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The Mode of Action of the Antitumour Agent GP 48,989* in the Rat†

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Abstract—*The transplantable mammary carcinoma (LMC_1) of John's Strain Wistar rat did not respond to either 50 μg tamoxifen daily or bilateral ovariectomy. This failure to inhibit growth, correlated with a very low level of [^{3}H]oestradiol binding capacity of tumour tissue (2.93 ± 1.17 fmole/mg cytosol protein) that was not significantly greater than for heart muscle.*

GP 48,989, which has been reported to inhibit hormone independent DMBA-induced mammary tumours did not alter the growth of LMC_1. It did reduce uterine and ovarian weights but without affecting oestrogen receptor concentrations or oestradiol-stimulated rises in uterine wet weight. In ovariectomized Sprague–Dawley rats serum LH levels were reduced when treated with GP 48,989 and/or oestradiol, but GP 48,989 did not increase uterine wet weight. It is suggested that GP 48,989 inhibits the release of gonadotrophin and provokes regression of mammary tumours by altering the endocrine status rather than by direct anti-timour action.

INTRODUCTION

ABOUT one third of cancers of the human breast respond to endocrine ablation or additive hormone therapy whilst combinations of chemotherapeutic agents can produce additional remissions [1]. Hormone responsiveness in human breast cancer has been correlated with the presence of the oestrogen receptor protein [2] and related tumour specific agents e.g., antioestrogens have been developed [3]. The treatment of "hormone independent", or oestrogen receptor-negative breast tumours with conventional chemotherapeutic agents is associated with a high incidence of side effects and it would be an advantage to develop new compounds which were selective for these tumours.

One compound of potential interest is GP 48,989, which inhibits the initiation and growth of 7,12 dimethylbenz(a)anthracene (DMBA)-induced tumours [4, 5]. Perhaps of greater significance though is the observation that GP 48,989 is apparently active against "hormone independent" DMBA-induced tumours [6]. As another attractive feature, the compound has low toxicity [5].

We have used a transplantable mammary adenocarcinoma (LMC_1), which arose spontaneously in a female Wistar rat of John's strain [7], in an attempt to quantify macroscopically and biochemically the antitumour activity of GP 48,989. The tumour has previously been shown to respond to chemotherapy and/or radiotherapy [8, 9]. Since however we were especially interested in the hormone-independent action of GP 48,989 on mammary tumours we first established that LMC_1 was unaffected by the antioestrogen tamoxifen or by ovariectomy of the host animal.

MATERIALS AND METHODS

Tumour growth assay

Full details of the histology, cellular kinetics, macroscopic growth and transplantation history of the LMC_1 tumour have been reported [7]. For this study, tumours of the 37th–40th generation were used. These were prepared using the method of Thomlinson [10]

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*5-Methyl-3-(2-methylallyl)-2-[(3-methyl-4-oxo-2-thiazolidinylidene)hydrazono]-4-thiazolidinone.

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by implanting "sausages" of tumour mince subcutaneously into the flank of 200–250 g isologous virgin females. Three mutually perpendicular tumour diameters were measured daily with calipers and rats were assigned randomly to experimental groups when their tumours first reached a mean diameter of 9 ±1 mm. Throughout experiments all rats were provided with food and water *ad libitum* and a 12 hr alternating light/dark regime (0600/1800).

The gross response of the tumours to treatment was determined from mean diameters by computation of the volume doubling times of the tumour up to 20 days from randomization [7]. The response of the tumour bearing host was determined by weighing (host plus tumour) at daily intervals. On each occasion, body-weight was expressed as a percentage of the weight of the animal at the start of the experiment after subtracting the weight of the tumour. The latter was determined using a calibration curve, established previously relating excised tumour weight to *in situ* diameter for LMC_1.

Tamoxifen and ovariectomy

To determine the hormonal dependency or otherwise of LMC_1 rats were assigned to 4 different treatment groups: (a) control; (b) 50 μg (per rat) of tamoxifen daily, (c) bilateral ovariectomy and (d) sham ovariectomy. All rats were subjected to anaesthesia with ether daily, at which times all experimental procedures and measurements were carried out.

Tamoxifen [1-(4-β-dimethylaminoethoxyphenyl)1,2diphenylbut-1-ene] was obtained from ICI Ltd (Pharmaceuticals Division). Solutions in peanut oil for subcutaneous injection were prepared as previously described [10]. GP 48,989 (Ciba–Geigy (Pharmaceuticals Division) Basle, Switzerland) was administered as a suspension in 2% methylcellulose and 0.9 NaCl.

