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High uptake of RSU 1069 and its analogues into melanotic melanomas

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Summary. RSU 1069 and RSU 1164 are electron affinic agents that contain a nitro group together with a weakly basic alkylating aziridine moiety, and they represent lead compounds in the development of dual-function, bioreductive, hypoxic cell radiosensitizers. We studied the pharmacokinetics of these drugs in mice carrying KHT sarcoma, Lewis lung carcinoma, and B16 melanoma. Following an i.p. dose of 80 mg/kg, absorption was rapid and the elimination $t_{1/2}$ was in the region of 30 min for both agents. Maximal tumour levels were 91, 16 and 19 μg/ml for RSU 1069 and 109, 26 and 28 μg/ml for RSU 1164 in the B16, KHT and Lewis lung tumours, respectively. In B16 melanoma these levels corresponded to tumour:plasma ratios of 3.8 for RSU 1069 and 3.7 for RSU 1164. Cellular uptake of RSU 1069, RSU 1164 and a related compound, RB 7040, was measured in vitro as a function of extracellular pH. Melanotic cells from both B16 melanoma and HX118, a human tumour xenograft, showed substantially greater accumulation of these weakly basic sensitizers than any other cell type examined. Ratios of intra-:extracellular concentration (Ci/Ce) for RSU 1069 were around unity and independent of pH for Lewis lung cells and HX34 amelanotic melanoma cells, whereas ratios of up to 3 and 5 were obtained in B16 and HX118 cells, respectively. The highest measured value of Ci/Ce was 15 for RSU 1164 in HX118 cells at pH 8.4; this compares with a ratio of 1.5 for HX34 cells at the same pH. These studies indicate that the high levels of uptake of the weakly basic sensitizers into melanotic melanoma in vivo is a cell-mediated phenomenon and may be due to a lower average intracellular pH in the melanotic cells.

Introduction

A number of clinical studies have suggested that radiobiological hypoxia might limit the effectiveness of radiotherapy in some types of human cancer, and the hypoxic cell radiosensitizer misonidazole (MISO) has been shown to improve the results of treatment at some sites, notably in carcinoma of the pharynx [11, 12]. However, the dose-limiting neurotoxicity of MISO is such that it precludes widespread clinical use. As a result, a number of different compounds are under investigation in an attempt to develop new, less toxic and/or more effective drugs for the sensitisation of hypoxic tumour cells. Among these are RSU 1069 and its alkyl-substituted aziridinyl analogues, which are more potent hypoxic cell radiosensitizers than MISO both in vivo and in vitro [1–3, 19]. RSU 1069 is a mixed-function radio- and chemosensitising agent consisting of an electron affinic 2-nitroimidazole together with an alkylating, weakly basic aziridine moiety in the N-1 side chain [1]. This agent is also a potent bioreductive agent, possessing powerful cytotoxic properties in its own right when reductively metabolised in an hypoxic environment [17, 18].

Experimental pharmacokinetic studies of RSU 1069 and its analogues have demonstrated marked selective uptake of the compounds into B16 melanoma cells, with tumour concentrations up to 4 times higher than those found in plasma or normal tissues [5]; the reason for this phenomenon is not understood. High tumour drug levels have been described for other basic 2-nitroimidazoles, such as pimonidazole (Ro 03-8799), but for that compound normal tissue levels were also found to be high [14]. In an independent pharmacokinetic study with RSU 1069, selective uptake into the KHT tumour was not demonstrated [24]. We therefore investigated the question as to whether the uptake of RSU 1069 and its analogue RSU 1164 into neoplastic tissues is tumour type-specific. We report the pharmacokinetics of RSU 1069 and RSU 1164 in mice carrying either KHT sarcoma or Lewis lung carcinoma, and the results are compared with our previously reported results for B16 melanoma [5].

