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RU 58668: Further *In Vitro* And *In Vivo* Pharmacological Data Related to its Antitumoral Activity

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Previous studies with the pure antiestrogen RU 58668 showed that this compound proved to be highly antiproliferative *in vitro*, and to be the only antiestrogenic compound so far known to induce long-term regression of MCF-7 tumours implanted into nude mice. In order to obtain more insight into the therapeutic potential of this molecule, we performed a new set of experiments *in vitro* and *in vivo* in comparison with tamoxifen and/or ICI 182,780. *In vitro*, 1 nM RU 58668 induced an accumulation of MCF-7 cells in G0/G1 phases of the cell cycle within 48 h and, in contrast to trans-4-hydroxy-tamoxifen, blocked the invasiveness of ras-transfected MCF-7 cells into the chick embryo heart during the three weeks of co-culture. An *in vivo* dose-effect relationship study showed that RU 58668 induced a regression of MCF-7 tumour with as low a dose as 10 mg/kg/week, and that such an effect can not be obtained either with a sublethal dose of adriamycin or with ICI 182,780, (2–250 mg/kg/week). This reduction in the tumour volumes accords with histological modifications of the tumours, which showed a decrease in the ratio of epithelial cells over the tumoral mass, and with a concomitant decrease in their regrowth potential when reimplanted into naive nude mice. Taken together, these results suggest a promising usefulness for RU 58668 in the treatment of metastatic breast cancer in women. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Partly agonistic antiestrogens, such as tamoxifen, have been widely used in the treatment of estrogen receptor-positive metastatic breast cancer for more than 20 years. In addition, partly successful attempts at treating non-mammary tumours which express the estrogen receptor have been reported with tamoxifen or derivatives [1–7]. The use of "pure" antiestrogens, without partial agonistic activity, however, has been suggested for many years [8], because the estrogenic component of tamoxifen could be directly linked to the occurrence of secondary tumours, especially at the endometrial level [9]. It could also be one of the parameters involved in the escape of advanced tumours from tamoxifen treatment, as suggested by *in vivo* studies in mice [10], and may even be directly involved in the stimulation of breast cancer metastasis [11]. In other respects, it has been postulated that mammary tumour growth could be stimulated by autocrine or paracrine growth factors which could replace the estrogenic stimulation [12, 13]. Under growth factor-stimulating conditions, mixed antiestrogens are poor inhibitors of cell growth *in vitro* [14, 15], even if an indirect effect of tamoxifen on tumoral growth has been described *in vivo*, via a decrease of circulating growth factors [16].

This led the ICI group to synthesize the first pure antiestrogens, exemplified by ICI 164,384 [17] and ICI 182,780 [14, 18], the latter being in phase II clinical trials [19, 20]. We recently described a new pure antiestrogen, 11β-[4-[5-[(4,4,5,5,5,-pentafluoropentyl)sulfonyl]pentyloxy]phenyl]-estra-1,3,5(10)-tri-

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ene-3,17β-diol: RU 58668 (scheme 1) [15, 21–23], which displayed improved *in vitro* antiproliferative activities when compared to ICI 182,780 and similar antiuterotrophic activities in mice or rats. When tested for their activities on MCF–7 tumours implanted in nude mice, RU 58668 was the only compound able to induce a long-lasting reduction (at least 25 weeks) of the tumour volume [23]. When administered monthly at at dose of 250 mg/kg as subcutaneous oily injections, tamoxifen and ICI 182,780 slowed down, or at the best stopped, tumoral growth without inducing a tumour regression [15]. In order to get more insight into the causes of this striking difference between the *in vivo* antitumoral activities of the two "pure antiestrogens", we performed a set of new experiments. First, the dose/antitumoral activity relationships of RU 58668 and ICI 182,780 were studied in order to compare the doses of the two compounds which would induce the maximal effect. Second, experiments on the regrowth ability of tumour fragments excised from animals previously treated with these two compounds and tamoxifen were carried out, along with histological examination of the tumoral tissues. In addition, *in vitro* experiments were undertaken to study the effect of RU 58668 on the kinetics and intensity of cell cycle modifications. Invasiveness of the very highly tumorigenic ras-transfected MCF–7 (MCF–7vht) in chick embryonic heart was also studied to evaluate the influence of this compound on the metastatic potential of MCF–7 nodules into non-tumoral tissues.