GP 48,989

Two experiments were undertaken. In the first, rats were randomized into four different groups: (a) control, (b) 50 mg/kg GP 48,989 (s.c.) daily, (c) ovariectomy and (d) ovariectomy and 50 mg/kg GP 48,989 (s.c.) daily. In the second experiment rats were randomized into: (a) control, (b) control but no anaesthetic or daily tumour measurements, (c) 50 mg/kg GP 48,989 (i.p.) daily and (d) 100 mg/kg GP 48,989 (i.p.) daily.

Oestrogen receptor assay

To determine the presence or not of oestrogen receptors in LMC_1 tumour tissue, animals from the tamoxifen experiment, either ovariectomized (Gp.c.) or sham ovariectomized (Gp.d.) were sacrificed 20 days after entry and the oestrogen binding capacity was determined on cytosols prepared from their tumour, heart and uterine tissues.

Tissues were powdered, using a tissue pulveriser (Thermovac) cooled in liquid nitrogen, and then homogenized in 1.5 m TED buffer [0.01 mole/l Tris (Sigma), 1.5 mmole/l EDTA (Sigma) and 0.15 mmole/l dithiothreitol (Aldrich) pH 7.4] by two 2-sec bursts of an Ultraturrax tissue homogeniser with ice/water cooling. Homogenates were centrifuged at 1200 ***g*** for 30 min at 4°C. Aliquots (150 μl) of supernatants were incubated (4°C) in duplicate with 50 μl TED buffer containing 5×10^{-6} mole/l diethylstilboestrol (British Drug Houses Ltd.) for 10 min. Buffer, 50 μl TED, containing 2.5×10^{-8} mole/16,7 (^{3}H) oestradiol–17β (41 Ci/mmole Amersham), was added to each tube and incubated at 30°C for 30 min. Tubes were cooled to 0°C in ice/water and 400 μl of a 0.5% dextran coated charcoal suspension was added and incubated for 25 min. Charcoal was separated by centrifugation at 1200 ***g*** for 10 min and specific tritium (^{3}H) counts determined as previously described [1]. Cytosol protein concentrations were determined [13] and results were represented as fmole (^{3}H) oestradiol binding/mg cytosol protein.

Ovariectomy and luteinizing hormone

In experiments separate from those described above, 180–200 g Sprague–Dawley female rats were ovariectomized under ether anaesthesia and 4 days later assigned to treatment groups; (a) control, (b) 100 mg/kg GP 48,989 (i.p.), (c) 5 μg/rat (s.c.) oestradiol–17β (Sigma Chemicals) in 0.1 ml peanut oil and (d) 100 mg/kg GP 48,989 (i.p.) and 5 μg/rat oestradiol–17β (s.c.). Each group was treated daily for 8 days, and then rats were killed by decapitation 2–3 hr after the last injection. Uteri were dissected out and weighed wet on a torsion balance. Blood was also collected and allowed to clot at 30°C for 15 min. Serum was obtained by centrifugation at 1000 ***g*** and stored at 20°C before assaying for luteinizing hormone (LH).

Serum LH was estimated by a double antibody radioimmunoassay [14]. Highly purified ovine LH (LER-1374A) was used for iodination and ovine LH (NIH-LH-S19) as

assay standard; the first antibody, GDN15, was raised in rabbits against ovine LH. In this assay rat plasma and pituitary LH preparations show parallel dilution curves to the ovine LH standard. There was negligible cross reaction with rat follicle stimulating hormone, growth hormone and prolactin.

RESULTS

Tumour assays

The pattern of LMC_1 tumour growth in tamoxifen treated and ovariectomized rats was the same as in control animals (Fig. 1). No significant differences in volume doubling times of the tumours were found for any 5-day period prior or subsequent to the initiation of treatment (Fig. 2). In all 4 groups in the tamoxifen experiment the bodyweight of rats declined to about 90% as the volume of the tumour increased but more sharply following ovariectomy or sham operation (Fig. 1). In terms of gross response, therefore, neither form of antioestrogen therapy produced any effect on either LMC_1 or its host. In accord with these results, the oestrogen binding capacity for LMC_1 tumour tissue from the ovariectomized and sham ovariectomized rats was not significantly different from that for heart tissue, ($P>0.05$) and both were very low in comparison to that for uterine tissue removed from the same animals (Table 1).

