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Original Paper

The Effect of Onapristone, a Progesterone Antagonist, on the Growth of Human Gastrointestinal Cancer Xenografts

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Onapristone is a progesterone antagonist which inhibits the growth of mammary tumours in mice. The effect of Onapristone and concomitant oestrogen (E_2) supplements on the growth of five human gastrointestinal cancer xenografts was examined. E_2 stimulated RD19 (gastric tumour) tumour growth in female mice and tumours grew less well when also treated with Onapristone ($P < 0.05$). Onapristone had no effect on male mice bearing RD19 tumours or mice of either sex bearing MKN45G (gastric) tumours. PAN-1 (pancreatic) tumours were significantly stimulated by E_2 (by 64% of control, $P = 0.02$) and Onapristone treatment inhibited E_2 stimulated growth (52% reduction of E_2 control, $P > 0.05$). C146 (colorectal) tumour growth was not stimulated by E_2 nor inhibited by Onapristone. E_2 stimulated formation of AP5LV (colorectal) tumour nodules (in lungs) (mean 38–52, $P = 0.001$). Onapristone significantly reduced the number of nodules (mean 32, $P < 0.05$) only in female mice not given E_2. Xenografts of some GI tumour cell lines grow at different rates in male and female mice. E_2 may cause additional growth stimulation and E_2 stimulated growth can be reversed by Onapristone to basal levels. © **1997 Published by Elsevier Science Ltd.**

Key words: Onapristone, progesterone antagonist, xenograft tumours, gastric, colorectal, oestrogen, male, female

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INTRODUCTION

ONAPRISTONE (ZK 98.299, Schering A. G., Berlin, Germany) [1] is a progesterone antagonist which inhibits the growth of mammary tumours in mice and rats [2]. In animal models, Onapristone is reported to have a novel mechanism of action mediated via the progesterone receptor (PgR) to which it binds. This induces differentiation of the tumour, leading to apoptosis and cell death [3].

Onapristone has the advantage of much lower glucocorticoid activity than another progesterone antagonist (Mifepristone) which has shown some promise as an endocrine therapy in patients with advanced breast cancer [4]. Clinical trials were recently established to assess the efficacy of Onapristone in patients with breast cancer and to establish if a similar mechanism of action could be identified in human breast cancer (Robertson, City Hospital, Nottingham, U.K.).

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We have previously reported that in *in vivo* studies using (GI) cancer cell lines, the sex of the mice was a significant factor affecting the rate of tumour growth [5]. In the present study, the effect of Onapristone on the growth of five GI tumour cell lines *in vivo* was evaluated, taking into account the sex of the animal and whether oestradiol (E) supplements were given. In the breast cell cancer line MCF7, PgR expression is upregulated by *in vitro* exposure to E_2 [6]. The GI cell lines used in these studies have been characterised for oestrogen receptor (ER) and PgR expression [7]. Low levels of both ER and PgR were detected in the GI cancer cell lines by enzyme immunoassay. E_2 supplements were given in an attempt to maximise PgR expression in the hope that Onapristone might be more effective.

MATERIALS AND METHODS

Cell lines

Five cell lines derived from primary human GI tumours in the Cancer Research Laboratories and Cancer Studies

Unit of Nottingham University were studied. These included two gastric cell lines, RD19 and MKN45G (the latter being a variant secreting high levels of gastrin derived from the parent line MKN45 [8, 9]), a pancreatic cell line PAN-1, and two colorectal cell lines C146 [10] and AP5LV, which colonises the lungs [11]. Cells were cultured to subconfluence in RPMI 1640 with 10% fetal calf serum (Both Gibco BRL, Paisley, U.K.), harvested using EDTA (0.025%), washed and resuspended in phosphate-buffered saline (pH 7.3) (OXOID, Basingstoke, U.K.) for injection into donor mice.

In vivo *models*

Nude mice (Harlan-Olac, Bicester, U.K.) and SCID mice (severe combined immunodeficient mice, bred in the Cancer Studies Unit, Nottingham, U.K.) were maintained in sterile isolators and used at 4–6 weeks of age. All animal work conformed to UKCCCR guidelines.