The tissue distribution of drugs can depend on their prototropic properties. This is particularly important when the pK_a of the drug is close to both the intracellular pH and the pH of the surrounding milieux. It has been proposed that for basic compounds this can result in the effective concentration of the ionized species in areas of low pH, such as might occur in tumours [8]. Therefore, to determine the basis for the previously reported difference in the uptake of RSU 1069 into the KHT and B16 tumours, we measured the pH dependence for the accumulation of RSU 1069 into these tumour cells and a variety of other cell types in vitro. Furthermore, cellular uptake of the RSU 1069 analogues RSU 1164 and RB 7040 was studied, since we had previously shown that these drugs but not RSU 1069 showed a substantial pH-dependent uptake into V79 cells in vitro [19, 20].

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Materials and methods

Compounds. RSU 1069 [NSC 347503, 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol] and the *cis*-2,3-dimethyl- and tetramethyl-aziridinyl derivatives RSU 1164 and RB 7040, respectively, were prepared by Drs. T. C. Jenkins and I. Ahmed as previously described [1, 3, 10]. Their general structure, substituents and pK_a and partition coefficient (P) values are shown in Fig. 1 and Table 1.

Tumour sytems. Male C57/B1 mice were obtained from the Medical Research Council (NIMR, Mill Hill, London). Male C3H mice were bred at the MRC Radiobiology Unit. Outbred male nu/nu mice were obtained from the Institute of Cancer Research (Chelsea, London).

B16 melanoma and Lewis lung tumour were obtained from uncloned cell lines routinely passaged in vivo in C57/B1 mice at the Radiotherapy Research Unit, Institute of Cancer Research (Sutton, Surrey). KHT sarcoma is similarly maintained by routine passage in C3H/He mice at the MRC Radiobiology Unit. Murine tumours were passaged in 6-week-old animals by the bilateral inoculation of tumour brei into the gastrocnemius muscle [15].

The two human xenografts HX34 and MX118 were originally established at the Radiotherapy Research Unit, Institute of Cancer Research (Sutton, Surrey) from biopsies taken from patients with metastatic melanoma (D. Courtenay and J. Mills, personal communication). HX34 is an amelanotic tumour and HX118 is melanotic. Xenografts were passaged by the s.c. implantation of 2 mm^3 tumour pieces in the dorsal region in 6-week-old nu/nu mice.

In vivo pharmacokinetics. Experiments were carried out when animals were 8–10 weeks of age, weighing 25–30 g and having pooled tumour weights of 200–500 mg. The compounds were injected i.p. in isotonic, aqueous sodium hydrogen carbonate at a dose of 0.08 mg g^{-1} in a volume of 0.010–0.012 cm^3 g^{-1}.

Table 1. Substituents and properties of RSU 1069 and some of its analogues

Drug	R_1	R_2	R_3	R_4	pK_a	P[a]
RSU 1069	H	H	H	H	6.04[b]	0.22[b]
RSU 1164	CH_3	H	CH_3	H	6.82[d]	1.67[c]
RB 7040	CH_3	CH_3	CH_3	CH_3	8.45[d]	6.40[c]

[a] Parototition coefficient values determined for unpronated bases (i.e. pH > 11)
[b] From Adams et al. [1]
[c] From Walling et al. [20]
[d] From O'Neill et al. [10]

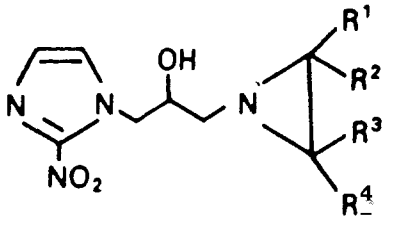

Fig. 1. General structure of radiosensitizers used

Animals were sacrificed at various times (2–120 min) after drug administration. Blood plasma and tissue samples (tumour, brain, muscle) were collected and homogenised as previously described elsewhere [4]. Samples were extracted in 4 volumes of methanol containing internal standard and centrifuged at 5,000 *g* for 5 min. Plasma supernatants were immediately analysed; tissue supernatants were freeze-dried and re-constituted in 0.2 cm^3 methanol before analysis. Drugs were assayed by reversed-phase high performance liquid chromatography (HPLC) as previously described [5].

