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Pharmacokinetic contribution to the improved therapeutic selectivity of a novel bromoethylamino prodrug (RB 6145) of the mixed-function hypoxic cell sensitizer/cytotoxin α-(1-aziridinomethyl)-2-nitro-1*H*-imidazole-1-ethanol (RSU 1069)

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Summary. RB 6145 is a novel hypoxic cell sensitizer and cytotoxin containing both an essential bioreductive nitro group and a bromoethylamino substituent designed to form an alkylating aziridine moiety under physiological conditions. In mice, RB 6145 is 2.5 times less toxic but only slightly less active than the aziridine analogue RSU 1069, giving rise to an improved therapeutic index. However, the mechanism for the enhanced selectivity is not clear. Reasoning that this may lie in a more beneficial pharmacokinetic profile, we investigated the plasma pharmacokinetics, tissue distribution and metabolism of RB 6145 in mice using a specially developed reversed-phase HPLC technique. An i.p. dose of 190 mg kg^{-1} (0.5 mmol kg^{-1}) RB 6145 produced peak plasma concentrations of about 50 μg ml^{-1} of the pharmacologically active target molecule RSU 1069 as compared with levels of around twice this value that were obtained using an equimolar i.p. dose of RSU 1069 itself. The plasma $AUC_{0-\infty}$ value for administered RSU 1069 was ca. 47 μg ml^{-1} h and that for the analogue RSU 1069 was ca. 84 μg ml^{-1} h. No prodrug was detectable. Another major RB 6145 metabolite in plasma was the corresponding oxazolidinone, apparently formed on interaction of the drug with hydrogen carbonate. The oxazolidinone initially occurred at higher concentrations than did RSU 1069, with the levels becoming very similar from 30 min onwards. Post-peak plasma concentrations of both RB 6145 metabolites declined exponentially, displaying an elimination $t_{1/2}$ of ca. 25 min, very similar to the 30-min value observed for injected RSU 1069. The plasma $AUC_{0-\infty}$ value for the metabolite RSU 1069 was about 1.3 and 1.6 times higher following i.p. injection of 95 mg kg^{-1} (0.25 mmol kg^{-1}) of the prodrug as compared with administration via the oral and i.v. routes, respectively. After i.v. injection, peak levels of the oxazolidinone metabolite were twice those observed following both i.p. and oral dosing and possibly contributed to the acute toxicity. After an i.p. dose of 190 mg kg^{-1} RB 6145, concentrations of RSU 1069 and the oxazolidinone metabolites rose to 40% and 33%, respectively, of the ambient plasma level in i.d. KHT tumours. The peak level of metabolite RSU 1069 was ca. 6 μg g^{-1} as compared with 10 μg g^{-1} following an equimolar dose of RSU 1069 itself; the tumour $AUC_{0-\infty}$ value for the metabolite RSU 1069 was some 35% lower. The $AUC_{0-\infty}$ in brain for RSU 1069 formed from RB 6145 was about 1.8 times lower than that obtained using an equimolar dose of the analogue RSU 1069. The hydrophilic oxazolidinone metabolite of RB 6145 showed tumour penetration similar to that of the metabolite RSU 1069 but was substantially excluded from brain tissue. About 34% of the delivered dose of RB 6145 appeared in the urine as the oxazolidinone and 12% as RSU 1069. We feel that the improved antitumour specificity observed for RB 6145 as compared with RSU 1069 may be explained at least in part by the more favourable tissue disposition of the metabolites, particularly the similar uptake of both the RSU 1069 metabolite and the oxazolidinone by tumour tissue, coupled with the lower brain exposure following prodrug administration.

Introduction

Hypoxic cells in solid tumours are relatively resistant to cell killing by radiation and some anticancer drugs [6]. Although various types of agents exhibiting electron affinity can enhance the radiation response of cells with diminishing oxygen supply in vitro, relatively few show activity at nontoxic doses in vivo [12, 22, 23]. Clinical trials of one of these compounds, the 2-nitroimidazole misonidazole, have revealed some therapeutic gain [13]. However, the drug could not be used at optimal doses because of neurotoxicity [7]. Approaches aimed at im-

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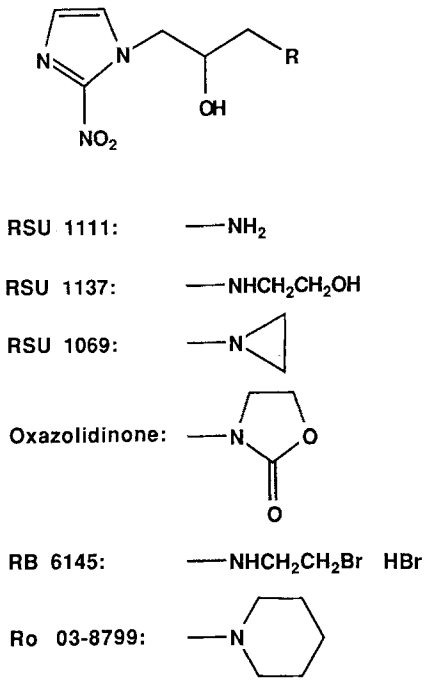

Fig. 1. Structures of nitroimidazoles

proving the therapeutic index of nitroimidazoles include the reduction of lipophilicity so as to restrict nervous tissue penetration and accelerate renal clearance, as in etanidazole [2, 3]; the incorporation of a lipophilic but basic moiety to increase tissue uptake as well as electron affinity, as in pimonidazole [16, 17]; and the reduction of electron affinity, as in nimorazole [14].

RSU 1069 [α-(1-aziridinomethyl)-2-nitro-1*H*-imidazole-1-ethanol] is a prototype mixed-function hypoxic cell sensitizer that contains a sensitizing nitro group and an additional alkylating aziridine function within the same molecule. It has been shown to be up to 1 order of magnitude more efficient as a radio- and chemosensitizer than misonidazole [1]. This activity is attributed to the alkylation of nucleophilic sites in DNA, followed by the bioreductive activation of the nitro group to generate cross-links [18]. Unfortunately, severe gastrointestinal toxicity was seen at relatively low doses in man such that the plasma concentrations achieved were considered to be too low for any appreciable radiosensitization [9].

The attractive molecular pharmacology of RSU 1069 has nevertheless led to the continued pursuit of equally potent but less toxic analogues. In an attempt to decrease the systemic toxicity of RSU 1069 but yet retain its sensitizing potency, a series of haloethylamino-substituted prodrugs has recently been designed to release the alkylating aziridine moiety under physiological conditions [10]. On the basis of its promising ability to maintain a relatively high degree of radiosensitization and bioreductive cytotoxicity combined with a comparitively low systemic toxicity, RB 6145 [α-([(2-bromoethyl)-amino]methyl)-2-nitro-1*H*-imidazole-1-ethanol hydrobromide] was chosen for detailed preclinical studies [4]. However, the mechanism for the improved therapeutic index was not clear.

This report provides comparative data on the pharmacokinetics and metabolism of RB 6145 and RSU 1069. The in vivo kinetics of the intramolecular conversion of the bromoethylamino-substituted prodrug to the active aziridine target molecule are described, as are those for the formation of the oxazolidinone derivative. The major intention was to elucidate whether the relatively low toxicity combined with the high radiosensitization and bioreductive cytotoxicity of the prodrug could be explained by an improvement in the distribution of the active product to the tumour relative to normal tissues. The experiments were carried out in C3H/He mice bearing implanted KHT tumours, in which much of the therapeutic and toxicological evaluation has been performed [4].