RD19, MKN45G, PAN-1 and C146 xenograft tumours were grown in nude mice from 10^7 cultured cells injected subcutaneously into the flank. The tumours were passaged *in vivo* prior to initiation of experiments. 10^4 cultured AP5LV cells were injected into SCID mice intravenously via the tail vein at the start of therapy.

Drug formulations

Onapristone and oestradiol were supplied by, and the dosing schedules advised by, Schering A. G. (Berlin). Drugs were formulated under aseptic conditions in vehicle (10% benzyl benzoate: 90% castor oil) and 100 µl given by s.c. injection at separate sites. The dose of Onapristone was based on the weight of the mice and given at either 10 mg/kg or 50 mg/kg six times per week. E_2 was given at 0.3 µg/mouse, three times per week. Vehicle alone was given in place of Onapristone or E_2 as a control so that all animals received the same total volume of vehicle.

Experimental design

Mice were randomised into groups for treatment with either Onapristone or vehicle, each either alone or in combination with E_2. For each treatment combination, there were two groups containing equal numbers of either male or female mice.

For MKN45G Onapristone treatment (0, 10 or 50 mg/kg of mouse weight) was initiated one week after tumour implant. All animals received E_2 from day 1 (n = 10/group).

For RD19, PAN-1 and C146 Onapristone (0 or 50 mg/kg of mouse weight) and E_2 therapy both started from day 1 following tumour implant (n = 7–8/group). Control groups (n = 4–7/group) not receiving Onapristone or E_2 supplements were included.

For AP5LV, Onapristone therapy (0 or 50 mg/kg of mouse weight) was administered either from day 1 or from day 7. Equal numbers of mice in each group were given E_2 from day 1 or no E_2 supplementation (n = 6/group).

Parameters measured

MKN45G, RD19, PAN-1 and C146 tumours grew in the flank of the mice at the injection site and tumour readings (two largest perpendicular diameters were measured using callipers, then multiplied together) recorded twice weekly by an observer blind to treatment. At termination, tumours were excised and weighed. Portions were fixed in formal calcium then embedded in paraffin wax. Blocks were prepared for frozen sectioning by cryopreservation in isopentane, and the remainder flash frozen in liquid nitrogen then stored at −70°C to preserve receptor content. Cytosols were prepared for analysis of PgR levels.

AP5LV is a human tumour of colorectal origin, but when injected i.v. into mice, it lodges and grows in the lungs. At termination, lungs were excised and the number of tumour nodules counted. Tumour burden was also determined by histology using paraffin embedded material. The cross-sectional area of lung occupied by tumour nodules was quantified by image analysis of haematoxylin/eosin stained sections.

Tumour weights at termination, and tumour readings at various time points during the experiments were compared for the treatment groups in each experiment and analysed statistically using the Mann–Whitney test. In the case of AP5LV, numbers of nodules visible at termination were compared between treatment groups. Analysis of variance (ANOVA) was applied to individual experiments (nesting animals in treatment groups for tumour reading data). Data from the whole series of experiments were examined in a single ANOVA to compare treatment parameters. There was close correlation between tumour weights and final tumour readings. Tumour weight data are shown in the figures and tumour readings are referred to in the text where relevant. In all analyses, differences were regarded as significant if $P < 0.05$.

Receptor status of GI tumour cells

PgR were measured using an enzyme immunoassay kit (Abbott Diagnostics, Maidenhead, U.K.). In four experiments (MKN45G, RD19, PAN-1 and C146), cytosols were made from 2–3 tumours pooled from each treatment group. As there was no randomised control group without E_2 in the MKN45G experiment, cytosol was also prepared from MKN45G donor tumours which had not been exposed to E_2. Cytosols were assayed on at least two occasions. AP5LV was not assessed since cytosol made from lung-containing nodules would have contained a high proportion of non-tumour tissue.

