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The Pyrimido-pyrimidine Derivatives RA233 and RX-RA85 Affect Cell Cycle Distribution of Two Murine Tumour Cell Lines

ROSEMARIE B. LICHTNER,*†§‖ GILLIAN HUTCHINSON* and KURT HELLMANN‡

**Cancer Chemotherapy Department, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2, U.K. and ‡Radiotherapy and Oncology Department, Westminster Hospital, Horseferry Road, London SW1, U.K.*

Abstract—*The pyrimido-pyrimidine derivatives RA233 and RX-RA85, which are potent inhibitors of platelet and tumour phosphodiesterases, were developed as antitumour agents. Clinical as well as animal studies suggest a tumour type specific, although moderate, antitumour activity for RA233. In our search for more potent congeners of RA233, we found that RX-RA85 was cytotoxic for cultured B16 melanoma and Lewis lung carcinoma cells at drug concentrations above 4 μg/ml whereas RA233 concentrations up to 400 μg/ml were tolerated. When tested for their effects on cell cycle distribution, RX-RA85 was 100-fold more potent than RA233 in producing an increase in the proportion of cells in S and G_2 + M phase in 3LL cells. Progression of 3LL cells through the cell cycle was delayed for 5 h by RA233 treatment, whereas RX-RA85 was ineffective. In contrast, B16 cells responded poorly to either drug. The effects of both compounds were not only tumour cell specific but also dependent on the stage of tumour cell growth (drugs added to synchronously vs. asynchronously growing cultures). In the case of RX-RA85, the potency to affect tumour cell cycle distribution was highly dependent on tumour cell number, making the potential of this drug as an antitumour agent somewhat limited.*

INTRODUCTION

THE pyrimido-pyrimidine analogues RA233 and RX-RA85 were developed originally as potent phosphodiesterase (PDE) inhibitors [1] and antiplatelet drugs [2]. Because platelets are thought to be involved in blood-borne metastasis [3], these drugs were tested as antimetastatic agents for a number of animal tumours with conflicting results [4]. However, RA233 treatment in a pilot clinical trial resulted in a reduction of metastasis formation [5] and in two independent recent trials it prolonged statistically significant survival in patients with limited non-small cell lung cancer, while it was ineffective in patients with extensive non-small cell lung cancer or with other types of cancer [6, 7]. These results suggest that RA233 has direct, tumour-specific effects. Nevertheless, RA233 might not be the optimal drug since its effects seem to be overwhelmed by tumour burden [8]. Therefore, in our search for more potent congeners of RA233 we tested the pyrimido-pyrimidine compound RX-RA85 in several *in vitro* systems. We have already shown that RX-RA85 affects growth and cytoskeletal organization of cultured tumour cells at 100-times lower concentrations than RA233 [9–11]. In this report we investigate the effects of RX-RA85 on cell cycle distribution of two mouse tumour cell lines in comparison with RA233, an extension of the data which had been reported recently [12].

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†Present address: German Cancer Centre, Department of Immunology and Genetics, 69 Heidelberg, F.R.G.
§To whom requests for reprints should be addressed.
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MATERIALS AND METHODS

Tumour cell lines

B16 melanoma and Lewis lung carcinoma (3LL) cells were grown in 100 mm diameter Petri dishes in Eagle's minimum essential medium in the presence of antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and supplemented with 10% calf serum (B16) or 10% foetal calf serum (3LL) and incubated at 37°C in a humidified atmosphere (90% air, 10% CO_2). The mean doubling time was 16.8 h for B16 and 14.4 h for 3LL cells, respectively.

Compounds

The drugs were pyrimido-pyrimidine derivatives and potent PDE inhibitors and antiplatelet drugs [1]. They were obtained from Dr. Karl Thomae GmbH, Biberach, F.R.G. The drugs were solubilized in 0.1 N hydrochloric acid and diluted with double distilled water.

*RA*233

2,4,6-Trimorpholino-pyrimido (5,4-d)-pyrimidine is a moderately potent PDE inhibitor ($EC_{50} = 2 \times 10^{-5}$ M) measured on platelet PDE [1] and 10^{-6} M measured on tumour cell PDE [13].

*RX-RA*85

4-(1-Oxidothiomorpholino)-8-phenethylthio-2-piperazino-pyrimido (5,4-d)pyrimidine shows a 10^4 times better inhibitory activity on platelet PDE ($EC_{50} = 2 \times 10^{-9}$ M) [1].