Materials and methods

Drugs. The prodrug RB 6145 [α-([(2-bromoethyl)amino]methyl)2-nitro-1*H*-imidazole-1-ethanol hydrobromide] was obtained from the Medical Research Council (MRC) Radiobiology Unit (Didcot, Oxon, UK) and the Warner-Lambert Company (Ann Arbor, Mich. USA). RSU 1069 [α-(1-aziridino-methyl)-2-nitro-1*H*-imidazole-1-ethanol; NSC 347 503], its aziridine hydrolysis product RSU 1137 [α-([(2-hydroxy-ethyl)amino]-methyl)-2-nitro-1*H*-imidazole-1-ethanol] and the *N*-dealkylated amine metabolite RSU 1111 [α-(aminomethyl)-2-nitro-1*H*-imidazole-1-ethanol] were supplied by the MRC. The internal standard Ro 03-8799 [α-(1-piperidinomethyl)-2-nitro-1*H*-imidazole-1-ethanol hydrochloride] was provided by Roche Products Ltd (Welwyn Garden City, Hertfordshire, UK). The metabolite 3-[2-hydroxy-3-(2-nitro-1*H*-imidazole-1-yl)propyl]-2-oxazolidinone was synthesized from RB 6145 according to a previously published method [20], and its identity was confirmed by mass spectrometry. The chemical purity of all drugs was found to be >99% using reversed-phase HPLC. The structures are shown in Fig. 1.

Drug administration. RSU 1069 and RB 6145 were dissolved in phosphate-buffered saline (pH 5) at 4° C and kept on ice and used within 20 min. RSU 1069 was injected i. p. at a dose of 110 mg kg^{-1} in a volume of 0.02 ml g^{-1} body weight and RB 6145 was given in the same way at a dose of 190 mg kg^{-1}; both doses were equivalent to 0.5 mmol kg^{-1}. In some experiments, half of the RB 6145 dose was injected in the same volume to compare the pharmacokinetics for different routes of administration, viz. i. p., i. v. via the tail vein and p. o. These doses lay within the pharmacologically active range [4, 10].

Mice and tumours. Adult inbred female C3H/He mice weighing 22–28 g were used for plasma pharmacokinetic experiments. Mice were obtained from our own breeding colony and were allowed laboratory chow and water ad libitum.

The KHT sarcoma was grown i. d. in the mid-dorsal pelvic region of the mice [11]. Tumour-bearing animals were used for tissue disposition studies after the tumours had attained a diameter of 6–8 mm.

Sample preparation. Using diethyl ether anaesthesia, blood samples (approx. 0.8 ml) were taken by cardiac puncture into heparinized syringes. Samples were immediately centrifuged at 3000 *g* for 1 min to obtain plasma. Aliquots (350 µl) were deproteinized by the addition of 630 µl methanol and then stabilized with 100 µl 0.1 M phosphate buffer (pH 3). Prior to analysis, 120 µl internal standard solution (Ro 03-8799 at 0.1 mg ml^{-1} in methanol) was added. After thorough vortexing and centrifugation (3000 *g*, 3 min) 50 µl aliquots of the clear supernatant were taken for duplicate analysis. Samples were protected from light and handled at 4° C. Biological material was stored at –20° C and analyzed within 24 h. Procedures used for the collection of tissue and urine samples were adapted from the method described for RSU 1069 [21]. Briefly, tissue was rapidly excised and immediately frozen in a methanol-dry ice bath. It was then homogenized with 2 vol. 0.1 M phosphate buffer (pH 5) and aliquots (100 µl) were extracted with 200 µl

