. The filters were hybridized with the probe in a solution containing 2×10^6 cpm/ml over 1–2 days. After a series of washes with SSPE/SDS washing buffers at 50°C the PgR mRNA species were identified by autoradiography, with an intensifying screen, for 1 to 5 h at −70°C.

Protein estimation

The protein concentration of the cytosols were determined by the method of Lowry *et al.* [34] using bovine serum albumin as a standard.

RESULTS

Animal studies

Short-term (24 h) treatment with danazol and progesterone provoked down-regulation of PgRs (assayed by the SSD method) in the rat uteri [Fig. 1(a)]. These receptor levels were unchanged by ethisterone treatment as compared with control values, which were 61 ± 9.9 fmol/

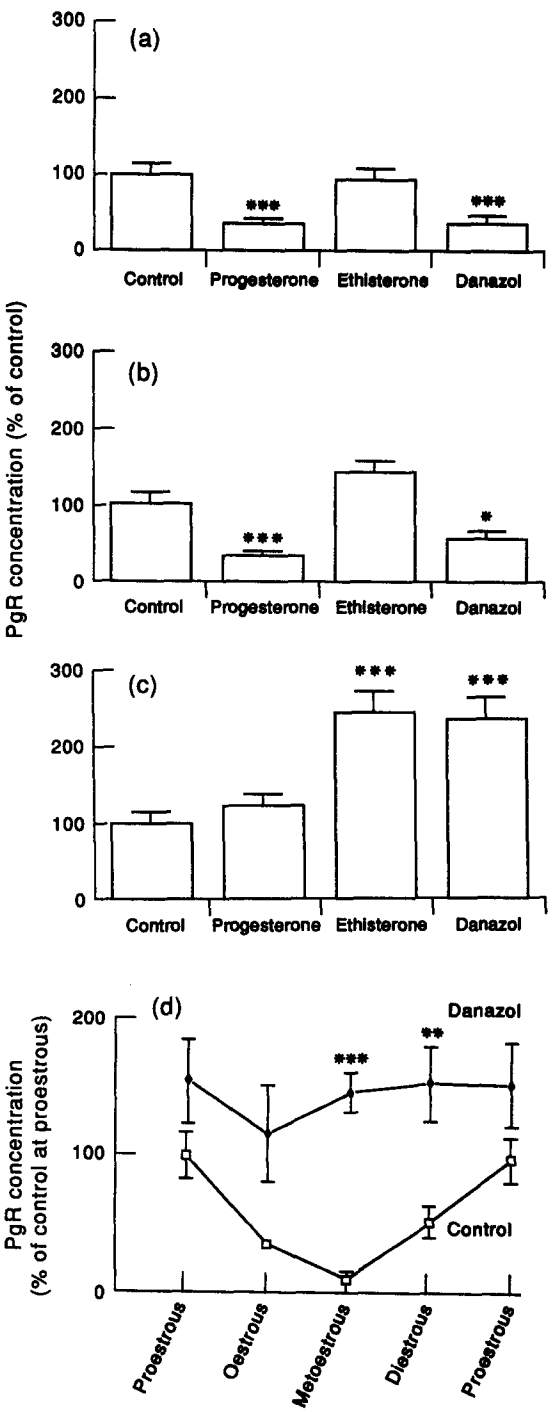

Fig. 1. (a, b and c) Twelve-week-old female rats were treated by subcutaneous injections with danazol, progesterone or ethisterone for (a) 1, (b) 7 or (c) 14 days. Twenty-four hours following the final injection, uteri were individually removed, and PgR concentration was determined by SSD. Each treatment represents a mean ± SEM; $n = 20$ for each treatment or control group. (*** = $P < 0.001$, ** = $P < 0.01$, * $P < 0.05$ as compared with control). (d) PgR concentration throughout the oestrous cycle as assayed by SSD in uteri of female rats treated for 2 weeks with danazol. Each point is represented as mean % of untreated control at proestrous ± SEM. From proestrous to diestrous $n = 5$, 2, 3 and 10, respectively, for control group and $n = 5$, 7, 3 and 5, respectively for the treatment group.

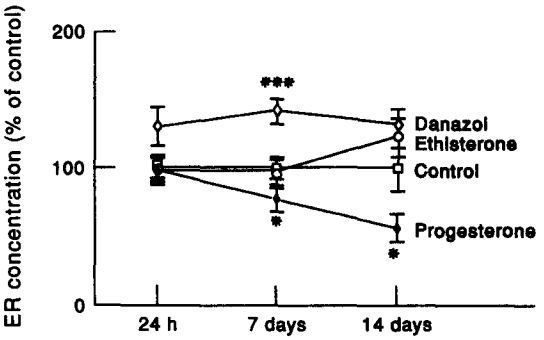

Fig. 2. ER concentration in rat uteri following steroid treatment at 1, 7 and 14 days as described previously. Each point, given as % of control, represents a minimum of 20 animals ± SEM.