Colony formation test

The inoculum was always derived from confluent cultures. 200 cells/5 ml (> 95% viability estimated by trypan blue) were seeded in 60 mm diameter Petri dishes and after 90 min incubation the substances or vehicle (100 μl) were added. In some experiments the substances were removed after 24 h by washing once with phosphate buffered saline (PBS) and addition of fresh medium (24 h exposure) while in others the drugs were left throughout the period of colony formation (constant exposure). The incubation was continued until day 9–10. The colonies were fixed and stained with Giemsa's and the number of colonies containing at least 50 cells were counted. The plating efficiency was 75–90% for the B16 and 40–60% for the 3LL cells.

Flow-cytofluorometry

The effect of RX-RA85 on cell cycle distribution of synchronized and asynchronously growing 3LL and B16 cells was studied by measurement of the DNA content using a Fluorescence Activated Cell Sorter (FACS). Tumour cells (5×10^5 or 10^6) from confluent cultures unless otherwise stated were seeded in 100 mm Petri dishes and at the indicated time points 100 μl of solubilized substance was added, while the controls received vehicle. The cells were detached with 2 mM EDTA in PBS. The tubes were transferred to ice, the cells spun down and about 10^6 cells carefully fixed with 80% alcohol. After 30 min on ice the cells were centrifuged and then stained with mithramycin for DNA with a modification of the method described by Crissman and Tobey [14]. Briefly, fixed cells were stained for 30 min at room temperature with 50 μg/ml mithramycin and 20 mM $MgCl_2$ in 25% aqueous ethanol. Samples were analysed for DNA content using a FACS II (Becton Dickinson Fluorescence activated cell sorter). Histograms obtained were analysed for cell-cycle distribution on a computer (Tektronix Model 4052) with a program developed at the Imperial Cancer Research Fund. This program normalizes cell numbers to the highest peak, e.g. G_{1+0} or G_2 + M. The resulting histograms were plotted with a Tetronix Model 4662.

RESULTS

Colony formation test

We compared the effects of RA233 and RX-RA85 on growth of B16 and 3LL tumour cells *in vitro* (Table 1). Constant exposure to RA233 or RX-RA85 resulted for both tumour lines in a dose-dependent inhibition, RX-RA85 being about 100–200 times more effective. For a particular drug the two cell line had comparable EC_{50}s. However, when the drugs were present for 24 h the tumour cell lines behaved differently with B16 cells being less responsive to treatment with either drug.

Flow cytofluorometry

The effects of RA233 and RX-RA85 on the cell cycle distribution of two different mouse tumour lines were investigated on synchronized and asynchronously growing cells. Partial synchronization was achieved by growing the cells to confluency without changing the medium, thus causing inhibition of cell proliferation by nutrient deprivation [15]. Seeding these cells in fresh medium induced after a lag period a synchronous wave of DNA synthesis [15, 16]. The variable factor is the dur-

Table 1. *Inhibition of colony formation after exposure of* 3*LL or B*16 *cells to RA* 233 *or RX-RA* 85

Tumour	Substance	EC_{50} for 24 h exposure (μg/ml)	EC_{50} for constant exposure (μg/ml)
3LL	RA 233	200	125
	RX-RA 85	0.9	1.3
B16	RA 233	>400	110
	RX-RA 85	2.8	1.5

