Biochemical Pharmacology, Vol. 35, No. 1, pp. 105–109, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00

RSU 1069, A NITROIMIDAZOLE CONTAINING AN AZIRIDINE GROUP

BIOREDUCTION GREATLY INCREASES CYTOTOXICITY UNDER HYPOXIC CONDITIONS

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The red-ox properties of nitroheterocyclic compounds have been established as important determinants of their biological properties [1, 2]. The correlation between one-electron reduction potential and the ability of nitro compounds to act as radiosensitizers of hypoxic cells is central to the development of agents useful as adjuncts in radiotherapy [3, 4]. A lead compound in this series has been misonidazole (1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol), which, although neurotoxic at doses necessary to achieve its maximal effect [5], has been shown to be of benefit in some clinical situations [6].

Drugs of higher therapeutic ratio than misonidazole have been developed but their improved efficacy is based on lower toxicity and/or improved tumour uptake [7, 8]. RSU 1069 (1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol) is a substantially more efficient radiosensitizer than misonidazole both *in vitro* and *in vivo* [9, 10] even though both compounds have similar one-electron red-ox potentials [9].

Structurally, RSU 1069 differs from misonidazole in that an aziridine replaces the methoxy group in the N1 side chain (Fig. 1). Aziridines are monofunctional alkylating agents which can react with cellular macromolecues such as DNA [11]. In order to establish whether the mechanism of the abnormally high sensitizing efficiency of RSU 1069 involves the alkylating properties of the aziridine group, studies have been carried out on the properties of this compound at the molecular, cellular and *in vivo* level. RSU 1069 shows very high toxicity towards hypoxic cells. This paper reviews the available toxicity data on RSU 1069 and examines the possible role of bioreduction in its modes of action.

THE DIFFERENTIAL TOXICITY OF NITRO COMPOUNDS TOWARDS HYPOXIC MAMMALIAN CELLS

Both hypoxic toxicity and radiosensitizing ability depend on electron affinity [12], although the mechanisms are different. Radiation sensitization is a fast free radical process whereas hypoxic toxicity arises from much slower temperature-dependent processes.

RSU 1069 shows abnormally high sensitizing efficiency and is much more cytotoxic than would be predicted on the basis of electron affinity. This toxicity is compared with that of misonidazole in Fig. 2. Under both aerobic and hypoxic conditions, RSU 1069 is more toxic than misonidazole suggesting that factors additional to electron affinity contribute to the mechanism(s) of cytotoxicity of RSU 1069.

For misonidazole, exposure of cells to concentrations of up to 40 mmol/dm^3 for 3 hr in air causes little cell killing, whereas cells exposed to 10 mmol/dm^3 misonidazole under nitrogen show a surviving fraction of less than 10^{-3}. The hypoxic toxicity of misonidazole is due to bioreduction which yields reduced nitro species. These may react with, and deplete, cellular thiols [13] and may also bind to DNA bases [14] and cause single strand breaks [15].

The toxicity of RSU 1069 in air is approximately 10-fold greater on a concentration basis than the toxicity of misonidazole even under nitrogen. Aerobic toxicity may be attributed to the alkylating

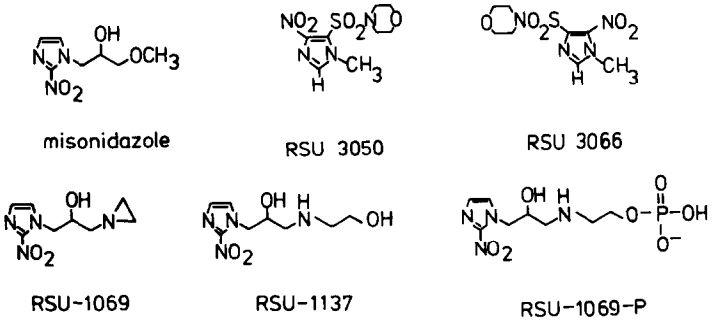

Fig. 1. Structures of misonidazole, RSU 1069, RSU 1137, RSU 1069-P, RSU 3050 and RSU 3066.