RESULTS

Effect of Onapristone on in vivo *tumour growth*

Gastric tumours. All of the MKN45G animals received E_2. There was no significant difference between any of the six treatment groups (males or females given high, low or no dose Onapristone) compared by Mann–Whitney analysis. ANOVA did not reveal any significant response to treatment with Onapristone in either sex (data not shown).

RD19 tumours in female mice were stimulated by E_2 significantly more than in male mice (Figure 1) whether or not Onapristone was part of the treatment ($P < 0.05$). In E_2 supplemented animals, Onapristone substantially reduced tumour load in female mice by 29%, but did not have the same effect in males. This sex–treatment interaction was significant (ANOVA $P < 0.001$). Tumour growth in male mice treated with E_2 or E_2 plus Onapristone was less than in male controls (79% and 74%, respectively). Inhibition was significant for tumour readings at 14 and 18 days ($P < 0.05$), but not at day 21 or for tumour weights (1.4 ± 0.1 and 1.0 ± 0.4 g, respectively).

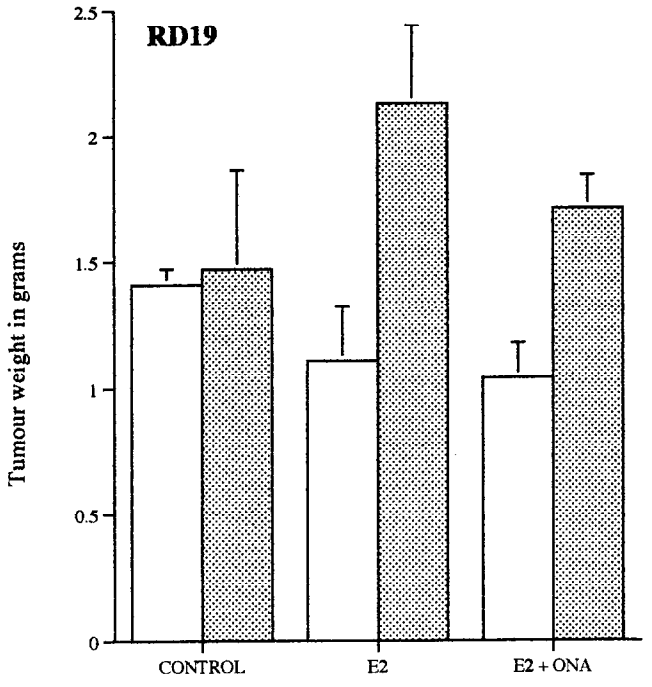

Figure 1. The effect of Onapristone (ONA) on mean weight ±SEM of RD19 xenografts at termination in male (open bars) and female (hatched bars) mice. Tumours were significantly larger in female than in male mice for groups given E_2 supplements (E_2 $P < 0.05$, E_2 + ONA $P = 0.006$: Mann–Whitney).