Data is presented as a concentration × time plot for each tissue in each tumour system; curves are hand-drawn. Statistical analysis and calculations of apparent plasma elimination half-life ($T_{1/2}$) and apparent volume of distribution (Vd) were carried out as previously described elsewhere [23]. The maximal tissue-to-plasma ratios obtained for tumour (T/P), brain (B/P), and muscle (M/P) were estimated from the curves at the time of maximal drug concentration in the tissue under study.

Drug uptake into cells in vitro. Single-cell suspensions were prepared for drug uptake studies using enzymatic disaggregation (37° C for 30 min) of finely chopped tumour material according to the following methods: B16 melanoma, 0.2% trypsin (Difco-Bacto); Lewis lung carcinoma, 0.1 mg/ml deoxyribonuclease (DNAase) (Sigma) plus 0.04 mg/ml trypsin; HX34 and HX118, 0.02% collagenase (Worthington) plus 0.02% DNAase plus 0.05% pronase (Calbiochem-Behring). Cells were exposed to the sensitisers at a concentration of 0.3 m*M* in phosphate-buffered saline (PBS) at the required pH for 1 h in suspension at 20° C. Samples were prepared and assayed by HPLC as previously described [10].

Results

Animal pharmacokinetics

Figures 2 and 3 show the plasma, tumour, brain and muscle pharmacokinetics for RSU 1069 and RSU 1164 in mice bearing KHT and Lewis lung tumours, compared with results from those carrying B16 melanoma.

From this data it is apparent that there are differences in peak plasma concentrations, which is further indicated by a comparison of the Vd values listed in Table 2. As the compounds were injected i.p., this was probably simply a reflection of the large differences in biodistribution found between the different tumour systems, which are described below. However, the shapes of the six plasma clearance curves, were very similar; in particular, no systematic differences were found between the two strains of mice ($t_{1/2}$ values are also listed in Table 2).

In contrast to the similarity of the plasma clearance curves, the tissue distributions of the compounds differed markedly between the systems. The maximal tissue/plasma ratios (T/P) obtained for tumour, brain and muscle are listed in Table 3. The T/P high values found with both compounds in B16 melanoma can be seen to reflect a rapid and selective concentration of the drugs in tumour tissue, with maximal tumour levels far exceeding the peak plasma concentrations. This phenomenon was not seen in either the KHT or Lewis lung tumours. In the latter systems RSU 1069 showed consistently low tumour levels;

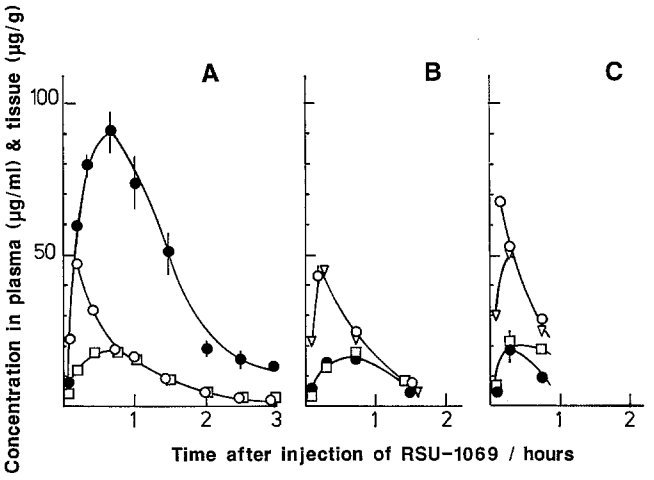

Fig. 2. Concentration of RSU 1069 as a function of time after i.p. injection of 0.08 mg g^{-1}. Tumour, ●; plasma, ○; brain, □; muscle ▽. *A*, B16 melanoma [5]; *B*, KHT sarcoma; *C*, Lewis lung carcinoma. Points represent mean values from 2–6 animals ± SE. Where no error bars are shown, errors lie within the dimensions of the plotted points. Three independent experiments were carried out in each tumour system