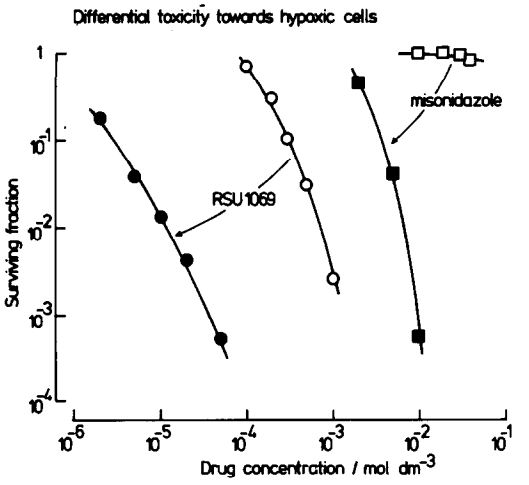

Fig. 2. Toxicity of misonidazole and RSU 1069 towards Chinese hamster V79 cells: open symbols, in air; closed symbols, in nitrogen. Cells in confluent cultures exposed to drugs for 3 hr at 37°: ■, □, misonidazole; ●, ○, RSU 1069.

properties of the aziridine group which, under nitrogen, acts in conjunction with the reduced nitro group to cause even greater cell kill. This differential toxicity is shown in Fig. 2. The importance of bioreduction in the cytoxicity of RSU 1069 and the nature of the co-operation between this and the alkylating action of the drug is discussed.

MOLECULAR STUDIES

In phosphate buffers at pH 7.0 and 37°, RSU 1069 hydrolyses to form the aziridine ring-opened product, RSU 1137 (Fig. 1). RSU 1069 also reacts with inorganic phosphate (P_i) to form the phosphorylated ionic product RSU 1069-P [16] (Fig. 1). The bimolecular rate constant for reaction with P_i, extrapolated to zero ionic strength is 1×10^{-3} dm^3/mol/sec (Table 1) [17]. The phosphorylation of RSU 1069 suggests that cellular nucleophiles may be important in the mode of action of the drug.

Since glutathione (GSH) is one of the most important cellular nucleophiles, the second order rate constant was determined for its reaction with the aziridine group of RSU 1069 at pH 7.5. In Table 1 the reactivity of RSU 1069 is compared with two GSH-reactive nitroimidazoles [18] that do not contain

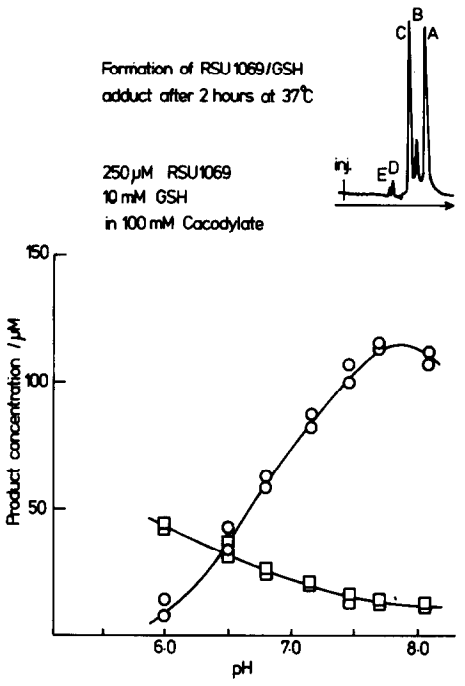

Fig. 3. pH dependence for the hydrolysis of RSU 1069 and its reaction with glutathione: ○, RSU 1069/glutathione adduct; RSU 1137, the aziridine ring opened hydrolysis product of RSU 1069. Inset: HPLC chromatogram of RSU 1069 incubated with glutathione at pH 7.5 (Beckman 344 HPLC system, 5 μm Spherisorb-CN column, 10% methanol in 10 mmol/dm^3 KH_2PO_4, pH 3.0 as mobile phase, at a flow rate of 1 cm^3/min, λ = 310 nm). Peak A = RSU 1069; B = RSU 1137; C = RSU 1069/glutathione adduct; D and E = minor products.

aziridine groups (RSU 3050 and RSU 3066, whose structures are shown in Fig. 1). Figure 3 shows that the extent of interaction of RSU 1069 with GSH rises with increasing pH to a plateau, or possible maximum, at around pH 7.6. Figure 3 also shows that hydrolysis becomes less important, relative to reaction with GSH, at pH values > 6.5.

