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A COMPARISON OF THE PHYSIOLOGICAL EFFECTS OF RSU1069 AND RB6145 IN THE SCCVII MURINE TUMOUR

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The physiological and therapeutic effects of the bioreductive agent RSU1069 (80 mg/kg i.p.) and its prodrug RB6145 (240 mg/kg i.p.) were investigated in the SCCVII tumour. Using laser Doppler flowmetry it was found that RSU1069 produced a significant 30% reduction in tumour blood flow 30 min after administration, while RB6145 had no effect. Tumour oxygenation, measured with an Eppendorf oxygen electrode, was unchanged by either agent except for a reduction in values less than 2.5 mmHg at 30 min after injection. Neither agent significantly altered tumour energy metabolism, assessed by ^{31}P magnetic resonance spectroscopy. Both agents significantly increased tumour glucose content by a factor of 1.6–1.7 at 30 min after injection, but had no effect on glucose-6-phosphate or lactate levels. Tumour growth was significantly delayed by heating (42.5°C, 60 min), and although neither RSU1069 nor RB6145 alone had any effect on tumour growth they produced a similar enhancement of the tumour response to heat. The therapeutic effects are consistent with the known conversion in vivo of one third of the pro-drug RB6145 to its active product RSU1069, however the physiological effects of the two agents in the SCCVII tumour are not identical.

RSU1069 (1) is a 2-nitroimidazole bioreductive agent which has been shown to have potent anti-tumour activity under hypoxic conditions (2, 3). However, clinical trials with this agent showed it to have considerable systemic toxicity (4) and so other agents of this type were developed to overcome this. RB6145 (5) is a pro-drug for RSU1069, which was prepared originally at the MRC Radiobiology Unit, and has been licensed recently to the pharmaceutical industry for clinical development. This agent is metabolised in vivo with 30% conversion to RSU1069, the other metabolite being 2-nitroimidazole-oxazolidin-2-one (5, 6). In experimental models, the administration of RB6145 at 3 times the RSU1069 dose (to give equivalent RSU1069 availability) results in a similar anti-tumour activity of the two agents, but the systemic toxicity of RB6145 is less (5, 7, 8).

The recent interest in the manipulation of tumour blood flow and physiology to improve conventional anti-cancer treatments (9, 10) has led to a number of experimental findings that certain chemotherapeutic and bioreductive agents themselves can significantly affect the physiology of solid tumours (11, 12).

The present study was designed to examine the effects of RSU1069 and RB6145 on the physiology of the SCCVII murine transplantable tumour, and to determine whether any physiological changes are reflected in altered therapeutic response. To carry this out, a single intraperitoneal dose of each agent was used, 80 mg/kg RSU1069, and 240 mg/kg RB6145, designed to give an equivalent RSU1069 availability in vivo, to allow a direct comparison of their effects on tumour blood flow, oxygenation status and energy metabolism, using a range of techniques routinely available for this purpose.

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Material and Methods

Mouse and tumour model

The SCCVII squamous cell carcinoma was used in all experiments. Details of its derivation and maintenance have been described elsewhere (13). Solid tumours were produced in 8–10 week old male or female C_3H mice, by injecting 2×10^5 cells in 0.001 ml culture medium, subcutaneously into the hind foot dorsum. Tumours were used for experiments at volumes of approximately 200 mm^3, determined from measurements of three orthogonal diameters according to the following formula:

$$\text{Volume} = \pi/6(d_1 \times d_2 \times d_3)$$

The implantation of the tumour in the hind foot dorsum permitted all experimental procedures to be carried out on non-anaesthetised mice. Where required, the mice were gently restrained in a specially designed jig which exposed the tumour-bearing legs. The legs were loosely attached to the jig with tape, without impairing the blood supply to the tumour.

RSU1069 and RB6145

RB6145 is metabolised in vivo to RSU1069 and an oxazolidin-2-one in the ratio 1:2 (5, 6), as shown in Fig. 1.

RSU1069 (α-(1-aziridinylmethyl)-2-nitro-1H-imidazole-1-ethanol) (MRC Radiobiology Unit, UK) was injected at 80 mg/kg in PBS. RB6145 (α-[(2-bromoethyl)amino]-2-nitro-1H-imidazole- 1-ethanol) (MRC Radiobiology Unit, UK) was injected at 240 mg/kg in acetate buffer, pH 5.4. Both agents were injected i.p. at 0.02 ml/g mouse body weight.