GP 48,989, whether administered s.c. or i.p., also did not affect the macroscopic growth and hence the volume doubling time of LMC_1 tumours (Table 2).

Animals treated daily with either 50 or 100 mg/kg GP 48,989 had reduced uterine and ovarian weights compared with control

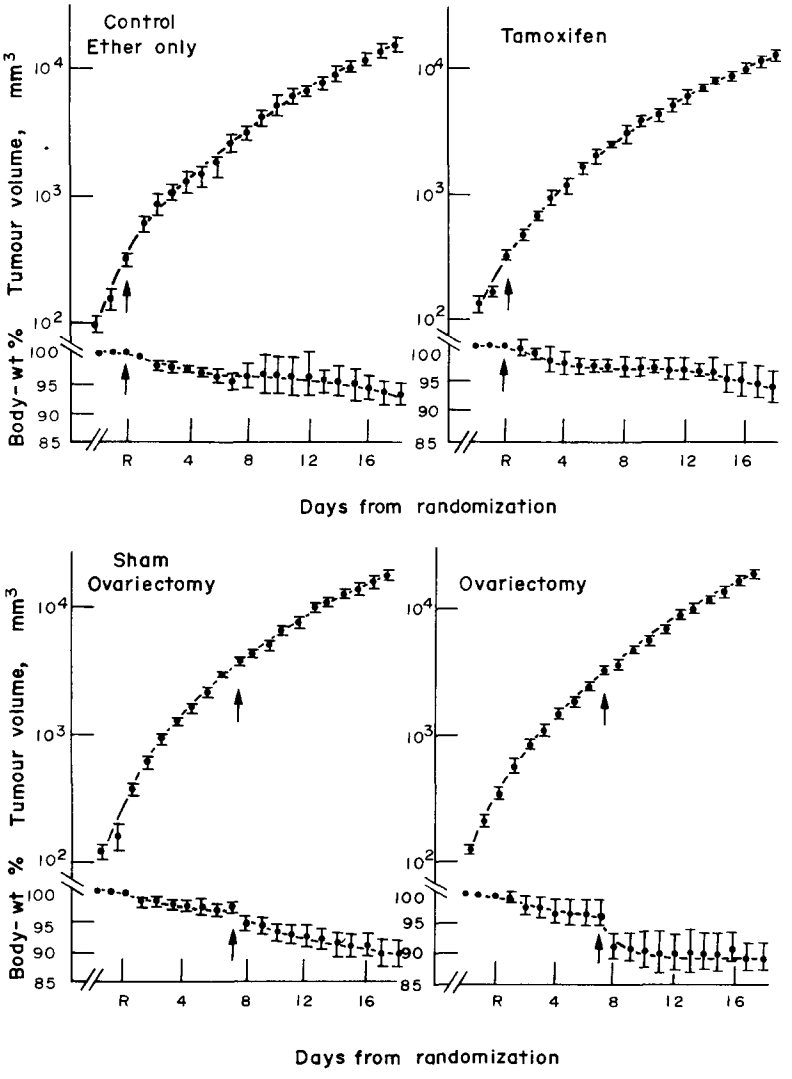

Fig. 1. Tumour growth and body weight after therapy or control treatment indicated, initiated at times shown by vertical arrows. All results are means ± S.E.M. for 10 rats per treatment group.

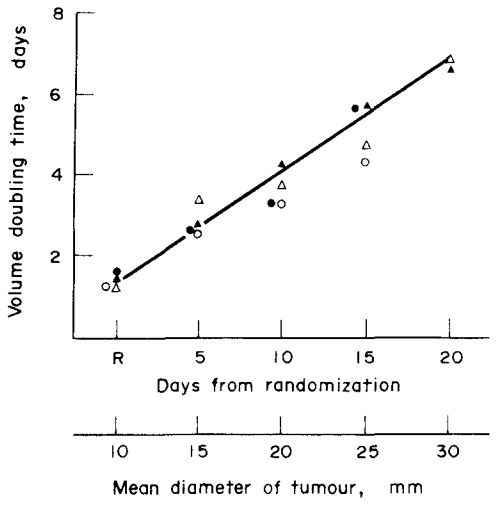

Fig. 2. Volume Doubling Time of LMC_1 after randomization and treatment. △ anaesthetic only; ○ sham ovariectomy; ▲ 50 μg tamoxifen s.c. daily; ● ovariectomy at R+7 days. Curve fitted to data points previously established [7] for the untreated LMC_1 tumour.