Table 2. Summary of plasma pharmacokinetic parameters

Drug	Mouse	Tumour	$t_{1/2}$[a] (min)	Vd ($dm^3\ kg^{-1}$)
RSU 1069	C57/Bl	B16	33 (30–35)	1.48
RSU 1069	C3H/He	KHT	33 (27–43)	1.46
RSU 1069	C57/Bl	LL	29 (22–40)	0.95
RSU 1164	C57/Bl	B16	32 (29–35)	1.71
RSU 1164	C3H/He	KHT	29 (27–31)	1.38
RSU 1164	C57/Bl	LL	32 (24–46)	1.43

[a] Values in parentheses represent 95% confidence intervals

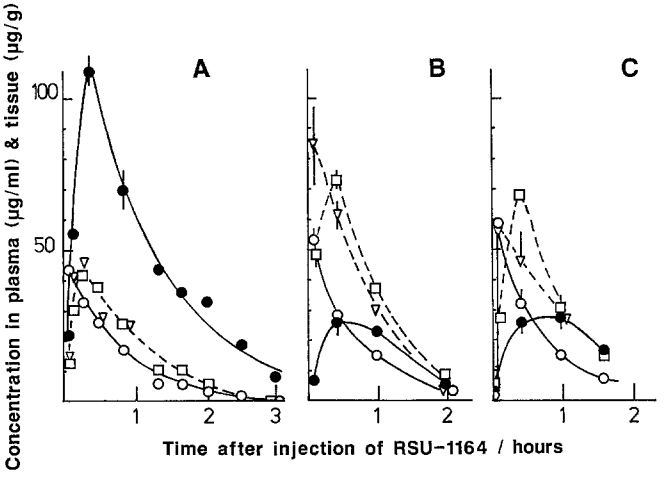

Fig. 3. Concentration of RSU 1164 as a function of time after i.p. injection of 0.08 mg g^{-1}. Tumour, ●; plasma, ○; brain □; muscle, ▽. *A*, B16 melanoma [5]; *B*, KHT sarcoma; *C*, Lewis lung carcinoma. Points represent mean values from 2–6 animals ± SE. Where no error bars are shown, errors lie within the dimensions of the plotted points. Three independent experiments were carried out in each tumour system

Table 3. Tissue/plasma concentration ratios for RSU-1069 and RSU-1164 in murine tumour systems

Tissue/plasma ratio	B16		KHT		LL	
	1069	1164	1069	1164	1069	1164
T/P	3.8	3.7	0.5	1.0	0.4	1.5
B/P	0.8	1.2	0.6	2.6	0.4	2.1
M/P	–	1.3	1.1	1.9	1.0	1.4

T/P values just in escess of unity were found for RSU 1164, but this was caused by slightly delayed clearance of the compound from tumour compared with plasma, not by selective uptake.

The patterns of drug distribution in normal tissues differed between the two compounds but did not seem to be much affected by the strain of mouse used. The hydrophilic compound RSU 1069 consistently showed normal T/P ratios close to or less than 1, whereas RSU 1164 showed some increased concentration in brain and muscle compatible with its greater lipophilicity. Differences between peak brain and muscle concentrations of RSU 1164 in B16-bearing mice and those in animals carrying the KHT and Lewis lung tumours are once again probably due to the high degree of rapid preferential uptake of this drug by the B16 tumour.

Influence of extra-cellular pH on cellular uptake in vitro

The pK_a of weak bases has been shown to be an important parameter in determinations of the cellular uptake of nitroimidazole radiosensitisers in vitro [6, 20]. To assess whether the finding of selective uptake of RSU 1069 and RSU 1164 into B16 melanoma is a cell-mediated phenomen, we measured the accumulation of these drugs into a variety of cell types in vitro. The cells used were melanotic melanoma cells from the B16 and HX118 tumours, amelanotic melanoma cell from the HX34 tumour, and Lewis lung carcinoma cells. The ratios of intra- to extracellular concentration (Ci/Ce) are plotted as a function of extracellular pH (pHe) in Fig. 4.