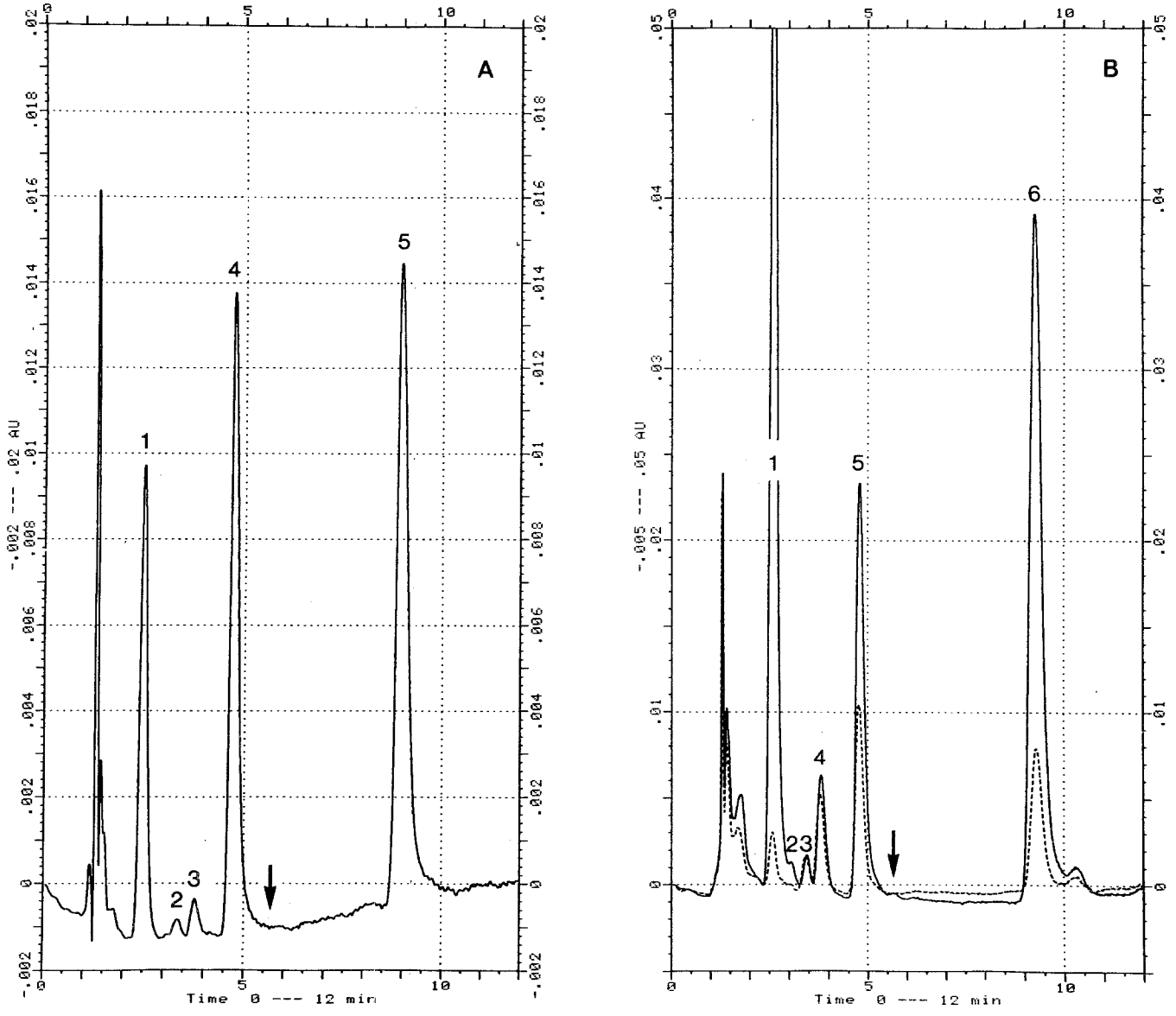

Fig. 2. A Representative chromatogram of a methanolic extract of blood plasma taken from a female C3H/He mouse at 45 min after an i.p. dose of 190 mg kg^{-1} RB 6145 and then spiked with 10 μg ml^{-1} Ro 03-8799 (*peak 5*). The sample contained 17 μg ml^{-1} oxazolidinone derivative (*peak 1*), traces of an unknown metabolite (*peak 2*), 1.1 μg ml^{-1} RSU 1137 (*peak 3*) and 17.3 μg ml^{-1} RSU 1069 (*peak 4*); the *arrow* denotes the retention time of RB 6145. **B** Representative chromatogram of urine (diluted 1 : 10) collected from female C3H/He mice ($n = 3$) for 24 h after i.p. injection of 190 mg kg^{-1} RB 6145 (*solid line*) or 110 mg kg^{-1} RSU 1069 (*broken line*) and then spiked with 50 μg ml^{-1} and 10 μg ml^{-1} Ro 03-8799 (*peak 6*), respectively. *Peak 1*, Oxazolidinone metabolite; *peak 2*, RSU 1111; *peak 3*, unknown metabolite; *peak 4*, RSU 1137; *peak 5*, RSU 1069; the *arrow* denotes the retention time of RB 6145. Chromatographic conditions: column, Waters Resolve CN Rad-Pak (10 μm); linear gradient, 3%–40% acetonitrile in 10 mM KH_2PO_4 (pH 3) run over 12 min; flow rate, 3 ml min^{-1}; injection volume, 50 μl; detection, absorbance at 325 nm

methanol containing 1.5 μg ml^{-1} internal standard. Samples were then processed as described above for plasma. Urine was collected on dry ice; 100 μl aliquots were mixed with 100 μl 0.1 M phosphate buffer (pH 5) and deproteinized by the addition of 700 μl methanol. To establish calibration curves, biological specimens were spiked with authentic standard solutions and analyzed alongside test samples.

High-performance liquid chromatography. Analysis of nitroimidazole levels in biological material was performed by reverse-phase HPLC. The modular HPLC system (Waters Associates, Milford, Mass., USA) comprised a Model 710B WISP automated sample injector equipped with a cooling unit set at 4° C, two Model 6000A pumps, a Model 660 solvent programmer, and a Model 990 photodiode array detector together with a Model APC IV Power Mate 2 personal computer (NEC, Boxborough, Mass., USA), a Model CP6 pinwriter (NEC) and a Model 990 plotter. Separation of analytes was achieved using a Waters Resolve cyanopropyl (CN) Rad-Pak column (10 cm × 8 mm internal diameter, 10-μm particle size) under compression from a Z-module (Waters) and protected by a Resolve CN precolumn. A linear gradient was run over 12 min from 3%–40% acetonitrile in 10 mM potassium dihydrogen orthophosphate (KH_2PO_4), adjusted to pH 3 with orthophosphoric acid (H_3PO_4), and delivered at a constant flow rate of 3 ml min^{-1}. For highest sensitivity, absorbance of the effluent was monitored at 325 nm and quantitation of all compounds was carried out by integration of peak area with reference to linear standard curves. Identification of analytes was based on experiments showing superimposition with authentic material using both the column described above and a Resolve octadecylsilane (C18) Rad-Pak column (10 cm × 5 mm inside diameter, 10-μm beads) together

with comparison of the recorded UV/visible spectra in the range of 220–500 nm.

Pharmacokinetic parameters. Elimination half-lives ($t^1/_2$) were obtained from post-peak concentration data using least-squares linear regression analysis and employing the equation $t^1/_2 = ln\ 2/k_{el}$, where k_{el} is the elimination rate constant given by *ln* plasma or tissue concentration vs time. For i.v. drug administration, the AUC value was calculated using the equation $AUC_{0-\infty} = c_0/k_{el}$, where c_0 is the extrapolated concentration at time 0. For i.p. and oral administration, values for AUC_{0-t} were determined using a computer programme based on the trapezoidal method (Multifit 2.0, Apple Macintosh, Cambridge, UK). The remaining AUC value extrapolated from t to infinity ($t-\infty$) was given by c_t/k_{el}, where c_t is the concentration at t.

Results

Chromatography and metabolites of RB 6145

Chromatography of nitroimidazoles was first attempted on several different HPLC packings, including octylsilane, octadecylsilane and phenyl, but peak shapes for prodrug RB 6145 and RSU 1069 were generally poor. Best resolution and peak shape for all analytes were obtained using a 10-µm Resolve CN column, whereas no baseline separation could be achieved on an end-capped Novapak 4-µm CN column. As an organic modifier, acetonitrile gave slightly sharper peaks than did methanol. The ideal pH of the mobile phase was 3; the addition of 1-heptane sulfate sodium salt (HSA) gave no improvement. Using a linear gradient of 3%–40% acetonitrile in 10 mM KH_2PO_4 buffer (pH 3) over 12 min, the analytes of interest were eluted with capacity factors of $k'_{Oxazolidinone} = 0.9$, $k'_{RSU\ 1111} = 1.4$, $k'_{RSU\ 1137} = 1.9$, $k'_{RSU\ 1069} = 2.7$, $k'_{RB\ 6145} = 3.2$ and $k'_{Ro\ 03\text{-}8799} = 5.9$. Chromatograms of control biological extracts revealed no significant interference peaks. No late eluted metabolite was detected.

Calibration curves for analytes were linear (plasma, $r > 0.9985$; tissues, $r > 0.9945$) over the concentration range of 0.05–100 µg ml^{-1} with zero intercepts. For an injection volume of 50 µl, detection limits for the various analytes in plasma were approx. 30 ng ml^{-1} at a signal-to-noise ratio of 3. The average extraction efficiency from plasma was ≥89% for the oxazolidinone derivative, RSU 1111, RSU 1137, RSU 1069 and the internal standard. The value for RB 6145 was lower but reproducible at 52%; this was mainly due to the formation of RSU 1069 despite the most stringent handling conditions.

Figure 2A shows a typical chromatogram of a methanol extract of plasma taken from a C3H/He mouse that had been given an i.p. dose of 190 mg kg^{-1} RB 6145 45 min previously; the large peak at $t_R = 4.8$ min corresponded to RSU 1069 (peak 4). Peak 1 was identified by co-elution with authentic material on two different columns (CN and C_{18}) as well as by comparison of the UV spectra as being the corresponding oxazolidin-2-one of RB 6145. The aziridine hydrolysis product RSU 1137 was also detectable at low concentrations (peak 3), as was an unidentified minor metabolite (peak 2) that eluted after 3.4 min. No RB 6145 was found.

Figure 2B depicts a chromatogram of pooled mouse urine (diluted 1:10) that was collected for 24 h after i.p. administration of 190 mg kg^{-1} RB 6145 (solid line) or of an equimolar dose of RSU 1069 (dotted line). After RB 6145 administration, five metabolites were detectable. Four of these were identified as being the oxazolidinone derivative of RB 6145 (peak 1), dealkylated prodrug RSU 1111 (peak 2), hydrolysis product RSU 1137 (peak 4) and RSU 1069 (peak 5); an additional unidentified metabolite (peak 3) eluted after 3.4 min, running close to RSU 1137. Again, no RB 6145 was detected. The same urinary metabolites were observed after RSU 1069 injection, albeit with substantially less oxazolidinone. Figure 3 depicts the photodiode-array UV/visible spectra of the three major urine metabolites after HPLC separation; the nearly identical spectra exhibited only one distinguished maximum at 325 nm.

