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INDUCTION OF MUTATIONS IN V79-4 MAMMALIAN CELLS UNDER HYPOXIC AND AEROBIC CONDITIONS BY THE CYTOTOXIC 2-NITROIMIDAZOLE-AZIRIDINES, RSU-1069 AND RSU-1131

THE INFLUENCE OF CELLULAR GLUTATHIONE

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Abstract—Incubation of the 2-nitroimidazole-aziridine, RSU-1069 [1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol], and its monomethylaziridine analogue, RSU-1131 [1-(2-nitro-1-imidazolyl)-3-(1-(2-methylaziridinyl))-2-propanol], with V79-4 mammalian cells for 2 hr under aerobic or hypoxic conditions induces mutations as measured at the hypoxanthine phosphoribosyl transferase locus. The ability of these agents to induce mutations is increased by a factor of 12–14 under hypoxic conditions. The increased cytotoxicity of these agents under hypoxic conditions was confirmed following a 2 hr incubation period. Decreasing the glutathione (GSH) content of the cells with buthionine-(*S,R*)-sulphoximine to <1% of the control generally results in an increase in the cytotoxicity and mutagenicity of these agents under both aerobic and hypoxic conditions. Since these agents do not modify the cellular GSH levels, it is inferred that the thiols partially detoxify through removal of a reactive metabolite of the agents, under hypoxic conditions, or removal of known DNA adducts, and not through their interaction with the agents themselves. Under aerobic conditions, the formation of mutations is consistent with the established monofunctional action of these agents whereas under hypoxic conditions the bifunctional action predominates for mutation induction, based upon the large differential aerobic:hypoxic effect. From a comparison of the number of mutations per lethal event, the effect of thiol depletion is more pronounced for cytotoxicity than for mutation induction by these agents. In summary, these agents are considered to be weak mutagens towards V79-4 cells under aerobic conditions when compared with other DNA alkylating agents, although they are more potent under anoxic conditions.

The compound RSU-1069 [1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol‡] is a more effective hypoxic cell radiosensitizer than misonidazole [1–3]. RSU-1069 has also been developed as a bioreductive agent whereby it is more cytotoxic towards hypoxic than aerobic cells on a concentration basis by about two orders of magnitude [3–5]. Potentiation of tumour cytotoxicity *in vivo* [5–8] has been demonstrated to be more effective with RSU-1069 than with misonidazole. Progressive methylation of the aziridine moiety of RSU-1069 decreases the differential hypoxic–aerobic cytotoxicity and reduces its ability to act as an alkylating agent and therefore bioreductive agent [9, 10]. Similarly, this progressive methylation reduces the extent of unscheduled DNA synthesis [11]. RSU-1069 and its alkyl-substituted derivatives are able to cause cell transformation using C3H $10T_{\frac{1}{2}}$ cells [12, 13]. Recently, the haloethylamino-compound, RB-6145, has been developed as a less toxic pro-drug for RSU-1069 [14, 15]. Both cellular and molecular studies indicate that RSU-1069 and its alkyl-substituted derivatives act as monofunctional alkylating agents under aerobic conditions whereas upon bioreduction they are converted into bifunctional agents [3, 4, 9, 16–20]. Studies *in vitro* with plasmid DNA [9, 16–18] have shown that RSU-1069 and its monomethyl-substituted aziridine analogue, RSU-1131 [1-(2-nitro-1-imidazolyl)-3-(1-2-methylaziridinyl))-2-propanol], induce a variety of DNA–base adducts, strand breaks and, under reductive conditions only, crosslinks [19, 20]. The efficiency of induction of DNA damage is reduced upon progressive methylation of the aziridine moiety of RSU-1069 [9, 12, 21]. Both RSU-1069 and RSU-1131 have been shown to induce single and double strand breaks (ssb and dsb) and adducts in cellular DNA following their incubation with V79-4 cells under both aerobic and hypoxic conditions [22–24]. Several of the DNA adducts are converted into ssb under conditions of high alkalinity [16, 23, 25]. The yield of ssb and especially dsb is significantly increased under hypoxia. In fact the induction of dsb by RSU-1069

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‡ Abbreviations: BSO, buthionine-(*S,R*)-sulphoximine; RSU-1069, 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol; GSH, glutathione; RSU-1131, 1-(2-nitro-1-imidazolyl)-3-(1-(2-methylaziridinyl))-2-propanol; ssb, single strand breaks; dsb, double strand breaks.

under hypoxic conditions is thought to be related to its increased cytotoxicity under hypoxia. The cellular DNA strand breaks induced by these agents under either aerobic or hypoxic conditions are not rejoined within 3 hr following drug treatment [24, 26] in contrast to radiation-induced DNA strand breaks. Although RSU-1069 has been shown to interact slowly with glutathione (GSH) [3], an important cellular nucleophile, it does not significantly modify the GSH levels within V79 cells [4]. The induction of dsb and crosslinks probably plays a significant role in determining the ability of these agents to act as hypoxic-selective cytotoxins [26].

The present study was undertaken to determine the hypoxic and aerobic cytotoxicity of RSU-1069 and RSU-1131 with V79-4 cells under conditions whereby the cellular GSH levels have been significantly decreased using buthionine-(*S,R*)-sulphoximine (BSO) [4]. Since these nitro-imidazole aziridines are thought to act through their ability to induce DNA damage, the mutation frequency of these agents has been determined in the presence and absence of BSO. From these studies, the potential role of GSH in modifying the various biological end points will be assessed with a view to extending our understanding of the mode of action of these bioreductive agents.