MATERIALS AND METHODS

Chemicals

Bromodeoxyuridine (BrdUrd), propidium iodide, trisodium citrate and 3,3′-diaminobenzidine were purchased from Sigma. Fluorescein isothiocyanate (FITC) was from Becton Dickinson and fetal calf serum (FCS) from Boehringer Mannheim, Germany. Unless otherwise stated, all the culture media were obtained from Gibco.

In vitro *studies*

Cell cycle analysis. MCF–7 cells (HTB 22 from ATCC) were seeded (5×10^5 cells) in Dulbecco modified essential medium (DMEM) without phenol red supplemented with 10% dextran charcoal-treated FCS into 25 cm^2 flasks. Concentrations of RU 58668 (0.01–1 nM) were added at the cell seeding time. Cell cycle analysis was performed as described by Khochbin *et al.* [24]. To isolate cells in S-phase, BrdUrd was added to the culture medium for 15 min at 37°C, cells were then trypsinized, washed in phosphate-buffered saline (PBS) and fixed with 70% ethanol. Incorporated BrdUrd was revealed with a monoclonal antibody anti-BrdUrd conjugated with FITC. To quantify the DNA content, cells were centrifuged and resuspended in a staining solution containing 7.6 μM propidium iodide and 4 mM trisodium citrate for 10 min at 37°C. Double-stained cells were analysed in the FAC Scan flow cytometer (Becton Dickinson). Dot-plots obtained after cytofluorometric analysis allowed the determination of labelled cells (S-phase) from unlabelled cells (G0/G1 and G2 – M phase).

Invasiveness of tumorigenic cells. The human breast cancer cell-line MCF–7ras, a generous gift of C. Sommers (Georgetown University, Washington, DC, U.S.A.) was established after the transfection of the Hras oncogene in the MCF–7 ductal adenocarcinoma cell-line [25]. A very highly tumorigenic MCF–7 line (MCF–7vht) was obtained by injecting monolayers of MCF–7ras cells (2×10^6/0.1 ml) subcutaneously near the mammary gland of 5-week-old female Swiss nude mice (Iffa Crédo, Les Oncins, France). After six weeks, one mouse was killed, the tumour was cut out, cut into pieces of 1–2 mm^3 and cultured in a 25 cm^2 flask with DMEM supplemented with 10% FCS. This process was performed twice.

The dose effect relationship of RU 58668, ICI 182,780 and trans-4-hydroxy-tamoxifen (4-OH-tamoxifen) on the growth of these cells, cultured as a monolayer in DMEM without phenol red, in the presence of 5% charcoal-stripped serum, has been carried out in 24 multiwells. The cell growth was evaluated by a fluorimetric DNA assay as previously described [15].

Furthermore, invasiveness properties of the MCF–7vht were evaluated, using the following protocol: cells were scraped from the plastic flask surface, the pellet was harvested in 1 ml of DMEM without phenol red, with 10% charcoal-stripped serum (FCS), 1% glutamine and 1% sodium pyruvate and placed on top of a semi-solid medium composed of 1% Noble agar (Difco) in distilled water at a 1:1 ratio and 2 × DME medium supplemented with 20% FCS in 60 mm Petri dishes, in order to obtain solid nodules. These nodules were subcultured once a week by cutting small pieces with microsurgical scissors. Fresh heart fragments were dissected from a 8-day embryonic chick and closely joined side by side to MCF–7vht nodules in culture medium with or without 0.1 nM RU 58668 or 4-OH-tamoxifen in the absence of estradiol. After a three-week culture, nodule-heart fragment complexes were fixed overnight in Bouin's fixative, embedded in paraffin and processed for histological examination. The presence of epithelial cells in chick embryonic heart was revealed by the three-step indirect method using murine monoclonal antibodies to cytokeratin gp 56 kD (KL1, Immunotech, France) as first antibody (dilution 1:400). The second antibody was a rabbit peroxidase-conjugated anti-mouse IgGs (Dakopatts Co. Inc., Denmark) (dilution 1:20). The third antibody was a pig peroxidase-conjugated anti-rabbit IgGs (Dakopatts) (dilution 1:20). The 3,3′-diaminobenzi-

dine was used as peroxidase-substrate-chromogen and Harris haematoxylin as counterstain.