Aqueous stability of RB 6145

RB 6145 disappeared monoexponentially at a rate that was dependent on the incubation conditions. The half-life in 0.1 M phosphate buffer (pH 7.4) was shown by HPLC to be ca. 7 h, 12 min and <2 min at 4°, 20° and 37°C, respectively. The major product was the ring-closed aziridine compound RSU 1069. The degradation of the prodrug was also monitored at 4°C in 0.1 M phosphate buffer (pH 5), i.e. under the conditions used for the preparation of RB 6145 for the pharmacokinetic experiments. The drug solution was stable for at least 20 min, the maximal interval between preparation and injections. After 6 days under the same conditions, there was 45% loss of RB 6145; the major products were RSU 1137 and RSU 1069. RB 6145 was very stable at pH 3, displaying no degradation at 4°C over 6 days. Based on this result, the biological samples were immediately stabilized by acidification, handled at 4°C and stored at –20°C. The stability of RSU 1069 has been reported in detail elsewhere [21]. In addition, we found that the drug was stable at 4°C in the pH range of 3–7.4 for at least 24 h.

Plasma pharmacokinetics

Figure 4A shows the plasma pharmacokinetics of the two major metabolites in C3H/He mice that had received 190 mg kg^{-1} RB 6145 i.p. No prodrug was detectable, even in plasma samples taken as early as 2 min after drug administration. The oxazolidinone analogue of RB 6145 reached peak plasma concentrations of 63.9 ± 24.8 µg ml^{-1} (mean ± 2 SE; $n = 5$) after 5 min. The intramolecularly alkylated (i.e. cyclized) aziridine product RSU 1069 achieved peak concentrations of 47.7 ± 9.8 µg ml^{-1} ($n = 5$) after 15 min. Figure 4B shows the plasma data obtained after an equimolar dose of RSU 1069 had been given by the i.p. route; absorption was relatively fast, giving peak levels of 96.6 ± 8 µg ml^{-1} ($n = 6$) after 15 min. Only small amounts of the oxazolidinone were detectable, reaching maximal levels of 0.3 µg ml^{-1} after 30 min (data not shown).

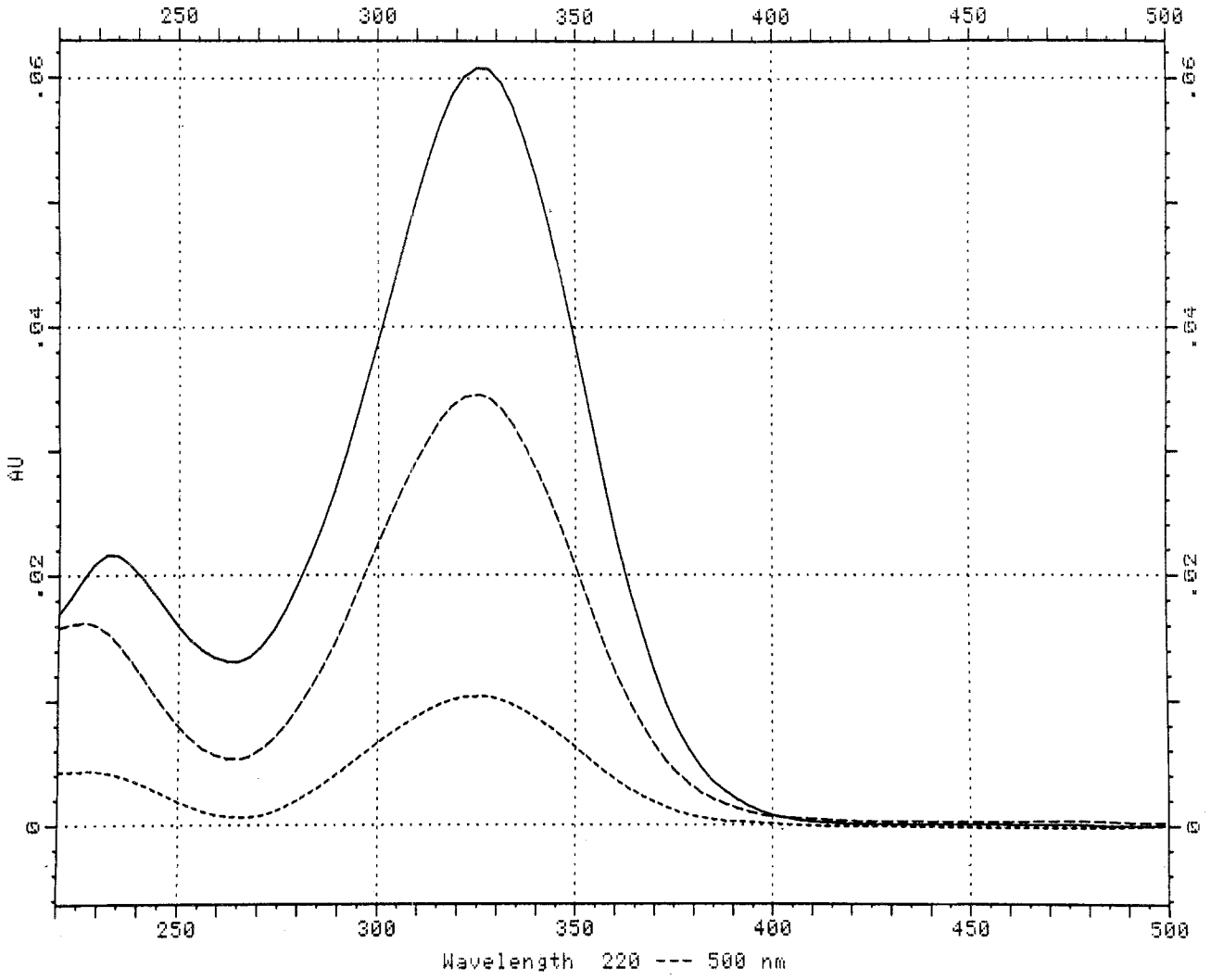

Fig. 3. Photodiode-array UV/visible spectra of the major mouse urinary metabolites of RB 6145 in mobile phase after HPLC separation as described in Fig. 2. *Solid line*, Oxazolidinone; *broken line*, RSU 1069; *dotted line*, RSU 1137

Post-peak plasma concentrations of the RB 6145 metabolites or the analogue RSU 1069 declined exponentially. The apparent mean half-lives for the oxazolidinone and the RSU 1069 metabolite were 24.5 and 24.9 min, respectively, as calculated by regression using pooled data ($n = 27$). The mean elimination $t^1/_2$ for the drug RSU 1069 was calculated to be 30.4 min ($n = 32$), which was almost identical with the value previously reported by us for male BALB/c mice [18]. The $AUC_{0-\infty}$ (mean ± 2 SE) value following i.p. dosing with RB 6145 was 46.8 ± 1.9 μg ml^{-1} h for the corresponding oxazolidinone metabolite and 41.1 ± 2.6 μg ml^{-1} h for RSU 1069 formed in vivo ($n = 27$); the $AUC_{0-\infty}$ value for the analogue RSU 1069 was double the latter value at 83.7 ± 2.1 μg ml^{-1} h ($n = 32$).