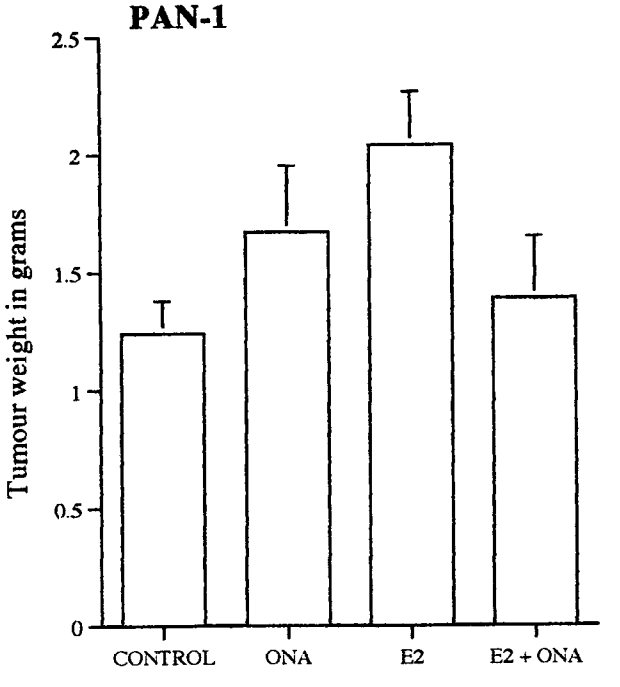

Figure 2. The effect of Onapristone (ONA) on mean weight ±SEM of PAN-1 xenografts at termination in all mice. Tumours were significantly larger in E_2 supplemented animals than in controls (E_2 versus control $P < 0.05$: Mann–Whitney). (Relative to controls, ONA weight +34%; E_2 + 64%; E_2 + ONA +12%.)

Pancreatic tumours. For PAN-1, male and female mice did not differ significantly in their response to the treatments given (ANOVA male versus female for tumour weights $P = 0.14$; tumour readings $P = 0.22$; data not shown). E_2 stimulated tumour growth significantly by 64% ($P = 0.02$) relative to controls. There was a trend for Onapristone to reduce tumour growth in E_2 supplemented animals (from 64% to 12%; $P = 0.06$ compared with E_2 treatment only). ANOVA of tumour weight data indicated that the interaction between E_2 and Onapristone was significant ($P = 0.02$). When the individual components of treatment were analysed further by ANOVA, the effect of E_2 was significant ($P = 0.005$) but that of Onapristone was not ($P = 0.22$). Comparing individual treatment groups (Figure 2), tumours in mice receiving Onapristone alone or Onapristone plus E_2 did not differ significantly from those in untreated controls (Mann–Whitney, $P < 0.05$). Tumour growth was stimulated by E_2 alone, but not if Onapristone was given at the same time (significance $P < 0.05$).

Colorectal tumours. For C146 Onapristone alone did not alter the rate of C146 tumour growth (Figure 3). E_2 produced a non-significant increase in tumour growth in females and inhibited it in males ($P = 0.04$). Mean tumour weights were greater for females than males in all four treatment groups. This male/female difference in response was significant in the groups receiving E_2 ($P = 0.002$) or Onapristone with E_2 ($P = 0.007$). Tumours in males receiving Onapristone with E_2 were larger than those in males receiving E_2 alone ($P = 0.022$), but not from controls. Tumours in females treated with Onapristone plus E_2 were not significantly different from those given E_2 alone but were larger than tumours from untreated controls. ($P = 0.04$). ANOVA confirmed that Onapristone and E_2 individually had no significant effect on tumour growth, but that the sex–E_2 interaction outlined above was significant ($P = 0.04$).

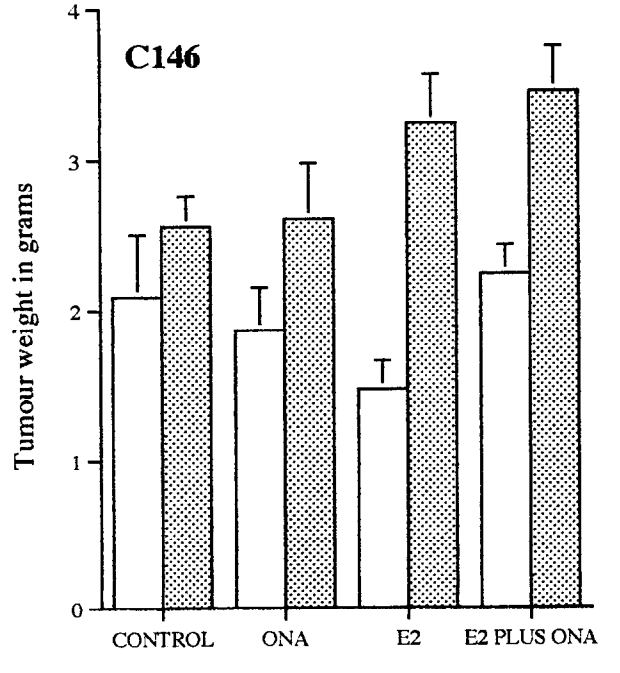

Figure 3. The effect of Onapristone on mean weight ±SEM of C146 xenografts at termination in male (open bars) and female (hatched bars) mice. Tumours were significantly larger in female than in male mice for groups given E_2 supplementation (E_2 $P < 0.05$, E_2 + ONA $P < 0.05$: Mann–Whitney).