Table 1. (^{3}H) oestradiol binding capacity (fmole/mg cytosol protein)

Treatment	Uterus	Tumour	Heart
Control (sham ovariectomy)	1080± 52.9 (6)	2.93±1.17 (10)	2.04±0.64 (10)
Ovariectomy	1189±103 (10)	2.1 ±0.78 (10)	3.2 ±0.49 (10)

Parallel incubates with 1000 fold excess of DES were undertaken to determine the contribution of non-specific [^{3}H] oestradiol binding. Number of rats from which samples assayed shown in parentheses.

groups in which the rats were anaesthetized and had their tumours measured daily (Table 3). In animals where no anaesthetic was used and their tumours were not measured daily although their uterine and ovarian weights, when killed at dioestrus, were higher than in measured control tumour bearing rats, this was not statistically significant. The uterine oestrogen capacity of animals treated with 100 mg/kg GP 48,989 (4.26±0.24 pmole/uterus, $n=8$) was not significantly different from the tumour measured controls (3.92±0.26 pmole/uterus, $n=8$).

Ovariectomized Sprague–Dawley rats

The administration of GP 48,989 (100 mg/kg i.p.) for 8 days did not increase the uterine wet weight or inhibit the uterotrophic action of daily administration of oestradiol (Fig. 3). The latter treatment did, however, lower the serum level of LH ($P<0.001$). A combination of oestradiol and GP 48,989 reduced serum LH levels below those determined after oestradiol alone ($P<0.05$) whilst GP 48,989 alone reduced it below controls but this was not significant because of the wide spread of values obtained.

DISCUSSION

The primary aim of this study was to investigate the direct anti-tumour properties of GP 48,989 in an hormone-independent

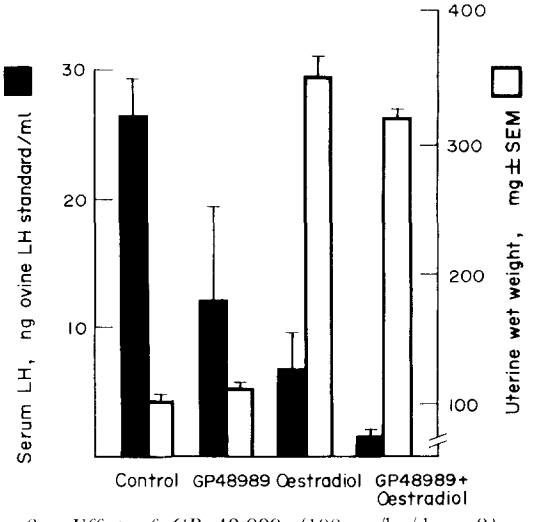

Fig. 3. Effect of GP 48,989 (100 mg/kg/day × 8), oestradiol (5 μg/day × 8) or a combination of the two on uterine wet weight and serum LH levels (8 ovariectomized rats/group).

Table 2. Volume doubling times of LMC_1 as a function of time after randomization

Time (days)	Control I (10)	'Ovex' (5)	Ovex + GP 48,989 50 mg/kg (s.c.) (10)	Ovex + GP 48,989 50 mg/kg (s.c.) (10)	Control II (5)	GP 48,989 (i.p.) 50 mg/kg (10)	GP 48,989 (i.p.) 100 mg/kg (10)
R	1.58±0.07	1.57±0.16	1.78±0.13	1.94±0.18	1.8 ±0.12	1.24±0.09	1.45±0.08
5	2.66±0.12	2.46±0.20	2.69±0.10	2.57±0.08	3.18±0.13	2.99±0.29	3.30±0.23
10	4.93±0.58	3.13±0.16	4.14±0.30	3.16±0.20	4.88±0.46	4.38±0.48	5.10±0.15
15	5.15±0.29	4.84±0.38	5.78±0.29	5.46±0.22	—	5.82±0.05	6.60±0.58

Ovariectomy (ovex) was undertaken 7 days after randomization. Number of rats per treatment group is given in parentheses.