In vivo *studies*

MCF–7 cell culture and tumour settlement. MCF–7 cells from ATCC (HTB 22) were routinely cultured and subpassaged in minimal essential medium (MEM) with phenol red in the presence of 5% FCS as previously described [15] in 75 cm^2 flasks. When subconfluent, the MCF–7 cells were trypsinized and resuspended in the above-mentioned medium at a density of 5×10^7 cells/ml. One hundred microlitres of this suspension was injected subcutaneously into the right mammary fat pad of four to five week old female balb/ca nude mice (Iffa Crédo). Tumour growth was stimulated by a weekly percutaneous administration of 5 mg/kg estradiol (E2) dissolved in 10 μl ethanol. When tumours reached 250–500 mm^3 (calculated as width^2 × length/2), the animals were killed and the tumours cut into small pieces for reimplantation. All the experiments were carried out with tumour fragments obtained from a single tumour.

Dose activity relationship of RU 58668 on MCF–7 tumours. Nude mice were subcutaneously (s.c.) implanted with 1–2 mm pieces of MCF–7 tumour, obtained as described above, and received a weekly administration of 5 mg/kg E2 percutaneously (p.c.) for five weeks. Animals were then randomized according to their tumour volume (week 0) and treated weekly with 0.25 mg/kg E2 alone, or with a weekly subcutaneous injection of 2, 10, 50 or 250 mg/kg of test compounds, suspended in arachis oil. A control group received E2+ the sublethal dose of 10 mg/kg adriamycin by intraperitoneal route in saline on weeks 0 and 3. The tumour volume was checked every week and, at the end of the experiment (week five), the animals were killed by cervical dislocation and the tumours and uteri were removed and weighed. The tumour evolution was calculated as the ratio: tumour volume on week five/tumour volume on week 0.

Incidence of a pretreatment with RU 58668 on tumour regrowth. After a five-week induction of the tumours, mice were randomized into four groups of five animals which received a weekly administration of 0.25 mg/kg E2 alone or along with two s.c. injections of 250 mg/kg RU 58668, ICI 182,780 or tamoxifen (one on week 0 and one on week five). On week 10, the animals were killed and each tumour was cut into 1–2 mm pieces which were reimplanted into five naive nude mice leading to groups of 25 animals bearing tumours previously treated by E2, E2+ RU 58668, E2+ ICI 182,780 or E2+ tamoxifen. The growth of these tumours was stimulated by a weekly administration of 5 mg/kg E2 for six weeks. The tumour growth was checked every week in order to evaluate the incidence of the previous treatment on their evolution into non-treated mice.

Histological studies. In order to correlate the regrowth profiles with histological parameters, a 14-week experiment, in which animals received 250 mg/kg test compounds on weeks 0, 5 and 10 along with 0.25 mg/kg E2 weekly, was carried out. On week 14, mice were killed and the tumours were removed and fixed in Bouin's solution, embedded in paraffin and processed for histological examination. Preparations were stained with Masson's trichrome technique in which collagen appears in green, cell nuclei in crimson red and cytoplasm in light brown.

RESULTS

In vitro *studies*

Effect on the MCF–7 cell cycle. Table 1 presents the compared fractions of MCF–7 cells in G0/G1, in S and in G2 + M phases, either untreated or treated

Table 1. Distribution of MCF–7 cells in the various phases of the cell cycle

		24 h			48 h		
		G0/G1(%)	S(%)	G2/M(%)	G0/G1(%)	S(%)	G2/M(%)
Control		45	35	19	52	27	21
	10^{-9}				41	35	23
4-OH-tamoxifen	10^{-8}				48	31	21
	10^{-7}				49	28	23
	10^{-6}				55	23	22
	10^{-11}	44	34	22	53	24	22
ICI 182,780	10^{-10}	45	34	20	54	25	21
	10^{-9}	45	35	19	57	18	25
	10^{-11}	46	32	23	51	24	24
RU 58668	10^{-10}	49	31	21	54	22	24
	10^{-9}	50	29	21	59	16	25