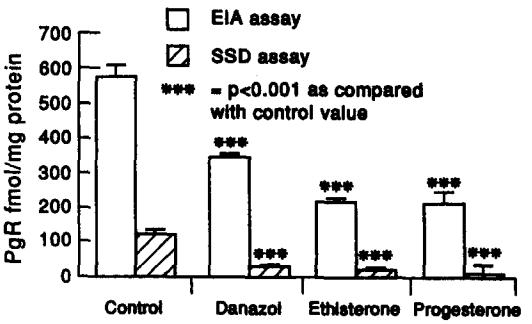

Fig. 4. MCF-7 cells were cultured for 7 days with danazol 10^{-6}M, ethisterone 10^{-7} M, or progesterone 10^{-8} M. PgR was assayed by SSD and EIA, each treatment representing the mean of 5 determinations carried out in duplicate ± SEM.

mg protein (mean ± SEM). PgR concentrations remained suppressed following 7 days treatment with danazol and progesterone [Fig. 1(b)]. However, when treatment was continued for 2 weeks a 2-fold induction of PgR ($P < 0.001$) was observed in the uteri of rats treated with danazol and ethisterone [Fig. 1(c)]. This induction was especially marked at the metoestrous phase of the oestrous cycle in these treated rats [Fig. 1(d)]. The PgR concentrations in uteri of rats treated for 2 weeks with progesterone returned to control values.

Oestrogen receptor concentrations decreased in the rat uteri after 2 weeks treatment with progesterone ($P < 0.05$ at 7 and 14 days), but increased ($P < 0.001$ at 7 days) with danazol treatment (Fig. 2). Ethisterone treatment did not significantly affect ER concentrations at any time point.

In vitro studies

In the breast cancer cell lines, MCF-7 and T-47D a similar response was observed after short-term exposure to progesterone, ethisterone and danazol. One hour exposure of MCF-7 cells to these compounds led to suppression of PgR concentrations 24 h later (Fig. 3). Exposure to these steroids was continued for 1 week and resulted in chronic suppression of PgR. This suppression was evident in the MCF-7 cells when PgR concentrations were assayed by both SSD and the EIA methods (Fig. 4). Longer incubation periods with danazol in both MCF-7 and T-47D cells maintained this chronic suppression of PgR (data not shown), apparently contradicting the data obtained from long-term administration of this steroid *in vivo* in the rat (Fig. 1).

Since *in vivo* the induction occurred following an initial period of suppression, T-47D cells were cultured in DMEM, 5% FBS for several weeks in the presence or absence (control) of danazol followed by a recovery period of 1 week, in danazol-free medium (DMEM with or without phenol red, containing 5% charcoal-stripped FBS), prior to harvesting. A 3-fold

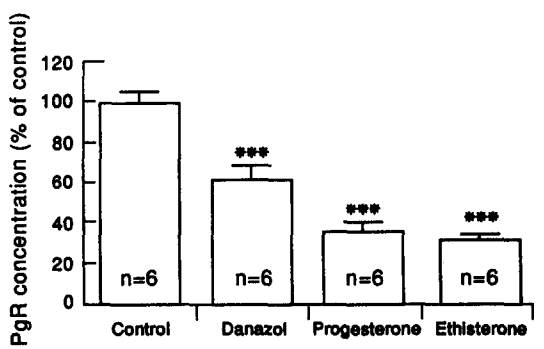

Fig. 3. MCF-7 cells were incubated for 1 h with danazol 10^{-8} M, progesterone 10^{-8} M or ethisterone 10^{-7} M. After this period cells were incubated for 24 h in steroid free medium (DMEM, 5% FCS). Cells were harvested and PgR determined by Scatchard analysis and plotted as % of control mean ± SEM.

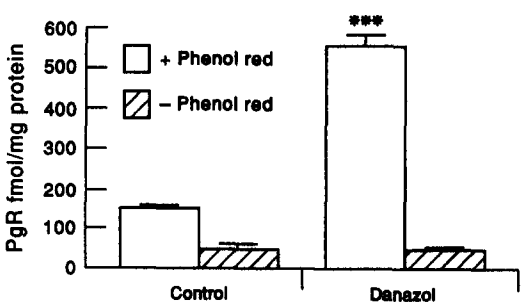

Fig. 5. T-47D cells routinely cultured with danazol 10^{-7} M were placed in media with or without phenol red indicator for 7 days. Untreated control cells were also incubated for this time course in the presence or absence of phenol red. PgR were assayed by SSD, each treatment representing a mean of 5 determinations carried out in duplicate ± SEM.