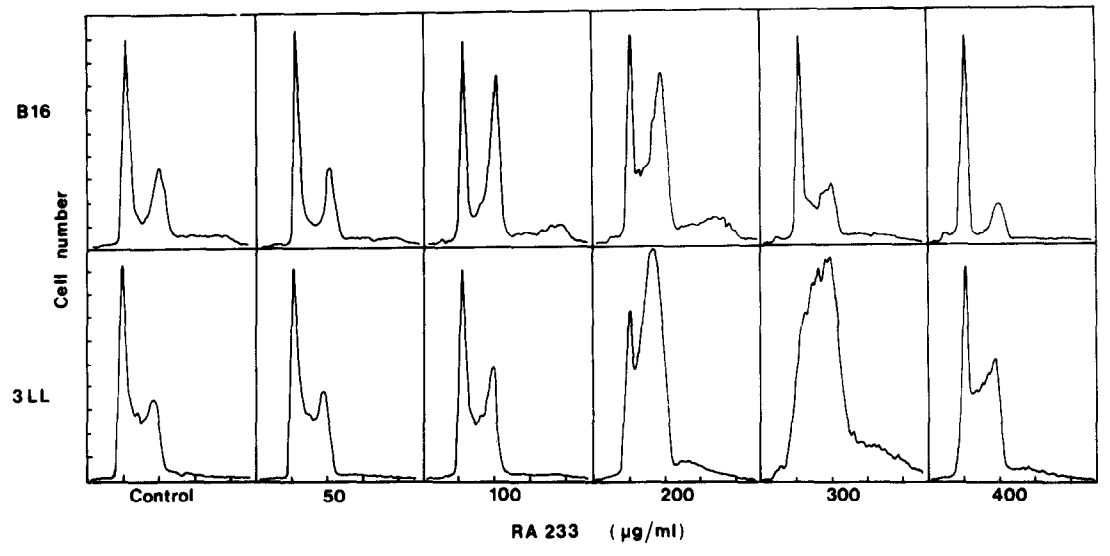

Fig. 1. DNA histograms of B16 (5 × 10^5 cells/plate) and 3LL (10^6 cells/plate) tumour cells, incubated for 24 h with increasing concentrations of RA233, added 1.5 h after seeding of plateau-phase cells.

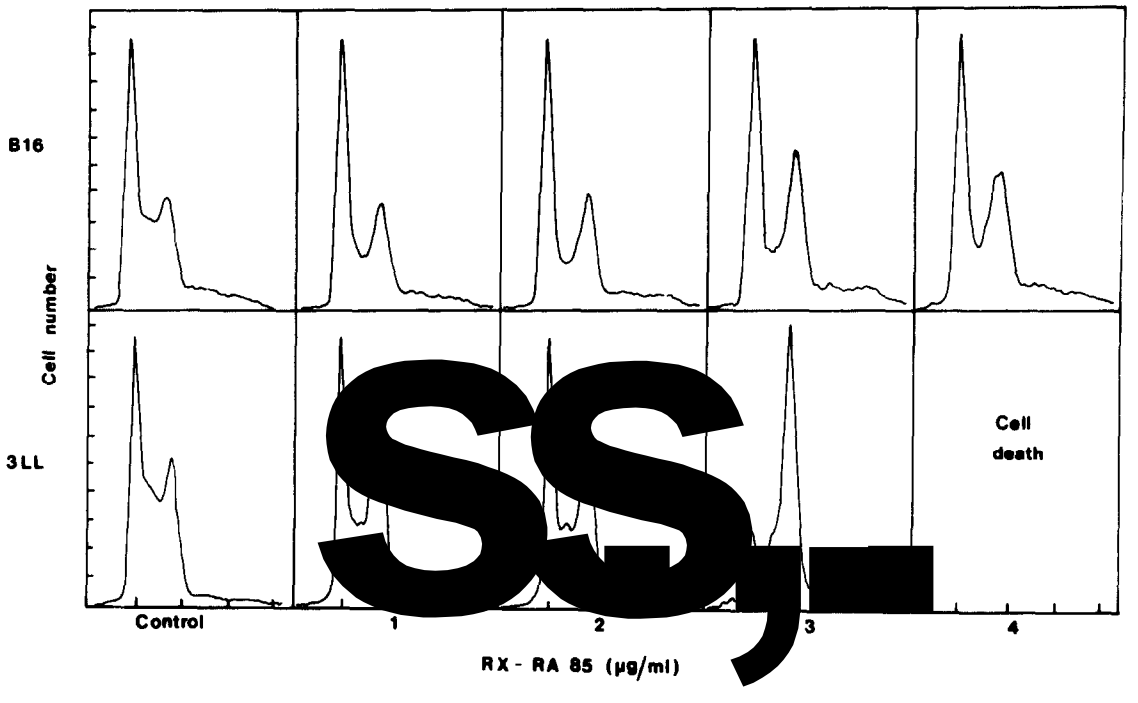

Fig. 2. DNA histograms of B16 (5 × 10^5 cells/plate) and 3LL (10^6 cells/plate) tumour cells incubated for 24 h with increasing concentrations of RX-RA85, added 1.5 h after seeding of plateau-phase cells.

ation of the lag phase which is highly dependent on the stage of growth of the cells used for seeding [17]. We tested the effects of different drug concentrations on both tumour cell lines during a 24 h incubation period. In Fig. 1 addition of RA233 to 3LL cells resulted in a dose-dependent increase of percentage of cells in G_2 + M phase. At higher concentrations (i.e. 400 μg/ml), the drug appeared to block the transit of cells from G_1 + G_0 to S phase. This might be due to inhibition of thymidine transport as it is reported for the pyrimido-pyrimidine derivative dipyridamole [18].