Mice that had been given 190 mg kg^{-1} RB 6145 as an i.v. bolus died within 1 min. No acute toxic signs were observed at half the dose. Figure 5 shows the comparative plasma pharmacokinetics of RB 6145 in C3H/He mice ($n = 28$) after an i.v. (panel A), i.p. (panel B) and oral (panel C) dose of 95 mg kg^{-1}. No parent prodrug was detectable following administration via any route. The pharmacokinetic parameters summarizing the results for i.v., i.p. and oral prodrug administration are given in Table 1.

Post-peak plasma clearance for the major metabolites was exponential in all cases. The apparent elimination $t_{1/2}$ for metabolite RSU 1069 appeared to be dependent on the mode of administration, being longest for the oral and shortest for the i.v. route (Table 1). Peak concentrations of the active metabolite RSU 1069 following oral administration of the prodrug were 29% lower than those measured for the i.p. route, and the latter were only 13% lower than those obtained for the i.v. route. The $AUC_{0-\infty}$ value for RSU 1069 after oral and i.v. dosing of RB 6145 was 76% and 64%, respectively, of that calculated for the i.p. route. In the case of the oxazolidinone metabolite of RB 6145, peak concentrations were about 2 times lower following oral and i.p. administration than after i.v. injection. The $AUC_{0-\infty}$ value for the oxazolidinone metabolite after oral administration was higher than that obtained following i.p. dosing and only slightly lower than that calculated after i.v. injection.

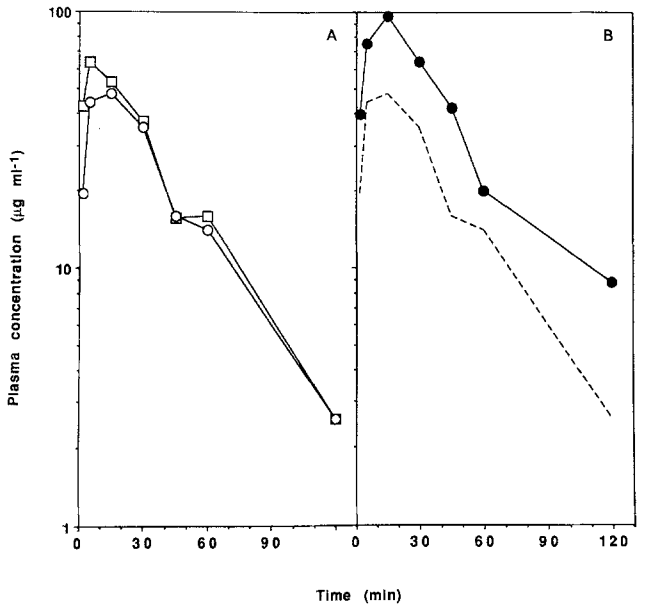

Fig. 4 A, B. Plasma pharmacokinetics of RB 6145 metabolites and RSU 1069 in C3H/He mice. **A** Mice receiving 190 mg kg^{-1} RB 6145 i.p.: □, oxazolidinone metabolite, SE 7%–26%; ○, in vivo-formed RSU 1069, SE 3%–30%. **B** Mice receiving 110 mg kg^{-1} RSU 1069 (●): SE 4%–25%. The *broken line* depicts data obtained for RSU 1069 as a metabolite of RB 6145 from panel A. Each point represents the average of 3 separate experiments involving 3–6 mice/point

Tumour and brain penetration

Figure 6 shows the comparative penetration of nitroimidazoles into KHT tumours after i.p. administration of 190 mg kg^{-1} RB 6145 (panel A) or of an equimolar dose of 110 mg kg^{-1} RSU 1069 (panel B). No RB 6145 prodrug was detectable in tumour tissue ($n = 29$), but penetration of the derived oxazolidinone and the RSU 1069 metabolite was quite substantial, reaching levels of 8.6 ± 0.7 and 6.3 ± 1.5 µg g^{-1} ($n = 10$), respectively, after 30 min. Expressed as a percentage of the ambient plasma concentration, the tumour level of the active metabolite RSU 1069 increased steadily from about 3% after 2 min to 40% after 2 h (Table 2). A similar time-course was observed for injected RSU 1069 (Fig. 6B): peak levels of 10.3 ± 1.7 µg g^{-1} ($n = 14$) were reached after 30 min and tumour/plasma percentages increased to 24% after 60 min. The major metabolite of RSU 1069 was RSU 1137, which reached maximal tumour concentrations of 0.6 µg g^{-1} after 45 min. The $AUC_{0-\infty}$ value in tumour for the RSU 1069 metabolite generated in vivo from RB 6145 was 7.8 ± 1.5 µg g^{-1} h ($n = 29$) as compared with 9 ± 1.3 µg g^{-1} h ($n = 29$) for the oxazolidinone after RB 6145 administration and 11.8 ± 0.8 µg g^{-1} h ($n = 39$) for the administered RSU 1069. These values represent 20%, 20% and 15%, respectively, of the relevant plasma $AUC_{0-\infty}$ figures.

Brain/plasma percentages obtained after RSU 1069 injection tended to be lower than the percentages of drug in tumour (Table 2). As far as absolute concentrations are concerned, a single i.p. dose of 110 mg kg^{-1} RSU 1069 gave peak levels of 7.6 ± 0.7 µg g^{-1} (mean ± 2 SE; $n = 12$) after 30 min (Fig. 7B). Maximal brain levels of RSU 1069 as a metabolite after an equimolar dose of RB 6145 were 2 times lower at 3.9 ± 0.4 µg g^{-1} h ($n = 10$) after 30 min (Fig. 7A). The mean $AUC_{0-\infty}$ value in brain tissue for the in vivo-formed RSU 1069 was 4 ± 0.9 µg g^{-1} h ($n = 27$) as compared with 7 ± 1.6 µg g^{-1} h ($n = 32$) for the injected drug, representing 10% and 9%, respectively, of the plasma $AUC_{0-\infty}$ value. Due to its hydrophilicity, the oxazolidinone metabolite of RB 6145 was almost totally excluded from the brain. Expressed as a percentage of the plasma exposure, the brain $AUC_{0-\infty}$ value was <4%.

Table 3 summarizes the apparently advantageous disposition of RSU 1069 following the administration of the RB 6145 prodrug as compared with the target aziridine

Table 1. Plasma pharmacokinetic parameters of RB 6145 metabolites in C3H/He mice given 95 mg kg^{-1} RB 6145 i.v., i.p. or p.o.