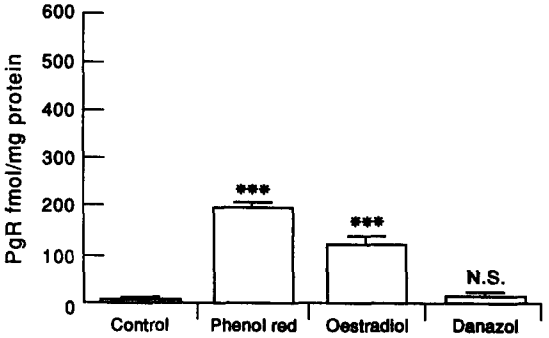

Fig. 6. T-47D cells were cultured for 1 week in phenol red free medium. The cells were incubated for a further week with danazol 10^{-7} M, oestradiol 10^{-8} M, DMEM containing phenol red or untreated (controls). PgR was assayed by SSD each treatment representing a mean of 5 determinations carried out in duplicate ± SEM.

increase in PgR concentrations above control levels ($P < 0.001$) was observed in those cells cultured in DMEM containing phenol red and previously treated with danazol (Fig. 5). However, this increase was not seen when phenol red was absent from the medium.

To more clearly define the contribution of phenol red, T-47D cells were cultured in phenol red-free medium (5% DCC FBS) for 2 weeks followed by exposure for 1 week either to phenol-red free medium alone (control) or to medium supplemented with phenol red (standard DMEM), oestradiol (10^{-8} M) or danazol (10^{-7} M) (Fig. 6). Phenol red induced PgR but only to those levels normally observed in T-47D cells routinely cultured in medium containing phenol red (100–200 fmol/mg protein) and well below those induced in the danazol-treated cells previously described (550–600 fmol/mg protein, Fig. 5). Oestradiol also induced PgR significantly but to a lesser extent, whereas danazol did not affect PgR concentrations.

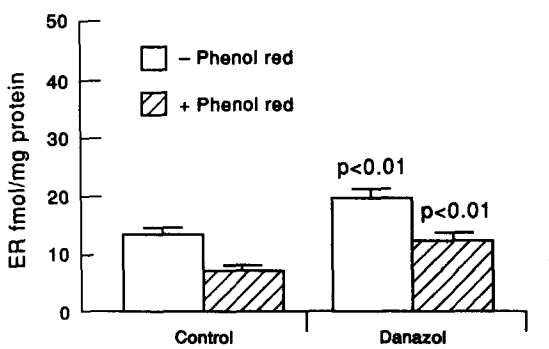

Fig. 7. T-47D cells routinely cultured with danazol 10^{-7} M were placed in media with or without phenol red indicator for 7 days. Untreated control cells were also incubated for this time course in the presence or absence of phenol red. ER were assayed by SSD, each treatment representing a mean of 5 determinations carried out in duplicate ± SEM.

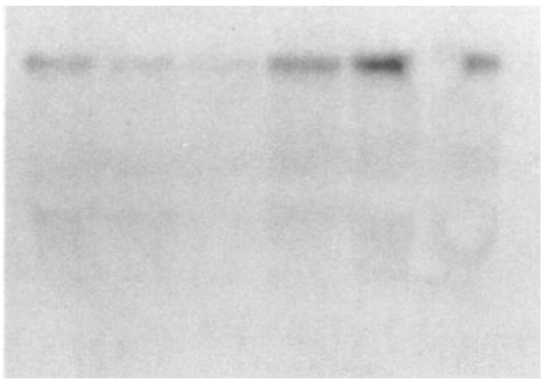

Fig. 8(a)

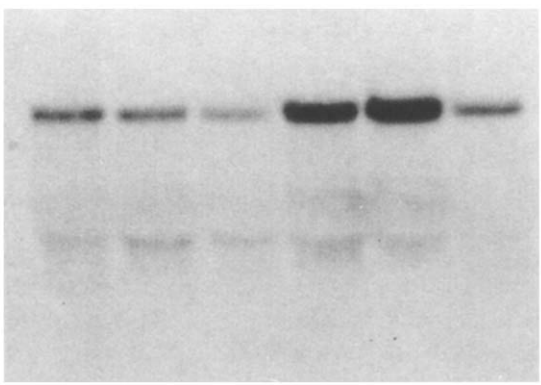

Fig. 8(b)

Fig. 8. T-47D cells were incubated with phenol red free medium for 3 days. Cells were further incubated with this medium supplemented with progesterone 10^{-6} M (a) or danazol 10^{-6} M (b). At certain time intervals flasks of cells were removed and RNA prepared. Subsequent hybridization with a radioactive DNA probe to the PgR allowed visualization of PgR mRNA by autoradiography at 80° for 1–5 h. Lane 1, control; lanes 2–6, 3, 6, 24, 30 and 48 h.

Small but significant ($P < 0.01$) induction of ER (assayed by the SSD method) was observed in the danazol-treated cells (Fig. 7), which was further enhanced with the absence of phenol red from the culture medium.