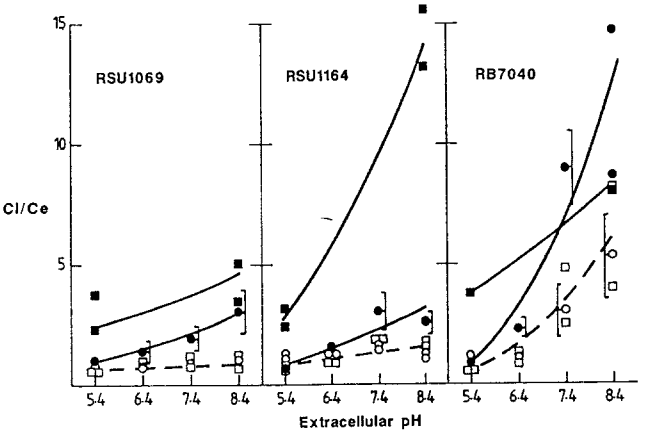

Fig. 4. Effect of extra-cellular pH on the ratio of intra-cellular (*Ci*) to extra-cellular (*Ce*) concentrations of RSU 1069 and its analogues. *Solid lines and solid figures,* melanotic melanomas; ●, B16 melanoma; ■, HX118 melanoma. *Dashed line and open symbols,* other tumours; □ HX34 amelanotic melanoma; ○, Lewis lung carcinoma. Symbols indicate individual determinations. Error bars are derived from three or more experiments

RSU 1069 does not accumulate intracellularly in HX34 melanoma or Lewis lung carcinoma cells (i.e. values of Ci/Ce remain around unity) within the pH range of 5.4–8.4; this is consistent with our previous work with V79 cells [20]. However, the uptake of RSU 1069 into both of the melanotic melanoma cell types, B16 and HX118, is clearly pH-dependent. Ci/Ce values of 1 at pH 5.4 and 3 at pH 8.4 were obtained for the uptake of RSU 1069 by B16 melanoma, whereas the corresponding data for HX118 melanoma were 3 at pH 5.4 an 4.2 at pH 8.4. Similarly, both B16 and HX118 melanoma cells show greater accumulation of RSU 1164 than either Lewis lung or HX34 amelanotic melanoma cells, which have similar levels of accumulation (Ci/Ce $\sim$ 1) at each of the pHe values. The greatest pH dependence for RSU 1164 is seen with HX118 cells, giving Ci/Ce values of about 15 at pHe 8.4. With RB 7040, Ci/Ce values increase with increasing pHe values for all four cell types. Again, Lewis Lung and HX34 melanoma show very similar levels of intracellular accumulation, and these are less than those of B16 melanoma and HX118 at pH values above 5.4.

Discussion

The main findings from the present work are:

1. The weak bases RSU 1069 and RSU 1164 are selectively concentrated in the B16 melanoma but not in the KHT or Lewis lung tumours or in the other murine tissues examined.
2. In vitro studies indicate that this selectivity is pH-dependent and may be a property of melanotic melanoma cells.

Our previous studies using V79 cells in vitro [20] have demonstrated that although the intracellular accumulation of RSU 1069 is independent of pHe, uptake of the methyl-substituted aziridinyl analogues increases with increasing pHe values. This is to be expected, given the pK_a values for these weak bases [21]. The data presented in this report for the uptake of RSU 1069, RSU 1164 and RB 7040 into Lewis lung and HX34 cells are entirely consistent with those of previous studies with V79 cells, since the Ci/Ce ratio increases with increasing pHe for RSU 1164 and RB 7040 but not for RSU 1069.