Parameter	Route of administration					
	i.v.		i.p.		p.o.	
	Oxazolidinone	RSU 1069	Oxazolidinone	RSU 1069	Oxazolidinone	RSU 1069
c_{max} (µg ml^{-1})	77.6±6.3 (70.8–86.1)	25.5±2.7 (23.6–29.4)	36.6±2 (33.9–38.7)	22.2±1.7 (21.1–24.8)	34.2±3 (30.1–36.9)	15.9±1.5 (13.8–17.1)
t_{max} (min)	≤2	≤2	5	5	5	5
$t_{1/2\beta}$ (min)	22.8 (21.5, 24.1)	17.1 (17, 17.1)	20.4 (19.4, 21.4)	21.1 (18.6, 23.6)	35.1 (29.7, 40.5)	27.8 (22, 33.5)
k_{el} (h^{-1})	1.8±0.1 (1.9, 1.8)	2.4±0.1 (2.5, 2.4)	2±0.2 (1.9, 2.1)	2±0.3 (1.8, 2.2)	1.2±0.2 (1, 1.4)	1.6±0.5 (1.2, 1.9)
$AUC_{0-\infty}$ (µg ml^{-1} h)	33.1 (30.8, 35.5)	10.6 (10, 11.2)	24.9 (24.5, 25.3)	16.6 (15.9, 17.3)	29.9 (29, 30.8)	12.6 (12.5, 12.6)

Parameters were obtained from 2 separate experiments, each involving 4 mice/point and 7 time points. c_{max} is given as the overall mean ± 2 SE, and the range for individual mice is also shown. Other data represent the mean of the 2 experiments, with the value for each also being indicated. t_{max} values were identical in both experiments. $t_{1/2\beta}$ represents the apparent half-life

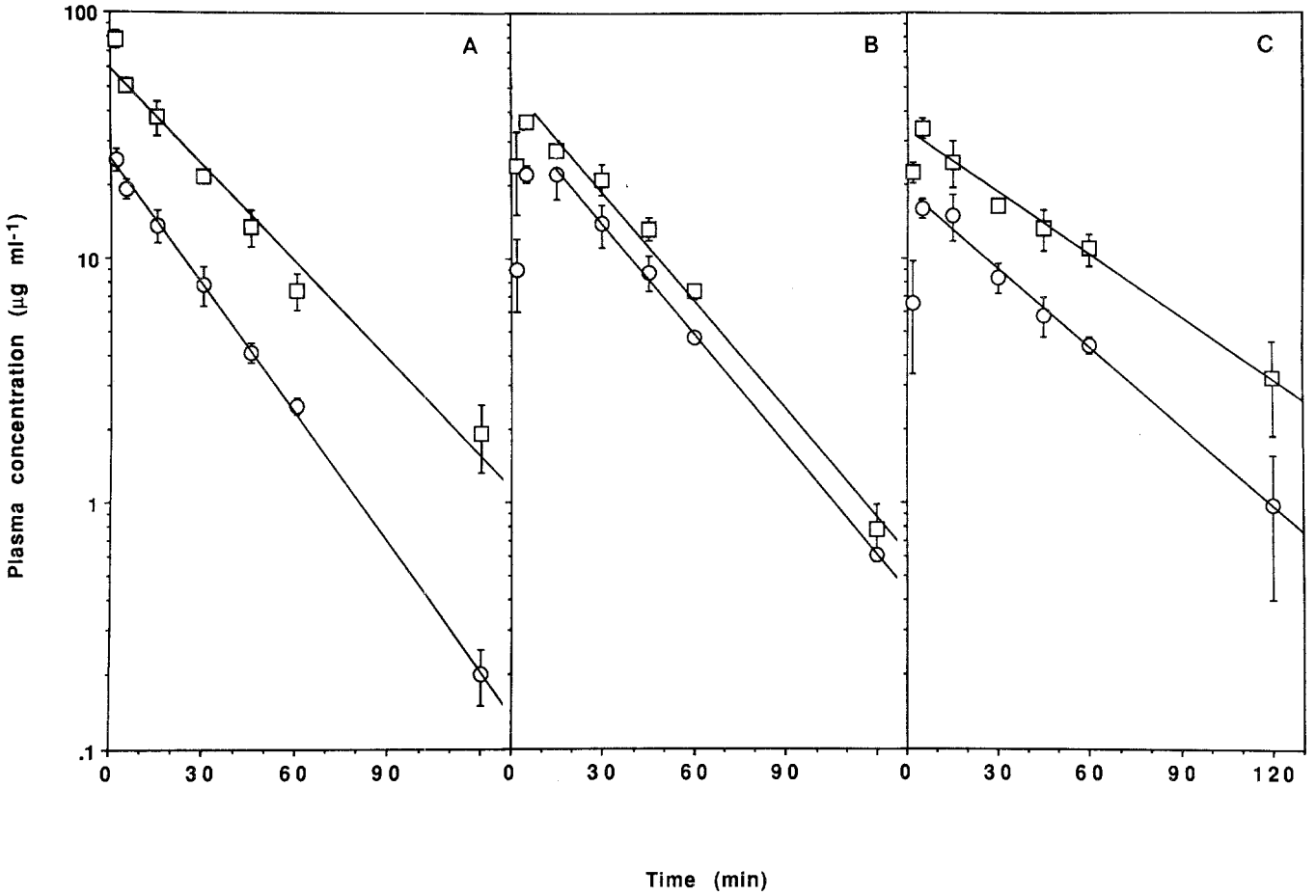

Fig. 5 A–C. Plasma pharmacokinetics of RB 6145 metabolites in C3H/He mice receiving 95 mg kg^{-1} RB 6145 **A** i.v., **B** i.p. and **C** p.o. □, Oxazolidinone metabolite; ○, in vivo-formed RSU 1069. Each point represents the average of 2 separate experiments involving 4 mice/point. Bars represent ± 2 SE

Table 2. Tumour and brain/plasma percentages obtained for RSU 1069 and the oxazolidinone in C3H/He mice given 190 mg kg^{-1} RB 6145 i.p. and values obtained for RSU 1069 in mice receiving an equimolar dose of 110 mg kg^{-1} RSU 1069 i.p.

Time (min)	KHT tumour/plasma (%)			Brain/plasma (%)		
	RB 6145 (190 mg kg^{-1})		RSU 1069 (110 mg kg^{-1})	RB 6145 (190 mg kg^{-1})		RSU 1069 (110 mg kg^{-1})
	Oxazolidinone	RSU 1069		Oxazolidinone	RSU 1069	
2	1.3± 0.5	3.3±0.3	2 ±0.6	≤0.2	≤0.5	≤0.3
5	3.8± 0.23	3.6±2.5	3.4±2.2	≤0.2	≤0.2	1.4±0.1
15	14.5± 0.9	12.1±1.6	7.4±2.9	2.1±0.4	5.7±1.2	5.2±0.9
30	22.9± 5.5	17.9±4.4	16.1±3.4	3.7±0.8	11.1±0.9	11.8±1.8
45	33 ± 3.7	25.6±3.5	15.2±4.4	4.4±1.3	15.5±5.9	14.2±2.7
60	31.4± 8.8	26 ±0.3	24.2±2.5	3.7±1.9	14.6±4.8	16.3±9.9
120	23 ±10.5	40 ±8.2	17.1±9.8	10.9±3.9	11.9±8.3	6.8±3.6

Data represent mean values ± 2 SE from 3–4 separate experiments involving 3–14 mice/point

molecule itself. In terms of both AUC and peak concentration, the reduction in the exposure of the tumour to drug was consistently smaller than that observed for plasma and brain.