Measurement of tumour blood flow

Tumour blood flow was measured using laser Doppler flowmetry (Laserflo Blood Perfusion Monitor, Model BPM 403, TSI Inc. St Paul, MN, USA), the technical details of which have been described elsewhere (14). A 0.8 mm diameter laser probe was inserted to a depth of approximately 1 mm into the tumour through a small incision in the skin over the tumour, then withdrawn slightly to ensure that there was no compression of the tumour under the probe tip. The agents were injected after a steady recording had been obtained.

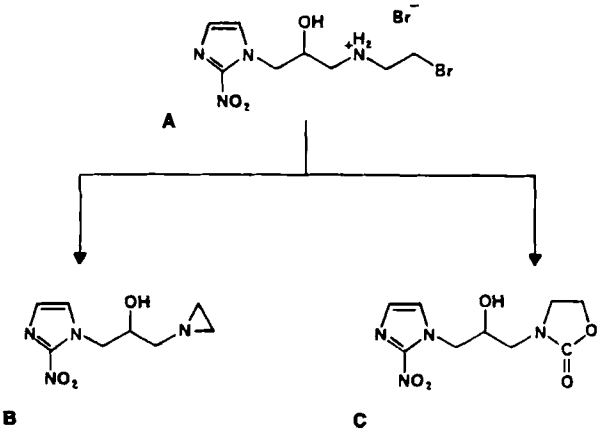

Fig. 1. The metabolism in vivo of RB6145 (compound A) to RSU1069 (compound B) and an oxazolidin-2-one (compound C) in the ratio of 1:2.

Measurement of tumour oxygenation

Measurement of tumour oxygen partial pressure distributions (pO_2) were made using a computerised fine needle polarographic oxygen electrode probe (Eppendorf, Hamburg, Germany), the details of which have been described previously (15). The needle was inserted into the tumour to a depth of approximately 1 mm, then moved automatically through the tissue in 0.7 mm increments, followed by a 0.3 mm backward step prior to measurement. The response time was 1.4 s, and 3–6 parallel insertions were made, giving a total of 35–100 measurements in each tumour.

^{31}P magnetic resonance spectroscopy (MRS)

MRS experiments were carried out using a 4.7 tesla, 30 cm horizontal bore magnet (Oxford Instruments, Oxford, UK) with a SISCO 200 spectrometer. A 7 mm diameter surface coil was placed over the tumour for Rf pulsing and signal collection. Acquisition parameters were set to minimise contamination of the tumour signal from underlying muscle. Each spectrum consisted of 256 scans with a 2 s delay, giving a total acquisition time of 8 min. A control spectrum was collected from each tumour prior to treatment. The mouse was removed from the magnet, the agent injected and the mouse returned to the magnet for subsequent collection of spectra. Spectra were analysed using an in-house baseline and Lorentzian curve-fitting programme. Data were expressed as changes in the ratio of the low energy, inorganic phosphate peak area to the sum of the areas of all peaks, or Pi/total.

Determination of metabolites

Tumours were excised from mice at 30 min after injection of the agent, weighed and homogenized in 4% perchloric acid. The homogenates were centrifuged at 10 000 g for 10 min and the supernatant fluids neutralised with 5M K_2CO_3. Glucose, glucose-6-phosphate and lactate were assayed using standard methods as described previously (16).

Hyperthermia treatment

Tumours were exposed to 42.5°C hyperthermia for 1 h by immersion of the tumour-bearing leg into a circulating

water bath (model TE 623, Heto, Birkerod, Denmark) stabilized to ±0.2°C of the adjusted temperature as described (17). The tumour response to treatment was assessed by measuring tumour volume on a daily basis, as described in 'Material and Methods', and calculating the time taken for tumours to reach 3 times the initial treatment volume.

Data analysis

All results are shown as mean values ±1 SE. Statistical analysis of the data was performed using Student's t-test, with $p = 0.05$ selected as the level of significance.

Results

The effects of RSU1069 injected at 80 mg/kg i.p. and RB6145 injected at 240 mg/kg i.p. on SCCVII tumour blood flow were determined using laser Doppler flowmetry. Fig. 2 gives the relative flow, as a percentage of the pre-treatment control values, with time after injection of the agent. RSU1069 reduced blood flow in the SCCVII tumour. This reduction was apparent within 20 min following injection and reached a maximal, 30% decrease by 30 min. Blood flow had returned to control levels by 45–50 min. In contrast, apart from a transient drop in flow within the first few minutes after injection, RB6145 produced a response which was identical to that seen with saline alone, suggesting that RB6145 was not affecting tumour blood flow.