As the reactivity of the aziridine group of RSU 1069 with GSH may influence the cytotoxicity of RSU 1069, it is pertinent to compare the reaction rate constant of RSU 1069 with GSH with those of other nitroimidazole sensitizers whose mechanism of action is known to involve depletion of cellular thiols [18] (Table 1). RSU 3050 has a higher reactivity with

Table 1. Second order rate constants for reaction of some nitroimidazoles with nucleophiles at 37° pH 7.5

Compound	Nucleophile	k_2 (dm^3/mol/sec)	Activity of GSH transferase (units/mg protein)	Ref.
RSU 3050*	GSH	11	27	[18]
RSU 3066*	GSH	0.83	0.08	[18]
RSU 1069†	GSH	3.9×10^{-3}	No activity detectable	
RSU 1069‡	P_i	4.2×10^{-4}	—	[17]

* In 50 mM phosphate.
† 100 mM sodium cacodylate.
‡ 50 mM phosphate. Extrapolation to zero ionic strength $k_2 = 1 \times 10^{-3}$ dm^3/mol/sec.

GSH than RSU 3066 and is also a more effective substrate for glutathione *S*-transferase. This is reflected by the fact that RSU 3050 has a much greater effect on cellular glutathione content than RSU 3066 [18, 19]. The reactivity of RSU 1069 with GSH is considerably lower than the reactivities for the other two compounds and there is no detectable activity of glutathione-*S*-transferase when RSU 1069 is the substrate. Measurements of total GSH levels in cells after treatment with RSU 1069 show no depletion to have occurred [20]. Suppression of intracellular GSH by RSU 1069 cannot be responsible for the abnormally high cytotoxicity of this drug.

Reaction with DNA has been demonstrated for both parent and radiation reduced RSU 1069, both of which bind to calf thymus DNA. Radiation-reduced RSU 1069 binds to a greater extent and more rapidly than does the parent compound. The binding ratio for reduced drug is approximately 3.5 times that of the parent RSU 1069 over 1 hr. In contrast, unreduced RSU 1137 (the aziridine ring opened hydrolysis product) does not bind to DNA, whereas the radiation reduced compound binds rapidly over the first hour with no further binding occurring thereafter [16]. The binding of parent RSU 1069 in air occurs via the aziridine group, and radiation reduction of the nitro group leads to the formation of a species that is capable of binding more rapidly.

A ^{14}C label in the 2-position of the imidazole is used in this laboratory for binding studies with RSU 1069 and RSU 1137. Therefore any isotopically-labelled bound material must include a C2 fragment. Radiation reduced 2-^{14}C misonidazole does *not* show any binding [16]. However, binding to DNA bases by a glyoxal-like species (the C_4, C_5 fragment) has been reported by others [14] and would not be detected in the present studies using 2-^{14}C labelled drug. No 2-^{14}C binding is observed following radiation reduction of misonidazole and this contrasts with the results for RSU 1137 which suggests the importance of the basic function in the N_1 side chain in the process of binding of [2-^{14}C]-imidazole fragments to DNA.

Both radiation-reduced and parent RSU 1069 induce single strand breaks in plasmid DNA [16]. Incubation of plasmid DNA with reduced RSU 1069 in the presence of either phosphate or deoxyribose 5-phosphate at concentrations greater than 0.35 mol/dm^3 prevents strand breakage, whereas 1.2 mol/dm^3 deoxyribose does not protect against such breakage [16]. From the observed protection against strand breakage by phosphate and deoxyribose-5-phosphate, and the instability of the aziridine group in the presence of inorganic phosphate, it has been proposed that the phosphate of DNA is one potential target for attack by the aziridine of RSU 1069 [16]. Alkylation of phosphate followed by a hydrolytic step can lead to single strand breakage.

