Pharmacokinetics and cytotoxicity of RSU-1069 in subcutaneous 9L tumours underoxic and hypoxic conditions

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Summary The acute toxicity, pharmacokinetics and hypoxic cytotoxicity of RSU-1069 were investigated using the subcutaneous (sc) rat 9L tumour model. The pharmacokinetics were studied after i.p. injection of RSU-1069 (20 mg kg\(^{-1}\) or 100 mg kg\(^{-1}\)). For both doses, the elimination of RSU-1069 followed first-order kinetics in both plasma and unclamped tumours. After 100 mg kg\(^{-1}\), the peak plasma concentration of RSU-1069 was 40 μg ml\(^{-1}\); the elimination \(t_1\) was 39.3 ± 11.1 min. After 20 mg kg\(^{-1}\), the peak plasma concentration was 3 μg ml\(^{-1}\); the elimination \(t_1\) was 47.8 ± 6.3 min. In unclamped tumours, the peak concentration was 50 μg g\(^{-1}\) with an elimination \(t_1\) of 36.1 ± 9.6 min for the 100 mg kg\(^{-1}\) dose, and 4 μg g\(^{-1}\) with an elimination \(t_1\) of 41.9 ± 6.1 min for the 20 mg kg\(^{-1}\) dose. The tumour and plasma elimination half-times were not significantly different (\(P > 0.2\)) for the two doses. Clamping the tumour 30 min after administration of 100 mg kg\(^{-1}\) of RSU-1069 decreased the tumour elimination \(t_1\) to 10.9 ± 1.4 min. After releasing the clamp, RSU-1069 returned rapidly to the unclamped tumour concentration. The unclamped tumour/plasma ratio reached a maximum of 4–6, then decreased to a constant value of about 2 for both doses, indicating that RSU-1069 accumulates in these 9L tumours. RSU-1069 kills hypoxic sc 9L cells more efficiently than oxic sc 9L cells; at a surviving fraction of 0.5, the SER was 4.8. For in vitro 9L cells, the SER was ≈ 50 when the comparison was between those treated in 2.1% \(O_2\) and those treated in < 7.5 × 10\(^{-5}\)% \(O_2\); it was ≈ 100 when the comparison was between those treated in 21% \(O_2\) and those treated in < 7.5 × 10\(^{-5}\)% \(O_2\). Tumours treated with RSU-1069 and clamped for various times exhibited biphasic cell-kill kinetics; at 50 mg kg\(^{-1}\), little additional cell kill was achieved after 40 min of clamping. Our data also indicate that RSU-1069 is 300–1000 fold more efficient than misonidazole or SR2508 for killing hypoxic sc 9L tumour cells in situ.

Materials and methods

In vivo tumour system

The maintenance of 9L tumour cells in vitro and the procedures for implanting 9L tumour cells subcutaneously in male Fisher 344 rats (250–300 g) have been described elsewhere (Wallen et al., 1980; Wheeler et al., 1984). Most of the tumours used in these experiments weighed 200–500 mg.

Drug storage and preparation

The RSU-1069 used in these experiments was supplied by the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment at the National Cancer Institute. The properies and activity of the RSU-1069 were confirmed using a recently synthesised lot, kindly supplied by Warner-Lambert (Dr M. Suto). RSU-1069 was stored at −79°C and dissolved at the appropriate concentration in sterile saline (0.85% NaCl) immediately before use.

RSU-1069 pharmacokinetics

Both a high (100 mg kg\(^{-1}\)) and a low (20 mg kg\(^{-1}\)) dose of RSU-1069 were used for the pharmacokinetic studies. The procedure for clamping the tumours and the preparation of plasma and tumour samples for analysis have been described in detail elsewhere (Wong et al., 1989). In the clamped experiments, RSU-1069 was administered 30 min before clamping, and the tumours clamped for periods of up to 2 h. The RSU-1069 concentration in the plasma and tumour samples was measured as a function of time after drug administration by an adaptation of the HPLC method described by Walton & Workman (1988). The HPLC instru-
mentation has been described in detail elsewhere (Wong et al., 1989). RSU-1069 was isocratically eluted from a Spherisorb phenyl column (Beckman Instruments). The mobile phase consisted of 85% potassium phosphate buffer (500 mM, pH 4.5) and 15% methanol at a constant flow rate of 1 ml min\(^{-1}\). RSU-1069 was monitored at 320 nm, and the lowest detectable quantity of RSU-1069 was 0.025 μg ml\(^{-1}\). The quantity of RSU-1069 in each sample was calculated by comparison to a standard calibration curve.