B16 cells showed a somewhat different sensitivity to the drug (Fig. 1). At low RA233 concentrations (<200 μg/ml), incubation for 24 h did not affect G_2 + M phase cells, whereas exposure to concentrations above 300 μg/ml RA233 resulted in a decrease in G_2 + M phase cells.

When various concentrations of RX-RA85 were added 1.5 h after seeding, at 3 μg/ml, 3LL cells showed an increase in G_2 + M phase cells similar to that described above for RA233 while B16 cells were much less affected (Fig. 2). These data show that B16 cells are clearly more resistant to treatment with these drugs.

Next, we investigated the effects of RX-RA85 and RA233 on the DNA content of the tumour cells as a function of incubation time. Figures 3 and 4

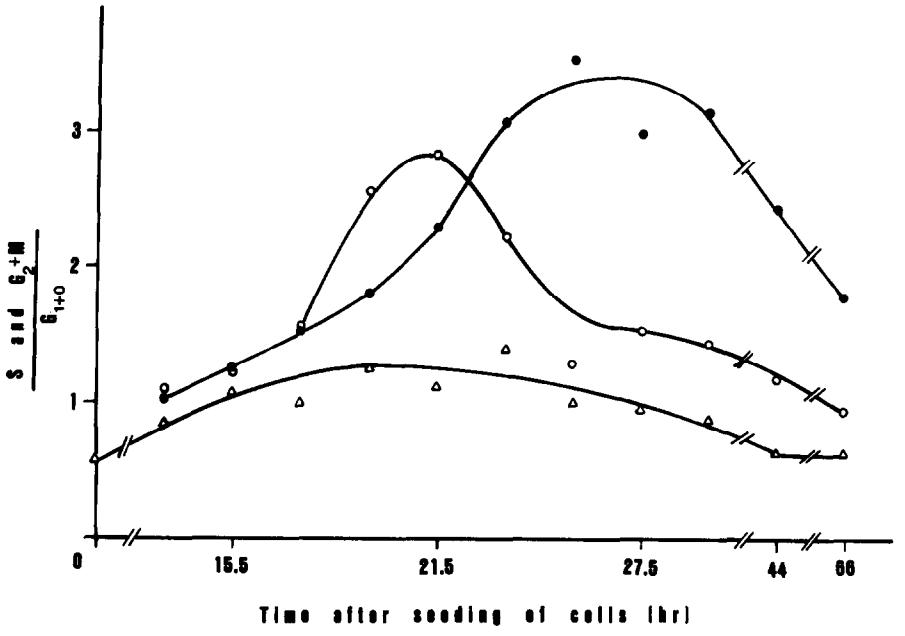

Fig. 3. The effects of RX-RA85 and RA233 on cell cycle distribution of 3LL tumour cells (10^6/plate) at different time points of incubation. The drugs were added 1.5 h *after seeding of plateau-phase cells. The histograms were cut out, weighed for each time point and the area under the S and G_2 + M peaks expressed as the ratio of* $\frac{S \text{ and } G_2 + M}{G_1 + G_0}$. △: *control*; ○: 3 μg/ml *RX-RA85*; ●: 200 μg/ml *RA233*.