Table 3. Rat uterine and ovarian weights after 21 days of GP 48,989

Treatment	Uterine wet weight mg ± s.e.	Combined ovarian weights mg ± s.e.
Unmeasured control	333.0 ± 32.8 (5)	66.8 ± 5.1
Measured control	253.6 ± 26.5 (5)	60.6 ± 3.2
GP 48,989 (50 mg/kg)	179.3 ± 5.5* (9)	42.0 ± 1.6†
GP 48,989 (100 mg/kg)	171.0 ± 4.5† (10)	41.3 ± 2.1†

Number of animals in each group is shown in parentheses.
*$P<0.01$, †$P<0.001$: for other values $P>0.05$; Comparison with measured control values by Student's *t*-test.

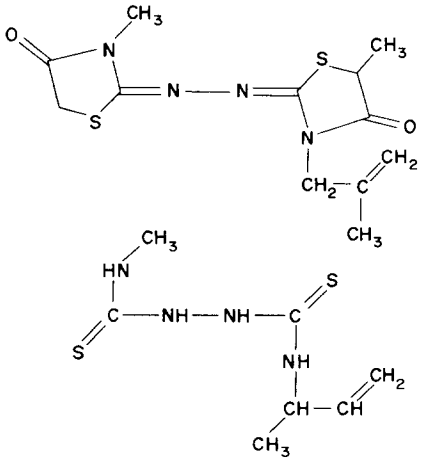

Fig. 4. Structure of GP 48,989 and the gonadotrophic inhibitor methallibure (ICI 33,828).

mammary cancer. As a test system we first established that the growth of LMC_1 in John's Wistar rats was not affected by either ovariectomy or tamoxifen treatment (Figs. 1 and 2). The inability of the tumours to respond to antioestrogenic treatment was consistent with their low oestrogen binding capacity (Table 1). In the DMBA-induced rat mammary carcinoma model, tumours with low levels of oestrogen receptors do not respond to tamoxifen therapy [15] or ovariectomy [16]. Similarly, in the clinic, breast cancers with low levels of oestrogen receptors are unlikely to respond to oophorectomy [2] or tamoxifen therapy [17–20].

GP 48,989 was inactive in the LMC_1 tumour system (Table 2) at doses previously reported to inhibit the growth of DMBA-induced mammary tumours [4, 5]. This result, coupled with the observation that uterine and ovarian weights were reduced during treatment (Table 3), suggests that the mode of action of GP 48,989 may not be due to a direct anti-tumour action, but results from a perturbation of the endocrine system. Furthermore, since GP 48,989 did not inhibit the uterotrophic actions of oestradiol or reduce uterine oestrogen receptor levels, then the compound cannot be said to be directly anti-oestrogenic in the uterus. The similarity of the structures of GP 48,989 (Fig. 4) and the inhibitor of gonadotrophic release, methallibure [21], led us to consider whether the reported antitumour effects were mediated through the pituitary.

Oestradiol, GP 48,989 and a combination thereof reduced the serum levels of LH in the ovariectomized rats (Fig. 2). Unlike oestradiol, GP 48,989 does not increase uterine wet weight and cannot be classified as an oestrogen, therefore it seems likely that oestradiol and GP 48,989 reduce circulating LH levels by different mechanisms. As GP 48,989 lowers serum LH levels then the anti-tumour activity in rats with DMBA-induced mammary tumours probably results from a disruption of the oestrous cycle.

The report [6] that GP 48,989 is active against some apparently "hormone-independent" DMBA-induced tumours is less readily explained. These may also depend upon other external endocrine influences, e.g., steroids from the adrenals may support the growth of DMBA tumours in ovariectomized rats and the release of prolactin may support their growth in rats treated with high doses of oestrogen. GP 48,989 might modify the release of other pituitary hormones. On the other hand, methallibure reduces thyroid function [22] and if GP 48,989 produces a similar effect then the resulting hyphothyroidism would perhaps be sufficient to alter homeostasis in ovarian independent DMBA-induced tumours and provoke regression.

In conclusion we have found that the reported anti-tumour agent GP 48,989 is inactive in a truly oestrogen-independent rat mammary tumour system. GP 48,989 reduces circulating LH levels and it seems more likely that the antitumour effects in the DMBA-induced rat mammary carcinoma model may result from a perturbation of the normal endocrine system rather than a direct action on the mammary tumour.

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