The Table summarizes the parameters calculated using the Eppendorf oxygen electrode in this tumour after administration of the bioreductive agents. The results show that almost all of the pO_2 parameters were unaffected by both RSU1069 and RB6145, although both agents did

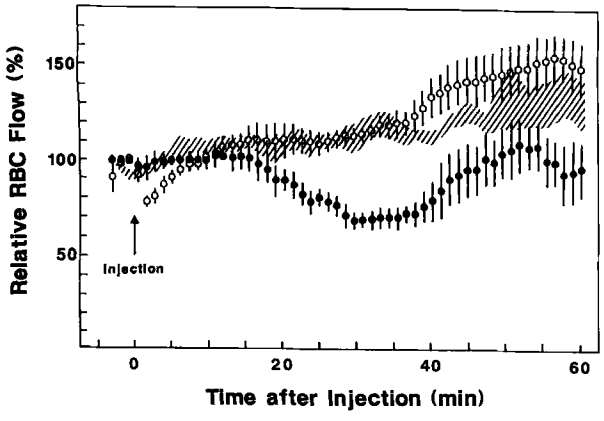

Fig. 2. Relative RBC flow against time for (●) RSU1069 at 80 mg/kg i.p. and (○) RB6145 at 240 mg/kg i.p. in the SCCVII tumour, measured using laser Doppler flowmetry. Points are means ±s.e. for 3-5 mice. Shaded region gives values for injection of saline alone.

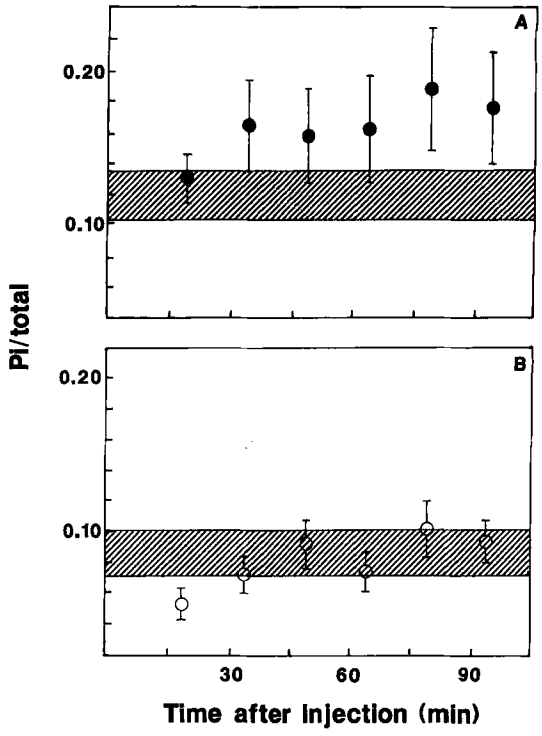

Fig. 3. Pi/total against time after injection of A: 80 mg/kg i.p. RSU1069 or B: 240 mg/kg i.p. RB6145 in the SCCVII tumour measured using ^{31}P magnetic resonance spectroscopy. Points are means ±SE for 5–8 mice and are plotted at the mid-point of the 8 min acquisition time. Shaded region gives Pi/total prior to treatment.

appear to reduce the percentage of pO_2 values ≤2.5 mm Hg, and this was significant for RSU1069 ($p < 0.05$).

The effect of these agents on phosphorus energy metabolism was assessed using ^{31}P MRS. Fig. 3a gives Pi/total with time after injection of 80 mg/kg i.p. RSU1069. There was a small increase in Pi/total over pretreatment control, but this was not statistically significant at any time after injection. For RB6145 at 240 mg/kg i.p. (Fig. 3b) there is clearly no effect on tumour phosphorus energy metabolism.

Fig. 4a gives the levels of glucose, 4b, glucose-6-phosphate and 4c, lactate in SCCVII tumours before and 30 min after injection of 80 mg/kg i.p. RSU1069 or 240 mg/kg i.p. RB6145. Only glucose levels were affected, and were increased to the same extent, 1.6–1.7 fold, by both bioreductive agents.