Urinary excretion

The 24-h urinary recovery data for both RB 6145 and RSU 1069 administration in C3H/He mice are summarized in Table 4. Consistent with the readily detectable levels of oxazolidinone metabolite in plasma following the administration of RB 6145, this was the major product excreted in urine. Some 12% of the RB 6145 dose was eliminated as RSU 1069 and 4%–5% was recovered as the hydrolysis product RSU 1137, whereas only traces of the *N*-dealkylated metabolite RSU 1111 were detectable. After the administration of an equimolar dose of RSU 1069 itself, urinary recoveries similar to those described above were seen

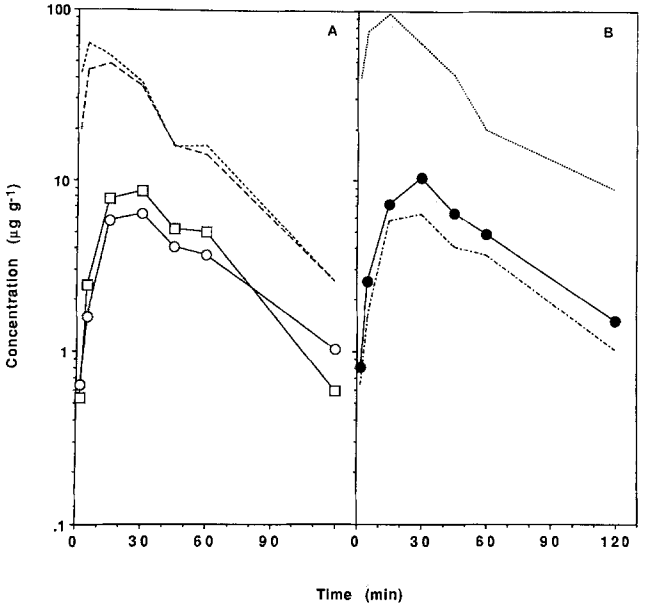

Fig. 6 A, B. Pharmacokinetics of RB 6145 metabolites and RSU 1069 in KHT tumours grown i.d. in C3H/He mice. **A** Mice receiving 190 mg kg^{-1} RB 6145 i.p. □, Oxazolidinone metabolite in tumour tissue, SE 3%–30%; ○, in vivo-formed RSU 1069 in tumour tissue, SE 1%–35%. The *dotted line* depicts the equivalent oxazolidinone metabolite time-course in plasma and the *broken line* represents the time-course of in vivo-formed RSU 1069 in plasma (data from Fig. **4 A**). **B** Mice receiving an equimolar dose of 110 mg kg^{-1} RSU 1069 i.p. ●, RSU 1069 in tumour tissue, SE 5%–34%. The *dotted line* depicts the time-course of injected RSU 1069 in plasma (data from Fig. **4 B**) and the *broken and dotted line* represents data obtained for RSU 1069 as a metabolite of RB 6145 in tumour tissue (data from panel **A**). Each point represents the average of 3–4 separate experiments involving 3–14 mice/point

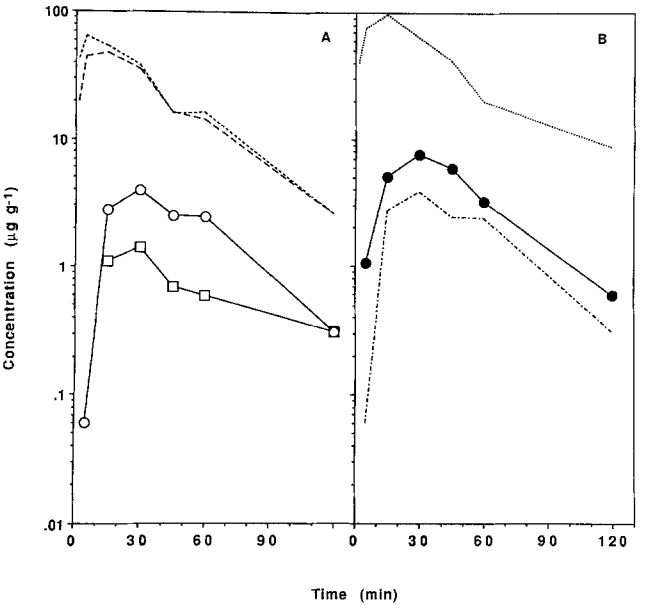

Fig. 7 A, B. Pharmacokinetics of RB 6145 metabolites and RSU 1069 in brain tissue. **A** Mice receiving 190 mg kg^{-1} RB 6145 i.p.: □, oxazolidinone metabolite in brain tissue, SE 6%–26%; ○, in vivo-formed RSU 1069 in brain, SE 3%–19%. The *dotted line* depicts the oxazolidinone metabolite time-course in plasma and the *broken line* represents the time-course of in vivo-formed RSU 1069 in plasma (data from Fig. **4 A**). **B** Mice receiving 110 mg kg^{-1} RSU 1069 i.p.: ●, drug in brain tissue, SE 5%–21%. The *dotted line* depicts the time-course of the drug in plasma (data from Fig. **4 B**) and the *broken and dotted line* represents data obtained for the RSU 1069 metabolite in brain tissue (data from panel **A**). Each point represents the average of 3–4 separate experiments involving 3–12 mice/point

Table 3. Comparison of exposure to the target aziridine molecule RSU 1069 following the administration of either RSU 1069 itself or the prodrug RB 6145 at a dose of 0.5 mmol kg^{-1} i.p.

Administered drug	RSU 1069 AUC			RSU 1069 peak concentrations		
	Plasma	Brain	Tumour	Plasma	Brain	Tumour
RSU 1069	83.7±2.1	7±1.6	11.8±0.8	96.6±8	7.6±0.7	10.3±1.7
RB 6145	41.1±2.6	4±0.9	7.8±1.5	47.7±9.8	3.9±0.4	6.3±1.5
RSU 1069/RB 6145	2.03	1.75	1.51	2.02	1.95	1.69

Data represent mean values ± 2 SE for pooled data from at least 2 independent experiments. The RSU 1069/RB 6145 ratio serves as a useful indicator of the reduction in exposure associated with the use of the prodrug: note that the decrease was greatest in the plasma and brain

for RSU 1069, RSU 1111 and RSU 1137. The oxazolidinone was also detected in urine, explaining a previously reported but unidentified metabolite [21]; however, the amount measured was 10 times lower than that observed following RB 6145 administration. This difference accounts for the lower overall urinary recovery. For both drugs, the remaining material most likely represents either products of reaction with biological nucleophiles or polar reduction products.

Discussion

The present study was undertaken to evaluate the pharmacokinetics, distribution and metabolism of the new hypoxic cell sensitizer RB 6145, a bromoethylamino-substituted 2-nitroimidazole designed to form RSU 1069 under physiological conditions [10]. As compared with more conventional nitroimidazoles, the incorporation of the alkylating aziridine moiety into RSU 1069 renders the latter much more potent as a hypoxic cell-specific radiosensitizer, chemosensitizer and cytotoxic agent [1]. However, its clinical use is limited due to severe dose-limiting gastrointestinal toxicity [9]. Interestingly, the RSU 1069 prodrug RB 6145 has been found to be almost as active but substantially less

Table 4. The 24-h urinary excretion as a percentage of the delivered dose in C3H/He mice given 190 mg kg^{-1} RB 6145 i.p. or an equimolar dose of 110 mg kg^{-1} RSU 1069 i.p.

Compound	Drug			
	RB 6145		RSU 1069	
	Experiment[a]		Experiment[a]	
	I	II	I	II
Oxazolidinone	39.6	27.5	3.4	3.3
RSU 1111	0.4	0.3	<0.1	<0.1
RSU 1137	4.2	3	5.2	5.5
RSU 1069	14.8	9.2	10.8	12.5
RB 6145	<0.1	<0.1	NA	NA
Total recovery	59	40	19.4	21.3

[a] Pooled data from 3 mice in each of 2 independent experiments
NA, Not applicable

toxic than RSU 1069 [4, 10]. Because of the potential clinical development of RB 6145 and related agents, we deemed it important to determine whether the therapeutic advantage of these drugs over the corresponding aziridine could be related to a more favourable pharmacokinetic profile. The experiments were carried out in C3H/He mice bearing KHT tumours such that the results could be directly compared with the therapeutic and toxicological data.