CELL SURVIVAL *IN VITRO*

The molecular studies indicate the importance of the aziridine moiety of RSU 1069 in regard to its reactivity with DNA and its cytotoxic properties. This has been supported by several *in vitro* experiments [20]. Firstly, experiments have been carried out with cells labelled with 5-bromodeoxyuridine (5-BUdR) prior to treatment with RSU 1069. Cells with 5-BUdR incorporated into their DNA are much more sensitive to the effects of DNA damaging agents, e.g. radiation [21] or alkylating agents [22]. Secondly, cells were exposed to 3-aminobenzamide (3-AB) during, and after, treatment with RSU 1069. 3-AB can inhibit ADP-ribosyltransferase; this enzyme plays a role in controlling the ligation step of the excision repair of damage to DNA [23] including that caused by many monofunctional alkylating agents. When combined with monofunctional agents, 3-AB potentiates their cytotoxicity [24]. Treatment of cells either with 5-BUdR prior to exposure to RSU 1069 or with 3-AB during and after exposure to RSU 1069, considerably increases the cytotoxicity of the nitroimidazole. This implicates monofunctional alkylation of DNA [20]. Radiation-reduced RSU 1069 reacts with plasmid and calf thymus DNA [16], suggesting that products of anaerobic metabolism of RSU 1069, occurring intracellularly, may be responsible for the additional cytotoxicity of this compound seen under hypoxic conditions. Results of an experiment carried out to test this hypothesis are shown in Fig. 4.

Hypoxic cells were exposed to a range of concentrations of RSU 1069 up to 50 μmol/dm^3 in the presence of 1 mmol/dm^3 misonidazole. This concentration of misonidazole alone is nontoxic under these conditions. The data in Fig. 4 show that misonidazole protects cells from the cytotoxic action of RSU 1069. This protection does not occur under aerobic conditions.

The rationale behind this experiment was as follows. Hypoxic cytotoxicity correlates with one electron reduction potential but, as mentioned, RSU 1069 is anomalous in that despite its similar reduction potential, it is at least 2–3 orders of magnitude more cytotoxic than misonidazole under hypoxic conditions. One way of implicating a metabolically

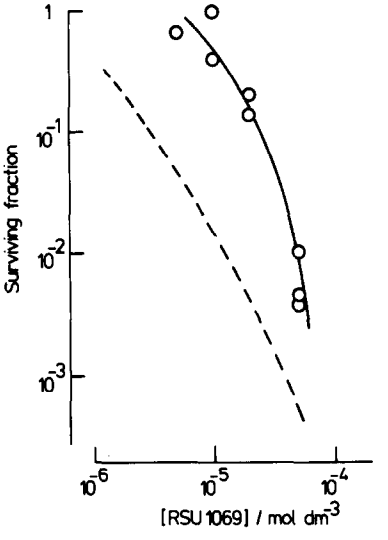

Fig. 4. Protection against the hypoxic toxicity of RSU 1069 by 1 mmol/dm^3 misonidazole. Confluent cultures of Chinese hamster V79 cells exposed to drugs for 3 hr at 37°. The dashed line represents the toxicity of RSU 1069 alone and is transposed from Fig. 2.

reduced product of RSU 1069 in the overall hypoxic cytotoxicity would be to show that inhibition of its metabolic reduction would reduce the toxicity. In the experiment, misonidazole is in great excess. It would be expected therefore that misonidazole could lower the efficiency of reaction of RSU 1069 with cellular reducing equivalents thereby leading to some protection against its cytotoxic effect. The observed partial protective effect of misonidazole shown in Fig. 4 is consistent with this proposal.

BIOFUNCTIONAL CHARACTER OF RSU 1069

It has been shown [20] that 3-AB does not potentiate the cytotoxicity of RSU 1069 under hypoxic conditions but does so under aerobic conditions. 3-AB increases the cytotoxicity of monofunctional alkylating agents [25, 26] but has a minimal effect on the cytotoxicity of bifunctional compounds except at supra-lethal doses [27, 28]. Therefore RSU 1069 may have bifunctional character due to the presence of the aziridine group and the nitro group which is reduced in hypoxia. Evidence in support is provided from studies using some DNA repair deficient mutants of CHO cells (Whitmore and Gulyas, in preparation). These include UV20 cells which are highly sensitive to mitomycin C and other bifunctional agents. After treatment with RSU 1069 in air, the UV20 cells are about five times more sensitive, on a drug concentration basis, than the parent CHO line. In contrast, the sensitivity of UV20 cells in nitrogen increases 100-fold relative to that of the parent cells.