The anti-tumour effects of RSU1069 and RB6145, either alone or when combined with hyperthermia, are summarized in Fig. 5. Neither agent alone had any effect on the growth of the SCCVII tumour. Heat alone significantly delayed tumour growth, the time taken for tumours to reach three times the treatment volume increasing by a little more than 2 days over that for untreated tumours. There was a further significant delay in tumour growth when either RSU1069 or RB6145 were given 30 min prior

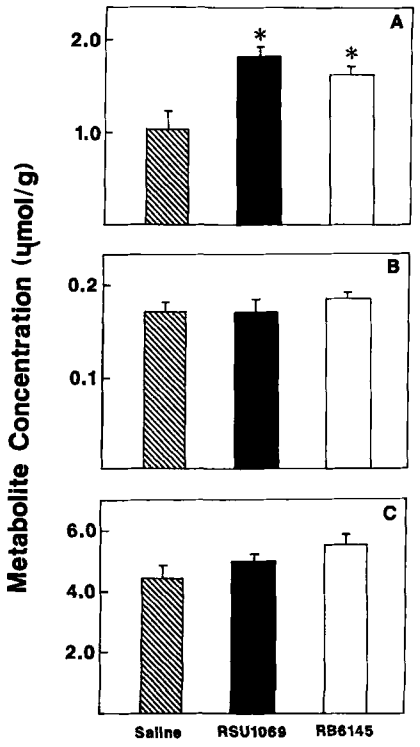

Fig. 4. The effect of saline, RSU1069 at 80 mg/kg i.p. or RB6145 at 240 mg/kg i.p. 30 min after injection on A: glucose, B: glucose-6-phosphate or C: lactate levels in the SCCVII tumour. Values are means ±SE for 4 mice.
* indicates significant difference from saline controls.

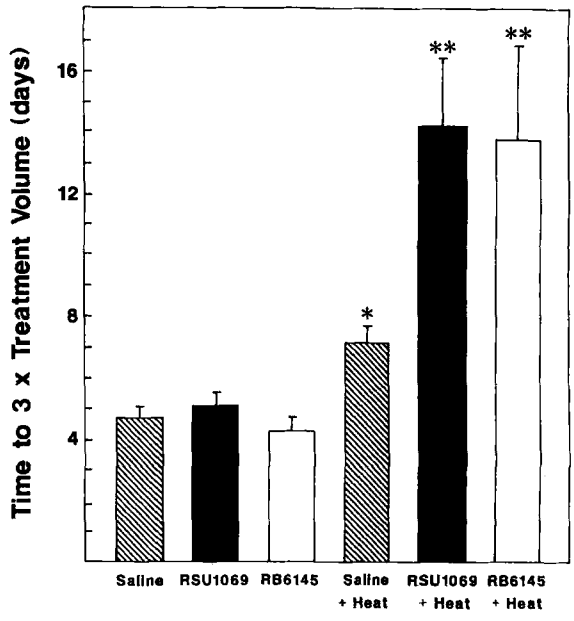

Fig. 5. The effect of saline, RSU1069 at 80 mg/kg i.p. or RB6145 at 240 mg/kg i.p. alone or injected up to 30 min prior to local heating at 42.5°C for 1 h on the growth of the SCCVII tumour. Values are means ±SE for 4–11 mice.
* indicates significant difference from saline controls.
** indicates significant difference from saline plus heat.

to local tumour heating, seen as an increase in time for tumours to reach 3 times treatment volume of 7 days for both agents, over that for heat alone.

Discussion

A single dose of 80 mg/kg RSU1069 significantly reduced blood flow by 30% in the SCCVII tumour. This reduction was similar to that obtained in the C_3H mammary carcinoma after i.p. injection of 100 mg/kg RSU1069 (18). These effects, when the agent was given i.p. occurred later and were less than the 70–80% reductions seen in the SCCVII and C_3H tumours almost immediately after i.v. administration of 100 mg/kg RSU1069 (11). These findings indicate a dependency on dose of the agent for tumour vascular response. Despite the decrease in blood flow in the SCCVII tumour, RSU1069 had a non-significant effect on energy metabolism measured by ^{31}P MRS. This agent did appear to reduce the pO_2 values less than 2.5 mmHg, but clearly had no effect on those values less than 5 or 10 mmHg, nor did it alter the median pO_2, suggesting that the apparent reduction in pO_2 values less than 2.5 mmHg may be an experimental aberration due to a group of control tumours with high levels of hypoxia.