Cells were untreated or treated with 0.01 to 1 nM of the pure antiestogens for 24 or 48 h or with 1 nM to 1 μM of 4-OH-tamoxifen for 48 h. At appropriate times, bromodeoxyuridine (BrdUrd; Sigma) was incorporated in cells in the S-phase. Incorporated BrdUrd was revealed with a monoclonal antibody anti-BrdUrd conjugated with fluorescein isothiocyanate (FITC; Becton Dickinson). To quantify DNA content, cells were resuspended in a staining solution containing 7.6 μM propidium iodide. Double-stained cells were analysed in the FAC Scan flow cytometer (Becton Dickinson). Dot-plots obtained after cytofluorometric analysis allow the determination of G0/G1, S-phase and G2 – M phase.

with 4-OH-tamoxifen, ICI 182,780 or RU 58668. Treatment of MCF–7 cells with 10^{-9} M RU 58668 for 48 h resulted in a 40% decrease of the number of cells in S-phase with a corresponding increase in the number of cells in G0/G1 phase. The experiment with RU 58668 was carried out three times. It is interesting to note that a reproducible and significant ($P < 0.05$) effect of RU 58668 on the cell cycle appeared after only 24 h. Only the presented experiment compared the three compounds at 24 and 48 h. In this experiment 4-OH-tamoxifen and ICI 182,780 seem to be inactive at 24 h. Low concentration (1 nM) of 4-OH-tamoxifen increased S-phase at 48 h. One micromole of that compound was required for reducing the cell number in S-phase. After a five-day treatment no difference could be observed between treated and untreated groups, all cells being accumulated in G0/G1 phases. This effect in the control group is related to the cell confluence.

Effect on in vitro proliferation of MCF–7vht. The ras-transfected MCF–7vht cell line, cultured in the presence of 5% stripped serum, showed a two to three times higher "spontaneous" proliferation rate than standard MCF–7 (data not shown). This allowed us to study the effect of test compounds on the growth of these cells, without any exogenous stimulating agent. Fig. 1 shows the activity of RU 58668, ICI 182,780 and 4-OH-tamoxifen after a seven-day culture of MCF–7vht cells. RU 58668 was 2.5 times more potent than ICI 182,780 and 40 times more potent than 4-OH-tamoxifen to inhibit the growth of these cells, with respective IC_{50}'s of 0.035 ± 0.010 nM, 0.09 ± 0.03 nM and 1.4 ± 0.6 nM (mean ± SEM of three or four experiments). Moreover, the two former compounds induced a nearly complete inhibition of cell growth (≥90%), whereas 4-OH-tamoxifen induced only a limited growth inhibition (50–60%) of the MCF–7vht cell line.

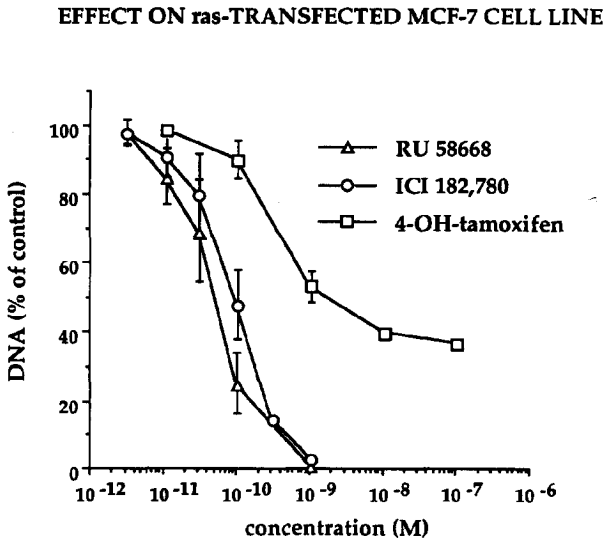

Fig. 1. *In vitro* activity of RU 58668, ICI 182,780 and 4-OH-tamoxifen on the MCF–7vht cells. The ras-transfected MCF–7vht cells were cultured in DMEM without phenol red in the presence of 5% charcoal-stripped serum and the indicated concentration of test compounds. Medium was changed every two to three days, and the DNA was assayed after eight days. Results are mean ± SEM of three or four experiments.