In contrast, the melanotic melanoma cells have different uptake patterns. B16 melanoma cells concentrate RSU 1069 intracellularly with increasing pHe. In addition, Ci/Ce values for pHe 6.4–8.4 are significantly higher for RSU 1164 and RB 7040 in B16 melanoma than for the amelanotic HX34 melanoma and Lewis lung carcinoma cells. The other melanotic melanoma, HX 118, shows the highest levels of intracellular accumulation of both RSU 1069 and RSU 1164 at pHe 5.4 and 8.4, respectively, the accumulation again increasing at elevated pHe. This suggests that the intracellular pH of melanotic melanomas is lower than that of either the amelanotic melanoma or Lewis lung carcinoma cells and/or that an additional mechanism exists within melanotic melanomas for concentrating these weakly basic compounds.

Other studies have shown that weak bases can concentrate in B16 melanoma and other melanin-containing cells, presumably by binding to melanin [9, 16]. However, in the present studies binding of the nitroimidazoles to melanin or other cellular material would not be observed, since the HPLC assay only measures free intracellular compound.

Uptake into both murine and human tumours has been shown to be increased relative to plasma for the weakly basic radiosensitiser pimonidazole (Ro-03-8799) [7, 8, 13]. Increased accumulation is also seen in some normal tissues [14]. Pimonidazole is structurally similar to and has a pK_a value close to that of RB 7040; both of these agents show Ci/Ce values substantially in excess of 1 for V79 cells held at pHe 7.4 [5, 20]. In addition, RB 7040 shows even greater accumulation in melanotic melanoma cells, and this is consistent with the clinical observation that pimonidazole is taken up by melanomas to a far greater extent than by other tissues or tumours [7].

In conclusion, the high levels of uptake of the weakly basic radiosensitisers into melanotic melanoma in vivo are wholly explicable on the basis of a cell-mediated phenomenon, which may be due to a lower average intracellular pH for the melanotic cells. If so, this may be exploitable in chemotherapy by the design of appropriate, weakly basic cytotoxic drugs for the treatment of melanotic melanomas.

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References

1. Adams GE, Ahmed I, Sheldon PW, Stratford IJ (1984) Radiation sensitization and chemopotentiation: RSU 1069, a compound more efficient than misonidazole in vitro and in vivo. Br J Cancer 49: 571–578
2. Adams GE, Ahmed I, Sheldon PW, Stratford IJ (1984) RSU 1069, a 2-nitroimidazole containing an alkylating group: high efficiency as a radio- and chemosensitizer in vitro and in vivo. Int J Radiat Oncol Biol Phys 10: 1653–1656
3. Ahmed I, Jenkins TC, Walling JM, Stratford IJ, Sheldon PW, Adams GE, Fielden EM (1986) Radiosensitization and toxicity in vitro and in vivo by analogues of RSU 1069. Int J Radiat Oncol Biol Phys 12: 1079–1081
4. Brown JM, Workman P (1980) Partition coefficient as a guide to the development of radiosensitizers which are less toxic than misonidazole. Radiat Res 82: 171–190
5. Deacon JM, Holliday SB, Ahmed I, Jenkins TC (1986) Experimental pharmacokinetics of RSU 1069 and its analogues: high tumour to plasma ratios. Int J Radiat Oncol Biol Phys 12: 1087–1090
6. Dennis MF, Stratford MRL, Wardman P, Watts ME (1985) Cellular uptake of misonidazole and analogues with acidic or basic functions. Int J Radiat Biol 47: 629–643
7. Dische S (1987) Radiotherapy using the hypoxic cell sensitizer Ro 03-8799 in malignant melanoma. Radiother Oncol 10: 111–116
8. Dische S, Saunders MI, Bennett MH, Dunphy EP, Des Rochers L, Stratford MRL, Minchington AI, Wardman P (1986) A comparison of the tumour concentrations obtainable with misonidazole and Ro 03-8799. Br J Radiol 59: 911–917
9. Lindquist NG (1973) Accumulation of drugs on melanin. Acta Radiol [Suppl] 325: 1–92
10. O'Neill P, Jenkins TC, Stratford IJ, Silver ARJ, Ahmed I, McNeil SS, Fielden EM, Adams GE (1987) Mechanism of action of some bioreducible 2-nitroimidazoles: comparison of in vitro cytotoxicity and ability to induce DNA strand breakage. Anticancer Drug Des 1: 271–280
11. Overgaard J, Hansen SH, Jorgensen K, Hjelm M (1986) Primary radiotherapy of larynx and pharynx carcinoma – an analysis of some factors affecting local control and survival. Int J Radiat Oncol Biol Phys 12: 515–521
12. Overgaard J, Hansen SH, Anderson AP, Hjelm-Handsen M, Jorgensen K, Sandberg K, Rygard J, Jensen RH, Petersen M (1987) Misonidazole as an adjuct to radiotherapy in the treatment of invasive carcinoma of the larynx and pharynx. In: Kärcher KH, Kogelnik HD, Szepesi T (eds). Progress in ra-