Several studies have shown that in order to change tumour metabolism and oxygenation significantly, blood flow reductions of 50% or greater are required (17, 19, 20). However, the finding that RB6145 had no effect on tumour blood flow was surprising, since 30% of RB6145 is

Table 1

The effect of RSU1069 (80 mg/kg) and RB6145 (240 mg/kg) on the oxygenation status of SCCVII foot tumours

Treatment	N/n[2]	Mean pO_2(mmHg)	Median pO_2(mmHg)	Inter-percentile range[3] (mmHg)	Values ≤2.5 mmHg %	Values ≤5 mmHg %	Values <10 mmHg %
Saline	10/581	7.4 ± 0.9[4]	6.0 ± 1.2	17.3 ± 1.4	37.5 ± 5.8	50.0 ± 6.5	67.0 ± 5.8
RSU1069	8/504	8.7 ± 0.8	6.3 ± 1.0	18.0 ± 1.6	20.3 ± 4.5*	48.7 ± 6.2	66.9 ± 5.2
RB6145	5/287	7.5 ± 1.2	5.2 ± 1.2	13.0 ± 1.9	19.6 ± 10.7	54.6 ± 11.6	73.2 ± 6.3

[1] Measurements were made with an Eppendorf oxygen electrode 30 min after i.p. injection of the agent. [2] 'N' represents the number of tumours and 'n' the number of measurements made. [3] The difference between the 10th and 90th percentiles. [4] Values are means ±1 SE.
* Significantly different from control values, using the result for each tumour as an independent observation.

metabolised in vivo to RSU1069 (5, 6), and a dose of RB6145 of 240 mg/kg was used in this study to give an equivalent in vivo level of 80 mg/kg RSU1069. One explanation for this may be a protective role for the oxazolidinone metabolite from RB6145, analogous to that for misonidazole over RSU1069 (3, 21). However, the oxazolidinone was not available in sufficient quantities for the appropriate experiments to be carried out. How RSU1069 is inducing a reduction in tumour blood flow in unclear, since it is not known to alter blood pressure in the mouse (18) as occurs with other physiological modifiers of blood flow (17, 22, 23).

Both RSU1069 and RB6145 increased tumour levels of glucose, an effect which is consistent with other nitroaromatic radiosensitizing agents, such as pimonidazole and misonidazole (16, 24). This increase in tumour glucose levels may be due to enhanced glucose production and glycogen degradation (16). However, this increase in glucose production occurred without any apparent increase in glycolysis, since there was no change in the levels of glucose-6-phosphate or lactate. This may have been a consequence of sampling at only 30 min after injection of the bioreductive agent, and changes in glycolytic activity may have been observed at later times (16).

The SCCVII tumour used in the present study was responsive to hyperthermia treatment, consistent with the results reported by others with the same tumour model (25). While RSU1069 and RB6145 alone had no effect on tumour growth at the doses used, they significantly increased the tumour growth time when combined with heat. This would be expected since several in vivo studies have demonstrated that other nitroimidazoles, such as misonidazole (26–29) and etanidazole (30) can interact with hyperthermia to increase tumour cell killing. Indeed, the interaction between RSU1069 and heat has already been demonstrated in the C_3H mammary carcinoma (18). The mechanisms responsible for the interaction between the bioreductive agents and heat are not clear, but may involve a drug-induced increase in the sensitivity of the tumours to heat or a heat-induced increase in the production of the toxic species of the agent. However, studies with the C_3H mammary carcinoma suggested that the main reason for the enhancement of damage when RSU1069 and heat were combined was that the heat treatment caused a substantial shutdown in tumour blood flow which resulted in a significant increase in tumour hypoxia, thus increasing the effectiveness of the bioreductive agent (18). Several studies have shown clearly that the anti-tumour activity of RSU1069 and RB6145 can be enhanced by increasing tumour hypoxia (31–34). The fact that identical effects were seen when both RSU1069 and RB6145 were combined with heat further supports the suggestion of a heat-induced increase in tumour hypoxia as a likely mechanism.

In conclusion, this study has shown that the effects of RSU1069 and RB6145 on tumour physiology are not the same. While RB6145 had no influence on tumour blood flow, RSU1069 significantly reduced it. If this effect were to occur in other tumours, in particular human tumours, and did so to a degree sufficient to reduce tumour oxygenation status, this would for example, produce serious adverse effects on tumour response to radiation. The fact that RB6145 had no effect on tumour blood flow makes it the agent of choice, and adds further support to the clinical testing of RB6145.

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