Effect on invasiveness of MCF–7vht in chick embryonic heart. Striking differences between 4-OH-tamoxifen and RU 58668 were also observed on the *in vitro* inhibition of MCF–7vht invasiveness in chick embryo heart. Fig. 2b shows chick embryonic heart after a three week contact with a nodule of MCF–7vht in the presence of RU 58668 at 10^{-9} M. No epithelial cells, stained with the specific epithelial cell antibody KL1 cytokeratin, had invaded the heart fragment. At the same concentration, 4-OH-tamoxifen (Fig. 2c) was unable to prevent invasiveness as shown by the presence of several stained cells in the embryonic tissue (see arrows). In the untreated-control group, embryonic tissue cannot be distinguish from MCF–7vht cells (Fig. 2a).

In vivo *studies*

Dose activity relationship of MCF–7 tumours implanted in nude mice. A dose activity relationship study was undertaken in order to determine the maximal tumour inhibitory effect of RU 58668 in this model. As shown in Fig. 3, the maximal antitumoral activity of RU 58668 and ICI 182,780 was reached at the dose of 50 mg/kg weekly: RU 58668 induced a regression of the tumours, whereas ICI 182,780 was only able to slow down the tumour growth (respective ratio of tumour volumes: 0.35 ± 0.05 and 1.72 ± 0.39, $P < 0.01$). A higher dose of the two compounds (250 mg/kg/week) did not induce significantly stronger effects. The regression of MCF–7 tumours induced by RU 58668 was observed at doses starting from 10 mg/kg/week. On the contrary, ICI 182,780 was not able to induce any regression of the tumours on that model, whatever the doses used. Nevertheless, in these animals, the two compounds displayed similar antiuterotrophic effects at equivalent doses (Fig. 4).

In order to test the sensitivity of these MCF–7 tumours to chemotherapeutic agents used in the clinic, we chose the established drug adriamycin. The dose used (two injections of 10 mg/kg) was the highest one compatible with the toxicity of the compound. In this model, adriamycin only led to a decrease in tumoral growth, without regression, an effect significantly smaller than that of 10 mg/kg/week RU 58668 (respective ratios of tumour volume at the end of the experiment: 1.56 ± 0.18 and 0.61 ± 0.07, $P < 0.01$). The relatively poor effect of adriamycin in this model shows that these tumours have a strong growing ability when implanted in nude mice.

Effect of RU 58668 on tumour regrowth. We have previously shown that RU 58668 induced the regression of E2-stimulated MCF–7 tumours implanted in nude