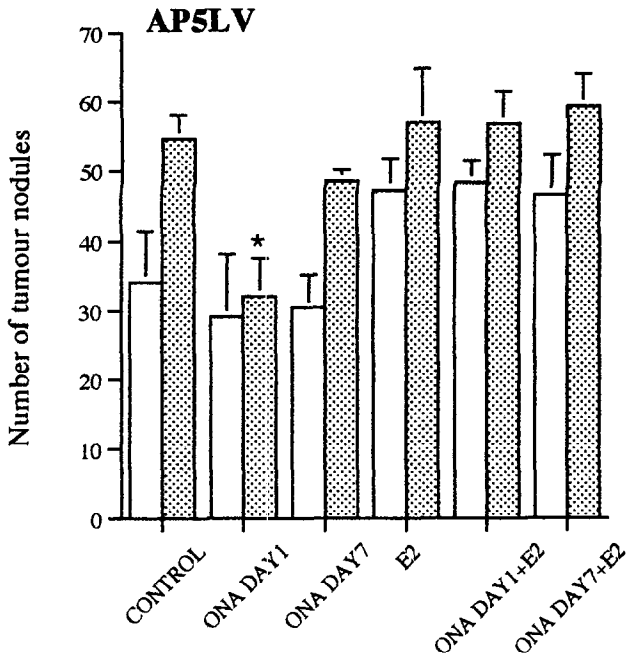

Figure 4. The effect of Onapristone treatment from day 1 or day 7 on the mean AP5LV nodule count ±SEM per lung in male (open bars) and female (hatched bars) mice ± E_2 supplementation. There were significantly fewer nodules (*Mann–Whitney $P < 0.05$) in female mice given Onapristone early (ONA day 1).

The AP5LV tumour burden was estimated by counting the number of nodules macroscopically visible on the lung surface (Figure 4). The effect of Onapristone was evaluated by ANOVA in two ways: firstly, whether Onapristone was present or absent, and secondly whether Onapristone was absent, present from day 1 or present from day 7. The number of nodules was not significantly influenced by Onapristone categorised in either of these ways and there were no significant interactions of Onapristone with sex or with E_2 influencing nodule number.

There were significantly more nodules in the lungs of female mice than in males (mean number of nodules 52.5 and 39, respectively, ANOVA $P < 0.001$). E_2 also significantly increased the nodule count (mean number of nodules with E = 52, without E = 38, ANOVA $P < 0.001$). Both sexes were therefore looked at separately, comparing either treatments with E_2 present or treatments without E_2 in order to evaluate the effect of the Onapristone given from day 1 or day 7 in more detail. Regardless of sex, the number of nodules formed during treatment with Onapristone from day 1 was reduced in comparison to all other treatments and significantly so ($P < 0.05$) when compared to all three treatments where E_2 was present. It seems that Onapristone must be given from day 1 in the absence of E_2 to achieve inhibition of AP5LV. This effect was more marked in female mice than in males.

Comparison of the five experiments

Onapristone was coded for ANOVA in three different ways: A = absent or present (at any dose at any time point), B = absent or present (at 50 mg/kg from days 0–2), C = absent or present (at 50 mg/kg at any time point). Onapristone did not significantly affect tumour growth in any of the three categories. E_2 was a significant factor in the analyses which include all cell lines, stimulating tumour growth in one or both sexes. Sex was highly significant in every analysis. Overwhelmingly, the effect was of greater tumour load in females than in males in most cell lines for most treatments.