Results of mRNA determination showed that short-term exposure to danazol (10^{-6} M) of T-47D cells cultured in the absence of phenol red results in an increase in PgR mRNA levels after 24 h (Fig. 8). A similar increase was seen with cells similarly exposed to progesterone (10^{-6} M). No discrepancy in loading levels of RNA was observed, as determined by visualization with ethidium bromide under ultraviolet light.

DISCUSSION

The present study was designed to follow up data obtained from an earlier clinical trial

[12, 13]. In this trial, danazol was shown to be capable of PgR induction in mastalgia patients who responded favourably to danazol therapy. Additionally, ER was induced during the course of treatment, although no relationship with response was seen. Our study attempted to reproduce these effects under laboratory conditions and to propose a mechanism by which this could occur.

Two weeks treatment of female Wistar rats with danazol and ethisterone (a major metabolite of danazol [28]) was shown to produce significant induction ($P < 0.001$) of PgR in the rat uterus [Fig. 1(c)]. This response differed from short-term studies [Fig. 1 (a and b)] where danazol was found to suppress PgR subsequent to 24 h and 7 days treatment when compared with controls. This suppression was not observed in animals treated with ethisterone [Fig. 1(a and b)], which did, however, produce induction of PgR, equivalent to that attained by danazol, in the uterus following 2 weeks treatment [Fig. 1(c)].

Thus, as with the clinical trial we have observed that danazol (and one of its metabolites) is capable of PgR induction, a typically oestrogenic response, despite the fact that danazol is unable to bind with the ER.

PgR induction has been reported elsewhere [35, 36] in the uteri of oophorectomized rats. Since anti-androgens have been shown to increase PgR concentrations [36], it is unlikely that a danazol-mediated androgen action would increase PgR. Some other mechanism must therefore be involved. In the present study danazol treatment was shown to induce PgR expression in the uteri of non-castrated rats with maximal effect at the metoestrous phase of the cycle [Fig. 1(d)]. Indeed, it would appear that the treated rats maintain their oestrous cycles (as determined by vaginal smears) but normal cycling of the PgR is abolished and PgR remain at the control proestrous concentrations throughout the cycle. The induction is a long-term response to danazol and occurs subsequent to an initial period of receptor suppression.

In contrast, chronic suppression of PgR was maintained in the breast cancer cell lines with increasing length of danazol treatment (data not shown). This was true when receptors were measured by SSD or the EIA method (Fig. 4), thereby eliminating the possibility that intracellular pools of steroid bind to any newly synthesized receptor, thereby making detection difficult. The EIA assay relies on recognition of epitopic sites located on the receptor surface and is capable of detecting receptors whether or not they are bound to endogenous steroid [37]. Despite the fact that higher concentrations of PgR are detectable by the EIA assay when compared with the ligand binding assay, strong down-regulation of this receptor assayed by EIA was still evident following exposure to danazol, ethisterone or progesterone. It is however notable that short-term studies indicated an induction of PgR mRNA in response to danazol (Fig. 8). The discrepancy between the mRNA and PgR assay data remains enigmatic. Further studies will be necessary to determine whether this is attributable to inhibition of translation or receptor recycling.

In T-47D cells, danazol induction of PgR was only observed if the cells were given a recovery period of 1 week following exposure to danazol (Fig. 5) in steroid-free medium containing phenol red. No PgR induction was observed if the recovery medium was phenol red-free. As phenol red has been previously shown to have an oestrogenic effect [38], experiments were conducted to assess the effect of phenol red alone on PgR expression in these cells. Although both phenol red and oestradiol were capable of inducing PgR (Fig. 6), the PgR concentrations observed were significantly lower than in cells pre-treated with danazol in the presence of phenol red. It is clear, therefore, that the inductive effect of danazol requires the presence of an oestrogenic stimulus.

Danazol treatment of both rats and cell lines was also shown to produce a concomitant increase in ER concentrations (Figs 2 and 6). This is contrary to the known action of progestins which are thought to down regulate ER mRNA and protein [39, 40]. However, it is in agreement with the increase in ER observed during danazol therapy in the clinical trial [11]. Hence, we can speculate that danazol's ability to induce PgR is a result of an increase in ER. Thus endogenous oestrogens could then act on this induced ER which would, in turn, lead to increased PgR. This may explain how danazol increases PgR even though it does not bind to ER and none of its metabolites are thought to possess oestrogenic activity. Furthermore, it is clear that in this case danazol does not act as a progestin since progesterone itself was unable to produce a similar induction *in vivo*.

In conclusion, these results show clear PgR induction by danazol at the molecular and receptor protein levels and provides an indi-

cation that this may be achieved by danazol inducing ER and acting synergistically with oestrogens. Further studies are required to determine the precise mechanism by which this occurs.

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