Using a modification of our earlier reversed-phase HPLC technique [21], we showed that RB 6145 is converted quite efficiently to the target molecule RSU 1069 in mice. This result was predicted by radiosensitizing and cytotoxicity studies [4, 10]. An i.p. dose of 190 mg kg^{-1} RB 6145 in mice produced peak plasma concentrations of around 50 μg ml^{-1} RSU 1069 as compared with about 100 μg ml^{-1} following an equimolar dose of RSU 1069. The partial inactivation of RB 6145 was found to be due to the formation of the oxazolidinone; this was almost certainly produced by reaction with hydrogen carbonate in the blood, as has been noted for other 2-haloethylamines [15] and for RB 6145 itself in aqueous solution [10]. The oxazolidinone was not detectable in tissue after the administration of RSU 1069 but occurred as a minor metabolite in plasma and urine. First-order elimination was observed for both RB 6145 metabolites, with an apparent half-life of approximately 25 min being recorded.

The prodrug itself was not detectable in biological specimens, even at very early sampling times. As previously reported the RB 6145 prodrug cyclizes to RSU 1069 in aqueous solution [10]. The intramolecular alkylation reaction is temperature- and pH-dependent. Based on these observations, the pH of the injection solution was adjusted to 5 and the drug was injected immediately to avoid degradation. The solution was stable at 4° C for the time required for drug administration. Under physiological conditions, the half-life of the prodrug was <2 min and is likely to be even shorter in vivo because of the formation of oxazolidinone in blood.

When the prodrug was given orally, peak plasma concentrations of the RSU 1069 metabolite were only 29%–38% lower than those measured after i.p. or i.v. injection of RB 6145. The plasma $AUC_{0-\infty}$ value for the RSU 1069 metabolite following oral administration of RB 6145 was 1.2-fold that obtained after i.v. injection and 76% of that calculated after i.p. injection. In contrast, the oral availability of RSU 1069 in C3H mice has previously been reported to be low at 20%, presumably as a result of extensive acid hydrolysis of the aziridine moiety in the stomach [21].

The present studies indicated that there was a suggestion of route-dependent elimination of the target metabolite RSU 1069, with the oral route producing a $t^1/_2$ value that was 1.6-fold that observed after i.v. injection of the prodrug. The AUC value for the oxazolidinone metabolite after oral administration of RB 6145 was slightly higher than that calculated following i.p. administration and only a little lower than that obtained after i.v. dosing. However, peak oxazolidinone levels following i.v. injection of the prodrug were about 2-fold those obtained after both oral and i.p. administration and possibly contributed to the acute i.v. toxicity. Tissue concentrations were not determined for oral dosing. The observation that systemic exposure to RSU 1069 was only slightly reduced (24%) probably explains the finding that radiosensitization and cytotoxicity were not markedly reduced when RB 6145 was given p.o. as opposed to i.v. An elucidation of the mechanism by which whole-body toxicity is decreased by a factor of 3 [5] requires further investigation. One possibility would be the avoidance of local toxic effects that may occur following i.p. administration.

After an i.p. dose of 190 mg kg^{-1} RB 6145, the penetration of the active metabolite RSU 1069 into KHT tumours was quite efficient, with peak levels of ca. 6.3 μg g^{-1} being observed at 30 min. The absolute peak tumour concentration for an equimolar dose of RSU 1069 was 10.3 μg g^{-1}, representing a tumour/plasma percentage of ca. 16% as compared with the 18% that was found after prodrug administration. The tumour AUC value for RSU 1069 was only 35% lower after the administration of RB 6145 as compared with RSU 1069 dosing; this disparity was smaller than the 2-fold difference in the relative plasma AUC values. It is noteworthy that the major oxazolidinone metabolite of RB 6145 gave a peak tumour concentration of around 9 μg g^{-1} and a tumour $AUC_{0-\infty}$ value that was somewhat higher than that calculated for the RSU 1069 metabolite. Taking into account the observation that RB 6145 is slightly but not markedly less active as a hypoxic cell radiosensitizer and cytotoxin [4, 10], it appears that these therapeutic activities may be predominantly related to similar levels of exposure to the RSU 1069 metabolite but may also involve a possible contribution by the oxazolidinone.

The oxazolidinone was shown to exhibit very weak in vitro radiosensitizing properties as compared with those of RSU 1069 [10], and the effectiveness of the former as a cytotoxic agent has not been reported. However, the situation may be quite complex after the administration of RB 6145 in vivo when both RSU 1069 and the oxazolidinone metabolite are simultaneously present in the tumour. At this time, a positive involvement of the oxazolidinone cannot be ruled out. Conversely, previous studies have shown that misonidazole may protect against the effects of

RSU 1069 [19], possibly by competing for nitroreduction, and it should be noted that a similar antagonistic effect might be possible for the oxazolidinone.

The precise timing of the achievement of peak tumour levels of RSU 1069 metabolites (30 min) is probably not as important as that involving conventional nitroimidazoles. Equal effectiveness has been seen on prodrug injection before or after irradiation [4]. In view of the molecular mechanism of RSU 1069's action involving cross-linking of DNA via the combination of aziridine and reduced nitro functionalities, its therapeutic activity might be expected to exhibit a greater dependence on AUC than on the drug concentration at the time of irradiation. The same lack of time dependence has been observed for RSU 1069, indicating that direct cytotoxicity towards hypoxic cells is the major mechanism of radiosensitization [8].

Besides providing one possible explanation for the similar therapeutic activity of equimolar doses of RB 6145 and RSU 1069, the pharmacokinetic profile also suggests a plausible rationale for the reported approximately 2.5 times lower toxicity of the prodrug [10]. This was paralleled by a reproducibly lower (ca. 2-fold) level of plasma and brain exposure to RSU 1069. The brain $AUC_{0-\infty}$ value was 4 $\mu g\ g^{-1}\ h$ after RB 6145 administration as compared with 7 $\mu g\ g^{-1}\ h$ following an equimolar dose of RSU 1069. Peak concentrations were also proportionately lower. Perhaps most importantly, the major RB 6145 metabolite, the oxazolidinone, was most efficiently excluded from the brain, presumably because its relatively high degree of hydrophilicity restricts its transport across the lipoidal blood-brain barrier. The same property likewise led to the preferential elimination of this metabolite via the kidneys in the present study. Thus, about 34% of the RB 6145 dose was eliminated in the urine as the corresponding oxazolidinone compared to 12% which was eliminated in urine as the RSU 1069 metabolite.

In reviewing the overall data, we feel that the comparative pharmacokinetic profile of RB 6145 indicates a therapeutic advantage for this drug over RSU 1069 and probably contributes to the nearly equivalent activity together with the substantially lower toxicity of the prodrug in mice. The benefits associated with RB 6145 administration include in particular a comparatively lower exposure to RSU 1069 in the plasma and brain as compared with the KHT tumour, whereas the oxazolidinone metabolite is excluded from the brain but not the tumour. The additional advantage afforded by oral administration of the prodrug may also have a pharmacokinetic basis, as has been suggested by preliminary studies using tritiated RSU 1069 [5]. Although the trends in the exposure parameters are consistent with the view that pharmacokinetics play a significant role in the promising therapeutic index of RB 6145, further studies are required to establish the precise quantitative relationships and to reveal the potential involvement of any additional factors. Taken together with the pharmacokinetic results reported herein the efficacy and toxicity data suggest that RB 6145 is an excellent candidate for clinical trial.

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