Since both sex and E_2 had such a strong influence on the outcome of treatment, the data were re-analysed to see the effect of Onapristone independently of these two factors. Tumours from mice treated with Onapristone were compared with control tumours (no E_2 supplement) and tumours from E_2 supplemented mice treated with Onapristone compared with tumours from E_2 supplemented mice in both cases in animals of the same sex. The results for tumour weight and tumour reading were similar (data not shown). The *P*-values by Mann–Whitney analysis for these comparisons attained significance in very few instances. Onapristone, when given as early therapy, inhibited AP5LV nodule formation in females ($P = 0.008$). With E_2 also present, Onapristone stimulated C146 in males ($P = 0.02$ for tumour weight and reading) and inhibited PAN-1 in both males and females ($P = 0.018$, $P = 0.047$ for tumour weight data only).

Measurement of PgR by EIA

There was no significant difference between PgR measurements on tumours from male or female animals which had received the same treatment, in particular between those that had or had not received E_2 supplements. Data for PgR measurements in cytosols prepared from C146 tumours are shown in Table 1 as an example. Mean values ± SEM for PgR are expressed in fmol/mg cytosolic protein. The PgR levels obtained for the tumour cytosols were also compared with PgR levels previously measured in cytosols prepared from the same cell line cultured *in vitro* (Table 2) either in E_2 depleted medium or in the presence of E_2 (nM).

In these *in vitro* conditions, all five GI cell lines expressed low levels of PgR with mean values of 2.3–11.2 fmol/mg. There was no evidence for upregulation of PgR by exposure to E_2 either *in vitro* or *in vivo*. PgR expression for MKN45G was very similar in cytosols prepared from cultured cells and from xenograft tumour whether or not E_2 was present (mean values 2.7–5.9 and 2.9–6.4 fmol/mg, respectively). RD19 and PAN-1 both expressed PgR in cytosols from cultured cells (mean values RD19: 8.5–10.6 and PAN-1:8.3–11.2 fmol/mg with and without E, respectively), but did not express measurable quantities in the tumour cytosols. PgR were expressed in C146 cytosols prepared

Table 1. Progesterone receptor status of C146 xenograft tumours measured by EIA

	Sex	$-E_2$	$+E_2$
−ONA	Male	1.6 ± 0.6 (n = 3)	3.4 ± 1.1 (n = 4)
	Female	−ve (n = 2)	4.7 ± 0.5 (n = 3)
+ONA	Male	−ve (n = 2)	−ve (n = 4)
	Female	−ve (n = 2)	−ve (n = 2)

PgR in fmol/mg protein given as mean ± SEM (n = number of determinations), −ve < 1.7 fmol/mg.
Significance (ANOVA) Sex $P = 0.849$ ONA $P = 0.001$ E_2 $P = 0.106$.

Table 2. Progesterone receptor status of GI cancer cell lines grown in vitro *or* in vivo*, with and without E_2 supplements*

Cell line	Without E_2 Mean ± SEM	(n =)	With E_2 Mean ± SEM	(n =)
MKN45G				
in vitro	6.4 ± 3.7	(n = 2)	5.0 ± 2.7	(n = 3)
in vivo	2.9 ± 0.3	(n = 2)	2.7 ± 0.5	(n = 12)
RD19				
in vitro	10.6 ± 4.4	(n = 3)	8.5 ± 3.0	(n = 6)
in vivo	–ve	(n = 2)	–ve	(n = 4)
Pan-1				
in vitro	11.2 ± 6.1	(n = 3)	8.3 ± 3.1	(n = 6)
in vivo	–ve	(n = 8)	–ve	(n = 8)
C146				
in vitro	8.3 ± 6.8	(n = 2)	2.3 ± 0.4	(n = 5)
in vivo	–ve	(n = 9)	2.4 ± 0.7	(n = 13)
AP5LV				
in vitro	4.5	(n = 1)	–ve	(n = 1)
in vivo	nt		nt	

PgR measured by EIA given as mean ± SEM fmol/mg protein (n = number of determinations; nt = not tested; -ve < 1.7 fmol/mg).

from cultured cells (mean values ± E_2 of 2.3 and 8.3 fmol/mg). PgR were also expressed at similar levels (mean values of 3.4 and 4.7 fmol/mg) by C146 xenograft tumours from E_2 supplemented mice but were not detected in any tumours exposed to Onapristone (Table 1). This reduction of PgR in the presence of Onapristone was highly significant (P = 0.001).