**Cell survival experiments**

The *in vivo* to *in vitro* colony formation assay has been described in detail elsewhere (Wallen et al., 1980; Wheeler et al., 1984). In one set of experiments, RSU-1069 was administered at 10–50 mg kg\(^{-1}\), and the tumours clamped for 2 h. In another set of experiments, 50 mg kg\(^{-1}\) of RSU-1069 was administered, and the tumours clamped from 10 min to 2 h. As controls, rats bearing unclamped tumours were treated with the same doses of RSU-1069. The colony formation assay was always performed 18–24 h after the drug treatment.

For the *in vitro* experiments, approximately 2 × 10\(^5\) 9L cells were plated in the central area of glass petri dishes as previously described (Koch, 1984) and incubated overnight at 37°C. Upon removal from the incubator, the dishes were cooled to 4°C and the medium removed. After rinsing with 1 ml of drug-containing medium, 1 ml of drug-containing medium was added. The dishes were then placed in leak-proof aluminium chambers (Koch & Painter, 1975) which were connected to a manifold and deoxygenated with a series of gas exchanges that took about 30 min (Koch et al., 1984). The oxygen concentration in the gas phase was monitored using a polarographic oxygen sensor (Koch et al., 1984). After deoxygenation, the chambers were rapidly brought to 37°C in a water bath and then placed in a 37°C incubator. The chambers were gently shaken to prevent gradients of oxygen, nutrients and RSU-1069. After the appropriate exposure time, the cells were trypsinised and plated for colony formation as previously described (Koch, 1984; Koch et al., 1984).

**Data analysis**

A complete description of the statistical analysis for the pharmacokinetics has been published (Wong et al., 1989). Briefly, the data were weighted by the inverse variance and analysed using the SAS nonlinear least-squares fitting routine. The exponential portions of these curves were compared using a t-test for the equality of slopes generated by the SAS program. All the *in vivo* to *in vitro* cell survival data are presented as the geometric mean ± 1 s.e.m. and have been corrected for cell yield as previously described (Rosenblum et al., 1976).

**Results**

**Pharmacokinetics**

The plasma pharmacokinetics of RSU-1069 at 100 mg kg\(^{-1}\) are shown in Figure 1a. The RSU-1069 peak plasma concentration of 40 μg ml\(^{-1}\) occurred approximately 25 min after i.p. injection. Elimination of RSU-1069 from plasma appeared to follow first-order kinetics with a half-life (t\(_h\)) of 39.3 ± 11.1 min. Clamping the tumour did not change the elimination kinetics of RSU-1069 from plasma (t\(_h\) = 41.4 ± 9.2 min, P > 0.9).

The distribution of RSU-1069 in unclamped and clamped tumours is shown in Figure 1b. In unclamped tumours, RSU-1069 reached its peak concentration of 50 μg g\(^{-1}\) in 30 min. Elimination of RSU-1069 appeared to follow first-order kinetics with a t\(_h\) of 36.1 ± 9.6 min. Clamping the tumour at 30 min decreased the elimination t\(_h\) of RSU-1069 to 10.9 ± 1.4 min (P < 0.01). The drug was undetectable beyond 120 min after clamping. Upon release of the clamp, the RSU-1069 concentration rapidly returned to the unclamped tumour level.

The peak plasma concentration of 3 μg ml\(^{-1}\) was reached about 10 min after injection of 20 mg kg\(^{-1}\) of RSU-1069 (data not shown). The peak plasma concentration after a dose of 100 mg kg\(^{-1}\) was about 13 times higher than that found after a dose of 20 mg kg\(^{-1}\). The elimination of RSU-1069 from the plasma after a dose of 20 mg kg\(^{-1}\) followed first-order kinetics with a t\(_h\) of 47.8 ± 6.3 min (data not shown). This elimination t\(_h\) was not significantly different (P > 0.4) from the t\(_h\) measured after a dose of 100 mg kg\(^{-1}\). RSU-1069 reached a peak tumour concentration of 4 μg g\(^{-1}\) 10 min after a dose of 20 mg kg\(^{-1}\) (data not shown). The peak tumour concentration after a dose of 100 mg kg\(^{-1}\) was also about 13 times higher than that found after a dose of 20 mg kg\(^{-1}\). The elimination of RSU-1069 from sc 9L tumours also followed first-order kinetics with a t\(_h\) of 41.9 ± 6.1 min (data not shown), which was not significantly different (P > 0.2) from the t\(_h\) measured after a dose of 100 mg kg\(^{-1}\).