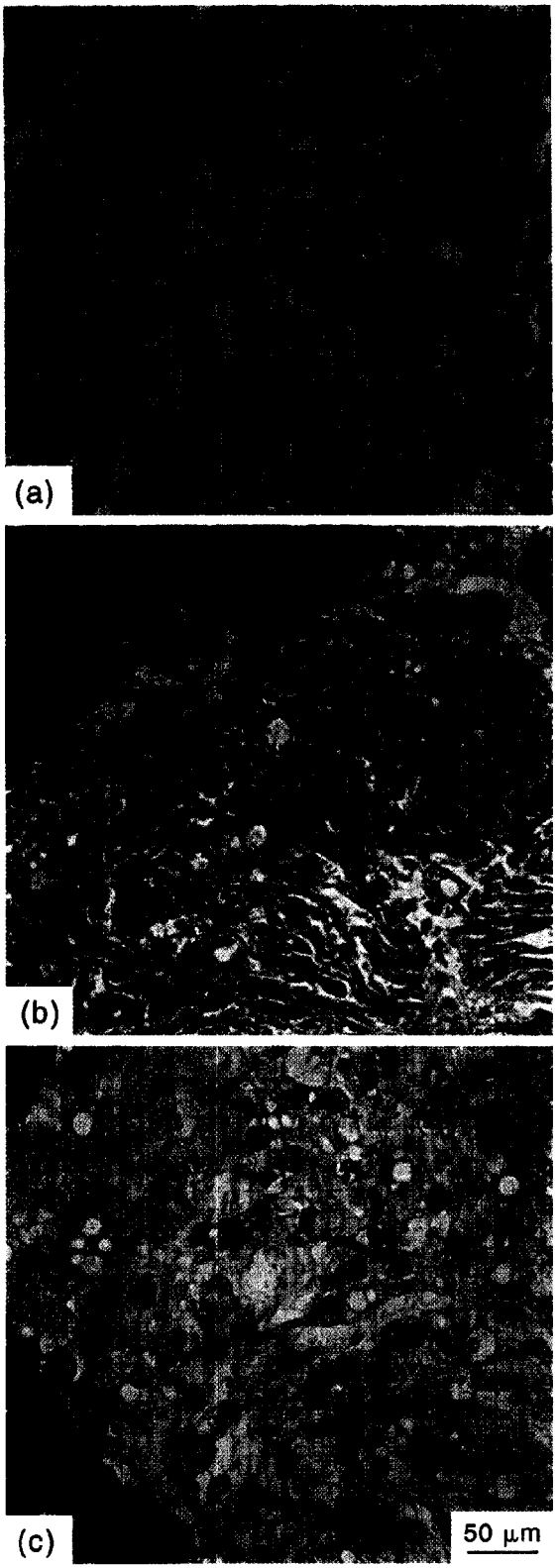

Fig. 2. Decreased invasiveness of MCF-7 transfected cells in embryonic chick heart. Embryonic chick hearts were maintained in culture closely with MCF-7vht nodules in the absence of estradiol, and treated with vehicle (2a), RU 58668 10^{-9} M (2b) or OH-tamoxifen 10^{-8} M (2c). After three-week culture, nodule-heart fragment complexes were fixed in Bouin's solution and included in paraffin. Epithelial cells were revealed by the three-step indirect method using murine monoclonal antibodies to cytokeratin gp 56 Kd (KL1, Immunotech, France) and appeared in brown (arrows) in photomicrographs of paraffin sections from embryonic heart (scale bar = 50 µm).

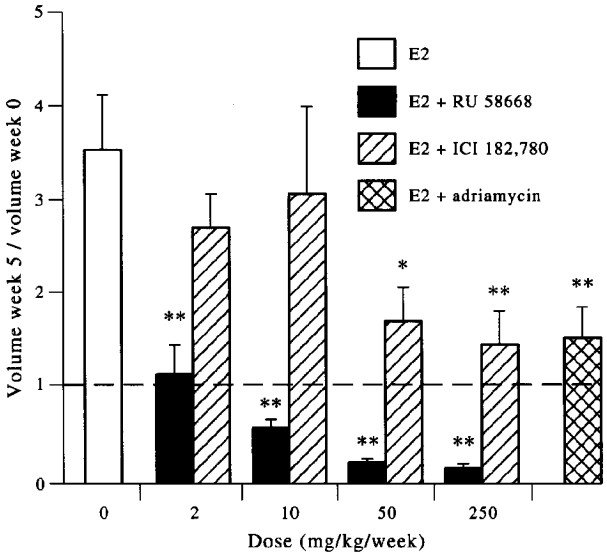

Fig. 3. Dose effect relationship of RU 58668 and ICI 182,780 on *in vivo* MCF-7 tumour evolution. After MCF-7 tumour settlement under estradiol, the animals received a weekly s.c. administration of the indicated doses of RU 58668 or ICI 182,780 along with 250 µg/kg estradiol for five weeks. The effect of these treatments was compared to that of i.p. injections of 10 mg/kg adriamycin on weeks 0 and three of the experiment. *$P < 0.05$; **$P < 0.01$ vs E2 (Mann–Whitney test).

mice [15, 23]. Similar results (Fig. 5) were obtained during the 10-week experiment described here; animals treated with RU 58668 plus estradiol showed a ratio: volume week 10/volume week 0 of 0.48 ± 0.11. Under the same conditions, the groups of animals treated with ICI 182,780 and tamoxifen led to respective ratios of 1.43 ± 0.28 and 2.66 ± 0.63. Control mice treated with E2 alone showed a ratio of 6.05 ± 1.09. In order to check if the RU 58668-treated tumours retain some growing ability after this 10-week treatment, 1–2 mm pieces of tumour from each of these groups were reimplanted into naive nude mice and stimulated for six weeks with 5 mg/kg E2 weekly, without any other treatment. The tumours from the different pretreated groups displayed different growth rates (Fig. 6), and significantly different weights at the end of the six weeks (Table 2). Tumours excised from animals treated with RU 58668 displayed the lowest regrowing ability and the

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