DISCUSSION

It has been reported that Onapristone inhibits the growth of mammary carcinomas by accelerating terminal differentiation and cell death by apoptosis [3]. It is believed that Onapristone effects these changes through its capacity to bind PgR. A further breast cancer study postulated that Onapristone inhibited tumour growth by inducing differentiation [12]. This was demonstrated by changes in tenascin expression and led to further work to determine the importance of other growth factors such as TGF-β and EGF [13].

In our studies, Onapristone had a modest effect on inhibition of GI tumour growth in some experimental situations. In the first two experiments (MKN45G and RD19) it became obvious that E_2 was affecting the rate of tumour growth. The initial protocol was, therefore, modified to include additional control groups to determine the extent of this phenomenom and thereby facilitate analysis of Onapristone efficacy. The initiation of Onapristone therapy was also brought forward to prolong treatment in such fast growing tumours. In this latter series of experiments, Onapristone had no significant effect on basal tumour growth rate in most of the GI tumour cell lines, although inhibition of AP5LV nodule formation by Onapristone's effect was more noticeable in mice not given E_2 supplements, where tumour take rate was lower. Onapristone was able to moderate hormone (E_2) stimulated tumour growth in female mice bearing RD19 tumours and to reverse the stimulation of tumour growth caused by E_2 in both sexes of mice bearing PAN-1 xenografts.

Low levels of PgR had been detected in cytosols made from cells cultured *in vitro* for all five GI cell lines used in these experiments [7]. The sensitivity of these cell lines to E_2 *in vivo* might lead one to expect that PgR levels could be manipulated, but the data do not support this hypothesis. PgR levels were not significantly upregulated by E_2 either *in vitro* or *in vivo*. The absence of PgR in the tumours of the animals given Onapristone with E_2 could be explained by downregulation of PgR. However, if this had been the case, the tumours from control animals treated with E_2 should have had detectable, if not increased levels of PgR. This was not the case for RD19 and PAN-1 where PgR were not detected in xenografts from either treated or control animals.

Interaction between cell lines, E_2 and Onapristone appears to be a complex process both to control and to interpret. In *in vitro* studies using low serum concentrations to reduce interference by other serum factors, such as growth factors, moderating effects on cell proliferation by interaction between progesterone and Onapristone in several of these GI cell lines was demonstrated (data not shown). Steroid hormones such as E_2 can indirectly effect tumour growth by regulation of growth factors such as IGF which may then function in autocrine pathways to regulate cell proliferation. The mechanisms involved have been extensively reviewed in the context of breast cancer [14].

These studies have shown that xenografts of some GI tumour cell lines grow at different rates in male and female mice and that E_2 often causes additional growth stimulation [5]. Such E_2 stimulated growth can be reversed by Onapristone to basal levels. In the conditions of our study, inhibition of tumour cell growth was moderate in comparison with that reported for mammary tumour models [15] and breast cell lines [16]. This may reflect the much lower level of PgR expression in the GI cell lines available for Onapristone binding. The two most promising avenues for future studies would be to extend the duration of Onapristone therapy, or to consider a combination therapy approach. Three weeks of Onapristone therapy may have been insufficient time to inhibit tumour growth. The experiment could be repeated using a slower growing tumour line, so that the duration of therapy could be extended to evaluate the effect of Onapristone on basal tumour growth. Schally and colleagues have suggested that the combination of a somatostatin analogue and a sex steroid hormone therapy, such as an anti-GnRH (gonadotrophin releasing hormone) analogue, is more effective than either alone [17]. Combining Onapristone with another therapy such as a somatostatin analogue should also be considered.

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