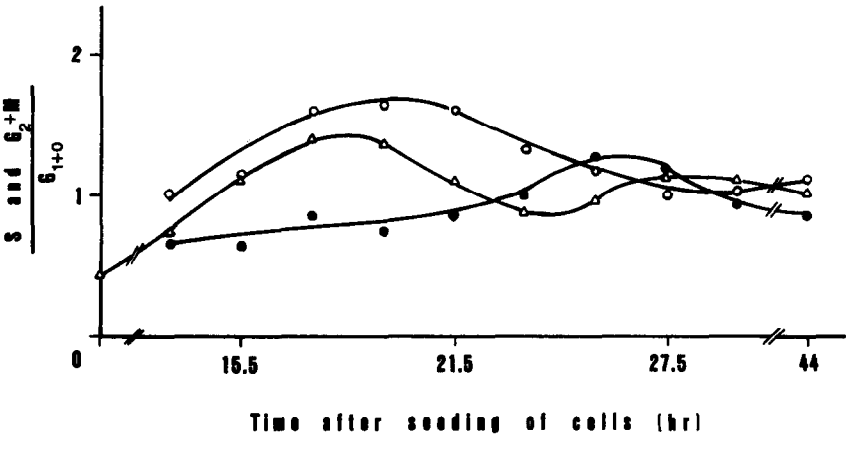

Fig. 4. The effects of RX-RA85 and RA233 on cell cycle distribution of B16 tumour cells (5×10^5/plate) *at different time points of incubation. The drugs were added* 1.5 h *after seeding of plateau-phase cells. The histograms were cut out, weighed for each time point and the area under the S and G_2 + M peaks expressed as the ratio of* $\frac{S \text{ and } G_2 + M}{G_1 + G_0}$. △: *control*; ○: 3 μg/ml *RX-RA85*; ●: 200 μg/ml *RA233*.

show representative time points from the corresponding experiments. When plateau phase 3LL cells were seeded into fresh medium the proportion of control cells in S and G_2 + M phase was elevated between 15.5 and 27.5 h (Fig. 3). Adding 3 μg/ml RX-RA85 resulted in a sharp increase in the proportion of cells in S and G_2 + M phase (1.5-fold) with the time course parallel to control cells, while after 66 h exposure to RX-RA85 the cell cycle distribution of treated and control cells were comparable.

Again, the response of B16 cells to RX-RA85 and RA233 was far less pronounced (Fig. 4). B16 cells grown to confluency and seeded into fresh medium synthesized DNA with a peak in the proportion of G_2 + M phase cells between 18 and 19 h. Adding 3 μg/ml RX-RA85 resulted in a slightly elevated proportion of cells in S and G_2 + M phase which persisted for a longer time than in the control group. The picture looked somewhat different when 200 μg/ml RA233 was added to B16 cells (Fig. 4). RA233 seemed to delay the onset of DNA synthesis in B16 cells.

Next, we investigated the sensitivity of 3LL cells in different growth phases towards these drugs. In pilot investigations we had noticed that asynchro-

nously growing B16 and 3LL cells were not or less sensitive to treatment with RX-RA 85 (data not shown). This observation prompted us to investigate the effects of RX-RA85 and RA233 on the more responsive 3LL cells seeded with different initial cell numbers and from different stages of growth. Figure 5 shows that the effect of RX-RA85 and RA233 on the cell cycle distribution of cells taken from the plateau phase depended largely on the seeding density. At an inoculum of 5×10^5 cells/dish almost all cells accumulated in G_2 + M phase. Peak II was still the predominant one at an initial cell inoculum of 10^6 cells, but at 2×10^6 cells the main percentage of cells was again restricted to peak I and the cell cycle distribution of treated cells was indistinguishable from the controls. In contrast incubating increasing numbers of cells with 200 μg/ml RA233 resulted in the accumulation of most of the cells in G_2 + M independent of the initial cell number inoculated (Fig. 5A).

The same treatment protocol was applied to cells taken from asynchronously growing cultures (Fig. 5B). Again the effect of treatment with 3 μg/ml RX-RA85 for 24 h on cell cycle distribution was dependent on the number of tumour cells seeded initially. Adding 200 μg/ml RA233 to cells taken from asynchronously growing cultures resulted in an increase of percentage of cells in G_2 + M phase. Again, this effect was independent of the cell number inoculated initially but far less pronounced than the effect on cells from plateau growth phase.