dio-oncology III. Proceedings of the 3rd International Meeting on Progress in Radio-Oncology, March 1985. ICRO, Vienna, pp 137–147
13. Saunders MI, Dische S, Fermont D, Bishop A, Lennox-Smith I, Allen JG, Malcolm SL (1982) The radiosensitizer Ro-03-8799 and the concentrations which may be achieved in human tumours: a preliminary study. Br J Cancer. 46: 706–710
14. Saunders MI, Anderson PJ, Bennett MH, Dische S, Minchington A, Stratford MRL, Tothill M (1984) The clinical testing of Ro 03-8799: pharmacokinetics, toxicology, tissue and tumour concentrations. Int J Radiat Oncol Biol Phys 10: 1759–1763
15. Steel GG, Adams K (1975) Stem cell survival and tumour control in the Lewis lung carcinoma. Cancer Res 35: 1530–1535
16. Stephens TC, Peacock JH (1982) Clonal variation in the sensitivity of B16 melanoma to mAMSA. Br J Cancer 45: 821–829
17. Stratford IJ, O'Neill P, Sheldon PW, Silver ARJ, Walling JM, Adams GE (1986) RSU 1069, a nitroimidazole containing an aziridine group: bioreduction greatly increases cytotoxicity under hypoxic conditions. Biochem Pharmacol 36: 105–109
18. Stratford IJ, Walling JM, Silver ARJ (1986) The differential cytotoxicity of RSU 1069: cell survival studies indicating interaction with DNA as a possible mode of action. Br J Cancer 53: 339–344
19. Walling JM, Stratford IJ, Adams GE, Silver ARJ, Ahmed I, Jenkins TC, Fielden EM (1986) Studies on the radiosensitizing and cytotoxicity properties of RSU 1069 and its analogues. Int J Radiat Oncol Biol Phys 12: 1083–1086
20. Walling JM, Stratford IJ, Adams GE, Stephens MA (1988) Dual function radiation sensitizers and bioreductive drugs: factors affecting cellular uptake and sensitizing efficiency in analogues of RSU 1069. Int J Radiat Biol 54: 641–649
21. Wardman P (1982) Molecular structure and biological activity of hypoxic cell radiosensitizers and hypoxia specific cytotoxins. In: Breccia A, Rimondi C, Adams GE (eds) Advanced topics on radiosensitizers of hypoxic cells. NATO Advanced Study Institutes Series A43. Plenum, New York, pp 49–76
22. Watts ME, Jones NR (1985) The effect of extracellular pH on radiosensitization by misonidazole and acidic or basic analogues. Int J Radiat Biol 47: 645–653
23. White RAS, Workman P (1980) Pharmacokinetic and tumour-penetration properties of the hypoxic cell radiosensitizer desmethylmisonidazole (Ro 05-9963) in dogs. Br J Cancer 41: 268–276
24. Workman P, Walton MI (1984) Pharmacology of the mixed function radio- and chemosensitizers CB 1954 and RSU 1069. Int J Radiat Oncol Biol Phys 10: 1307–1311

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