Figure 2 shows the RSU-1069 data plotted as the tumour/plasma ratio. In rats with unclamped tumours, the peak tumour/plasma ratio exceeded four in about 45 min after a 100 mg kg\(^{-1}\) dose and then decreased to two over the next 4 h (Figure 2a). The tumour/plasma ratio decreased to 0.1 about 90 min after clamping. After releasing the clamp, the RSU-1069 concentration rapidly returned to the same ratio as that found in the unclamped tumour. The variation of the tumour/plasma ratio as a function of time after a dose of 20 mg kg\(^{-1}\) was similar to that observed after a dose of 100 mg kg\(^{-1}\) (Figure 2b). The ratio reached a maximum value of six in 60 min and then decreased to a value of two over the next 2 h.

![Figure 1 Pharmacokinetics of RSU-1069](image-url)
RSU-1069 cytotoxicity

The ability of RSU-1069 to kill sc 9L tumour cells in vivo underoxic and hypoxic conditions is shown in Figure 3a. No cell kill was observed when the tumours were clamped for 2 h without an RSU-1069 treatment (Wheeler et al., 1984). When rats were treated with increasing doses of RSU-1069, a substantial difference in the kill was observed between sc 9L cells from tumours left unclamped (oxic) and those from tumours that were clamped for 2 h (hypoxic). At a dose of 50 mg kg⁻¹, the surviving fraction was about 0.6 and 0.001 under oxic and hypoxic conditions, respectively (Figure 3a). At a surviving fraction of 0.5, the sensitiser enhancement ratio (SER) was 4.8. After a dose of 50 mg kg⁻¹, a biphasic cell kill curve was observed as a function of the extent of the clamping period (Figure 3b). Most of the cell kill was achieved in the first 40 min after clamping. This is consistent with the 10 min elimination half-life measured for RSU-1069 in the clamped tumours (Figure 1b). By comparison to our previous work (Wong et al., 1989; 1990), RSU-1069 kills hypoxic sc 9L tumours 300–1000 fold more efficiently than either misonidazole (MISO) or SR-2508 (Table I).

The ability of RSU-1069 to kill 9L cells in vitro underoxic and hypoxic conditions is shown in Figure 4. The SER in vitro depended on the oxygen concentration. The SER was \( \approx 50 \) for 9L cells treated in an atmosphere of 2.1% \( \text{O}_2 \) compared to those treated in an atmosphere of \( < 7.5 \times 10^{-3}\% \text{O}_2 \). For 9L cells treated in an atmosphere of 21% \( \text{O}_2 \), the SER was \( \approx 100 \).

Discussion

Pharmacokinetics

The high dose of RSU-1069 (100 mg kg⁻¹) was selected in order to obtain an accurate measurement of the pharmacokinetics in both unclamped and clamped tumours with minimal systemic toxicity (LD₁₀₀ \( \approx 125 \text{ mg kg}^{-1} \)). The low dose of RSU-1069 (20 mg kg⁻¹) was selected to provide comparative pharmacokinetic data where the cell survival in unclamped and clamped tumours was \( \approx 100\% \) and \( \approx 25\% \), respectively (Figure 3a).

Both the low and high dose of RSU-1069 gave a similar elimination \( t_i \) in plasma and in tumours, but the peak concentrations differed by a factor of \( \approx 13 \) instead of \( \approx 5 \). These results are in contrast to those observed in mice by Walton & Workman (1988), where a 37% increase in the elimination \( t_i \) and a 2 fold difference in the volume of distribution was observed after administration of i.p. doses of 50 and 100 mg kg⁻¹. Although the exact reason(s) for these differences are unknown, it is possible that the elimination of
RSU-1069 (renal and/or hepatic in rats may not have been saturated at the 100 mg kg\(^{-1}\) dose. If that were the case, no differences in the elimination \(t_1\) between the two doses would be expected. On the other hand, the distribution (absorption or protein binding) could be saturated at the high dose of 100 mg kg\(^{-1}\); thereby, resulting in a higher than expected peak concentration accompanied by a longer distribution phase. The observation that it took 10 min to reach the peak concentration at 20 mg kg\(^{-1}\), and about 25 min to reach the peak concentration at 100 mg kg\(^{-1}\) supports this contention.