Data in Fig. 5 provide further support for the hypothesis that RSU 1069 is bifunctional in character. In these experiments cells were exposed to various concentrations of RSU 1069 in air for 1 hr, then washed free of RSU 1069 and either plated for colony formation or rendered hypoxic for 3 hr at 37° in full growth medium prior to plating. Clearly, the cells given the post-incubation treatment in nitrogen are much more sensitive than those not receiving this treatment (Fig. 5). One interpretation of these findings is that in air, RSU 1069 binds to cellular DNA components via reaction of the aziridine group. Subsequently, incubation in nitrogen allows reduction of the nitro group to take place thereby producing a second binding function *in situ*. Thus the increased cytotoxicity observed on post-incubation in nitrogen would result from two processes, i.e. reduction of the nitro group following alkylation via the aziridine group. Cooperation between these two mechanisms would be a plausible explanation of the high cytotoxic efficiency of RSU 1069 under hypoxic conditions provided a mechanism exists whereby reduction of the nitro group of the drug bound to DNA can take place.

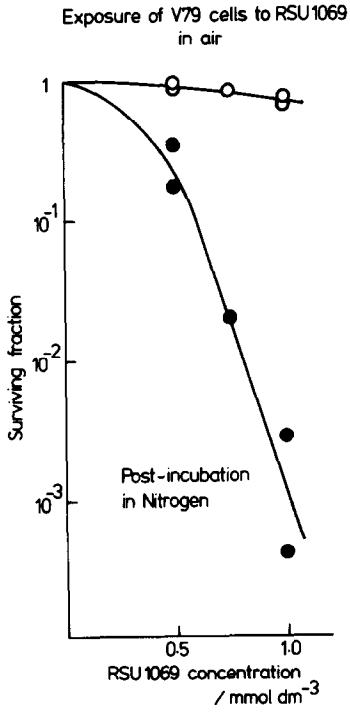

Fig. 5. Exposure of confluent cultures of V79 cells to various concentrations of RSU 1069 for 1 hr at 37° in air (open symbols). The closed circles are survival data for cells exposed to RSU 1069 for 1 hr, washed twice with phosphate buffered saline then, with fresh medium added, incubated for a further 3 hr in nitrogen.

TUMOUR CELL SURVIVAL *IN VIVO*

Figure 6 shows the interaction between radiation and RSU 1069 in the anaplastic MT tumour in WHT/Cbi mice treated *in situ* and assayed *in vitro*. RSU 1069 administered to tumour-bearing mice at various times before irradiation results in substantial radiosensitization. The maximum increase in cell killing that is achieved when the compound is administered 10–60 min before irradiation is equivalent to an enhancement ratio of 1.9 (0.38 mmols/kg^{-1}) [9]. Another interesting feature of the data is that some sensitization is still observed when drug administration and irradiation are separated by an interval of up to 3 hr (the half life of RSU 1069 in mice

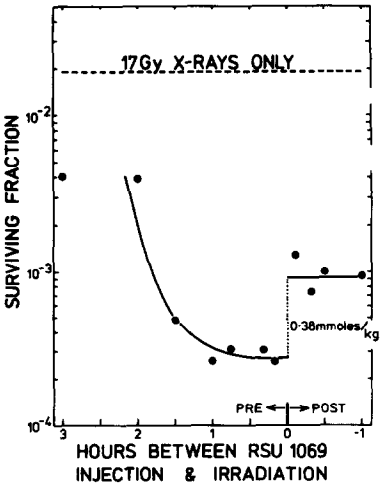

Fig. 6. Effect of RSU 1069 in combination with 17 Gy X-rays on the survival of MT tumour cells irradiated *in vivo* and assayed *in vitro*. Dependence of survival on time between drug administration and irradiation.

is only about 30 min ([29], Deacon and Holliday, unpublished data). When mice are given 0.38 mmol/kg RSU 1069 in the absence of radiation, tumour cell survival is reduced by only about 50%. This is unlikely to account for the sensitization observed at 2–3 hrs. RSU 1069 administered up to 1 hr post-irradiation still results in substantial cell killing of the radiation resistant hypoxic cells. This could be a manifestation of the very high hypoxic toxicity of RSU 1069 demonstrated *in vitro* [20].

CONCLUSIONS

Evidence is provided for the likely mechanisms operating in the cytotoxic actions of RSU 1069 under aerobic and hypoxic conditions. In air, RSU 1069 appears to act as a monofunctional alkylating agent, with the potential to act in a bifunctional manner under hypoxic reducing conditions. *In vivo* RSU 1069 can act as a conventional electron affinic sensitizer but, in addition, appears to cause further killing of hypoxic cells in irradiated tumours as a result of direct cytotoxicity.

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