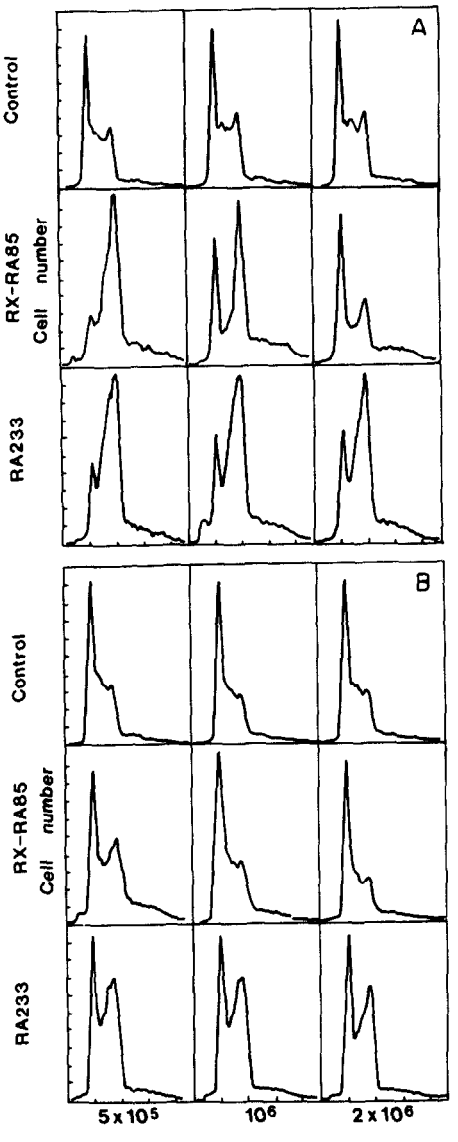

Fig. 5. DNA histograms of 3LL tumour cells taken from cultures in different stages of growth. 5×10^5, 10^6 or 2×10^6 cells were seeded, 3 μg/ml RX-RA85 or 200 μg/ml RA233 added after 1.5 h and after 24 h incubation the cell cycle distribution estimated. A. Cells taken from confluent cultures. B. Cells taken from asynchronously growing cultures.

DISCUSSION

Our experiments into the effects of pyrimido-pyrimidine derivatives on growth and cell cycle distribution of tumour cells showed that the drugs investigated increased the proportion of cells in S and G_2 + M phase and delayed the progression of cells through the cell cycle. Based on our previous study on the effect of these drugs on cytoskeletal organization of tumour cells [10], we hypothesize that drug interference with the reorganization of cytoskeletal structures caused mitotic inhibition. This is supported by the fact that the drug concentrations to yield these effects were comparable in both studies. Additionally, microtubule inhibitors such as nocodazole accumulate cells in mitosis [19].

In contrast to our previous investigation, however, we now find that the cytokinetic response of 3LL cells to RA233 was more sensitive than that of the B16 cells [12]. It is conceivable that with more than 1 year of subculturing, the tumour lines have changed their sensitivities towards RA233. This would not be surprising, as it has been reported by others that tumour cell lines with low or high passage numbers show different drug sensitivities [20]. In all our experiments 3LL cells have been more sensitive than B16 cells towards RA233 or the chemically related RX-RA85. This may be due either to different binding of substances to the cell membrane, different uptake rates, different enzyme affinities or different isoenzymes. The effects of the drugs tested are not only dependent on the tumour line but also on the stage of growth, confirming the observations of Biddle *et al.* [21]. This is also in agreement with the results of Chambers *et al.* [22], who showed that EMT6 cells in plateau phase were more sensitive to Adriamycin® compared to asynchronously growing cells and that cell numbers in relation to drug dose were crucial. Our investigation shows that this also applies to treatment of 3LL cells with RX-RA85 and may be due to drug metabolism or competition with non-specific binding sites on the cell membrane or within the cell. In the case of RA233 the activity is also dependent on the stage of growth of cells, but

independent of the cell number. The dependence of the effect of the PDE inhibitors tested on the stage of growth of cells could indicate that the activity of the inhibited PDE is dependent on stage of growth, thus it is known for example that for human lymphoid cells the activity of PDE fluctuates during the cycle [23].

The apparent dependence of the activity of RX-RA85 on tumour cell numbers and stage of growth makes the potential of this drug, unless as yet undisclosed advantages are revealed by further work, as an antitumour agent somewhat limited. Also, all animal experiments with RX-RA85 so far have shown no effect on primary solid tumour growth and, if anything, an increase in secondary Lewis lung tumours [4, 9] The increase in metastasis could be the result of damage to the vascular endothelial cells, thus enabling circulating tumour cells to adhere more readily to the exposed matrix [11].

RA233, on the other hand, does not damage vascular endothelial cells [24] and appears to be non-toxic generally [10, 12, 13, 21, 24–28]. At non-toxic concentrations (25 μg/ml) RA233 had pleiotropic and differential effects on cloned, cultured rat mammary adenocarcinoma cells, such as changes in cellular morphology, cytoskeletal organization, expression of p21 and interference with the growth promoting activity of EGF on these cells [13, 28]. When tested for its antimetastatic potency in this rat tumour model, RA233 treatment of tumour-bearing rats resulted in increased experimental metastasis while spontaneous metastasis was decreased, although moderately [24]. These investigations indicate that RA233 exerts its protective antimetastatic potency not via cytotoxicity but rather by modulating the growth potency of tumour cells.

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