RSU-1069 accumulated in the unclamped sc 9L tumours, as reflected by the high tumour/plasma ratio that peaked at 4–6 and remained at two for several hours after injection of both the low and high dose (Figure 2). It has been reported that the tumour/plasma ratio varies with tumour type. For example, the KHT fibrosarcoma has an RSU-1069 tumour/plasma ratio of 0.2–0.4 (Walton & Workman, 1988) while the B16 and HX 118 melanomas have an RSU-1069 tumour/plasma ratio of 3.7–4 (Deacon et al., 1986; Walling et al., 1989). A correlation between the ability to accumulate RSU-1069 and its relative antitumour activity has not been established (Cole et al., 1989). Hence, this phenomenon will complicate the interpretation of clinical pharmacokinetic and cytotoxicity data.

The RSU-1069 elimination \(t_1\) after a 100 mg kg\(^{-1}\) dose decreased significantly in clamped tumours (≈11 min) compared to that observed in unclamped tumours (≈36 min). Because these clamped sc 9L tumours are a closed system with no influx or efflux of the drug, this decrease in the elimination \(t_1\) probably results from the metabolic nitroreduction of RSU-1069 under hypoxic conditions. The elimination of RSU-1069 from clamped sc 9L tumours was nearly four times faster than the elimination \(t_1\) of MISO or SR-2508 from clamped sc 9L tumours (Wong et al., 1989; 1990). This difference in the elimination \(t_1\) was not predicted because the electron affinities of these three compounds are similar (Adams et al., 1984a). Other metabolic pathways, such as aziridine ring opening to yield RSU-1137 and aziridine ring removal to yield RSU-1111 (Walton & Workman, 1988), may be responsible for some of the disappearance of the parent compound.

**Clinical implications**

In a phase I clinical trial (Horwich et al., 1986), nongenotoxic side-effects, such as emesis, limited the RSU-1069 dose to less than 70 mg m\(^{-2}\) (≈1.75 mg kg\(^{-1}\)). In this clinical trial, a peak plasma level of 2–4 \(\mu\)g m\(^{-1}\) of RSU-1069 was achieved after a 70 mg kg\(^{-1}\) dose. In our study, a plasma level of 2–4 \(\mu\)g ml\(^{-1}\) corresponds to a 10–20 mg kg\(^{-1}\) dose. A 10–20 mg kg\(^{-1}\) dose reduces the surviving fraction of hypoxic sc 9L cells to only 0.5–0.25 (Figure 3a). In addition, the radiosensitising enhancement ratio has been estimated to be only 1.2 at this plasma level (Adams et al., 1984a). Therefore, the clinical usefulness of RSU-1069 as a killer of hypoxic cells or as a radiosensitisiser is limited, unless the drug-related side-effects can be overcome.

Because the aziridine ring in RSU-1069 can alkylate intracellular macromolecules (Stratford et al., 1985) to cause oxic cell cytotoxicity (Figure 3a), and metabolites of RSU-1069 that are formed under hypoxic conditions can potentiate the cytotoxicity of a number of alkylating agents, RSU-1069 may be an excellent potentiator of many alkylating agents (Adams et al., 1984b; Siemann et al., 1985). In fact, RSU-1069 may be the most efficient 2-nitroimidazole for killing hypoxic sc 9L tumour cells (Table 1) because it has both alkylating and chemopotentiation properties (Adams et al., 1984a, b) and is capable of self-potentiation when metabolised under hypoxic conditions. Currently, we are investigating the use of
small doses of RSU-1069 (<20 mg kg$^{-1}$) to potentiate
the cytotoxic effects of a number of alkylating agents (e.g.
BCNU, CCNU, cyclophosphamide ifosfamide, dacarba-
zine). As a chemopotentiator, RSU-1069 might be clinically
useful at low doses that avoid the previously described severe
side-effects.

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