MATERIALS AND METHODS

Chemicals. The synthesis, purification and physicochemical properties of RSU-1069 and RSU-1131 have been reported [1, 9]. Misonidazole was supplied by Dr C. E. Smithen. BSO was obtained from the Sigma Chemical Co. (Poole, U.K.) and all other chemicals were of AnalaR grade and used as supplied. All compounds were dissolved in complete medium to give the desired final concentration in contact with the cells. The solutions were freshly prepared for each experiment due to the known instability of the agents [14].

Culture conditions. Chinese hamster V79-4 cells were grown as a monolayer in Eagle's minimal essential medium supplemented with 2 mmol/dm^3 glutamine, 5 U/dm^3 penicillin, 0.005 g/dm^3 streptomycin and 10% foetal calf serum. Under these conditions the cells have a doubling time of 12 hr and a plating efficiency of about 80–90%.

Twenty four hours prior to treatment, 2×10^6 cells were plated in 90 cm^2 glass culture bottle and grown as a monolayer at 310°K. When required, 18 hr before treatment the culture medium was removed and BSO, dissolved in complete medium at a concentration of 1 mmol/dm^3, was added to the cells in culture.

Treatment conditions. After incubation for 24 hr, the cells were in logarithmic phase of growth. The medium was then removed and the required concentration of the agent, dissolved in 15 mL of fresh medium, was added to the cell monolayer. The cells were then incubated with the agent for 2 hr at 310°K. When anoxia was required, the cell monolayer was equilibrated with $N_2 + 5\%$ CO_2 for 20 min at 293°K prior to incubation with the drug in anoxia at 310°K. Immediately after treatment, the medium was removed and the cell monolayer washed twice with fresh medium, trypsinized, resuspended, counted and processed for both survival and mutations as described below.

Cell survival. The cell suspension was diluted with fresh medium and the cells plated at the appropriate concentration. After incubation for 7 days at 310°K, visible colonies were scored for survival. All experiments were performed using five different concentrations of the agent together with one control treated in the same way but in the absence of the agent. Five petri dishes, 6 cm diameter, were plated for each given concentration.

Cell mutation. Part of the cell suspension was used for mutation at the hypoxanthine phosphoribosyl transferase locus, using 6-thioguanine as the selective agent. For each concentration used, the cells were sub-cultured every 2 days in four 15 cm diameter petri dishes at a density of 1.5×10^6 cells/dish. After 8 days, for each drug concentration, five dishes (15 cm diameter) were seeded with 1×10^6 cells/dish in 20 mL of medium containing 5.0 μg/mL 6-thioguanine. The corresponding plating efficiency was simultaneously determined to evaluate the mutation frequency in terms of number of induced mutants per viable cell. The background frequency for the untreated cells was $(1.1 \pm 0.6) \times 10^{-5}$ mutants per viable cell.

GSH and protein determination. Cellular content of GSH was measured using the method proposed by Tietze [27] and the protein content was determined by the Lowry method [28].

RESULTS

Cytotoxicity of RSU-1069 and RSU-1131 in thiol depleted V79-4 cells

The cytotoxicity of RSU-1069 and RSU-1131, determined upon their incubation with V79-4 cells for 2 hr under either hypoxic or aerobic conditions at 310°K, is shown in Table 1, expressed in terms of the concentration of the agent required to reduce survival to 10%. The differential aerobic:hypoxic cytotoxicity of these agents (Table 1) and the greater toxicity of RSU-1069 compared with that of RSU-1131 is consistent with the previously reported differentials determined for a 3 hr incubation period [3]. The cytotoxicity of both RSU-1069 and RSU-1131 under both aerobic and hypoxic conditions is increased, on a concentration basis, as shown in Fig. 1 if the cells are pretreated with BSO, which reduces the level of non-protein sulphydryls and, in particular, GSH. Under aerobic conditions, the cytotoxicity of RSU-1131 is similar to that of RSU-1069 for BSO-treated cells. The greatest effect of BSO treatment is an ~38 fold increase in the cytotoxicity of RSU-1131 under hypoxia compared with that for untreated cells as shown in Table 2. Generally, the effect of BSO treatment is to increase the cytotoxicity of these agents by a factor of 3–6 as shown in Table 3. The level of GSH is reduced from 4–6 ng/mg protein to <0.02 ng/mg protein following BSO treatment. These values are consistent with those reported previously for V79-4 cells [29, 30]. As shown previously [4] RSU-1069 does not modify the cellular levels of GSH upon incubation with V79-4 cells for 2 hr under either aerobic or anoxic conditions. Under

Table 1. The effect of BSO upon the cytotoxicity of RSU-1069 and RSU-1131 after a 2 hr incubation with V79-4 mammalian cells under hypoxic and aerobic conditions

Compound	Cytotoxicity* (mmol/dm^3) Aerobic	Hypoxic	Differential
RSU-1069	1.3	0.026	50
RSU-1069 + BSO	0.45	0.005	90
RSU-1131	2.5	0.45	5.6
RSU-1131 + BSO	0.45	0.012	37.5

* Concentration of agent to reduce survival to 10%.

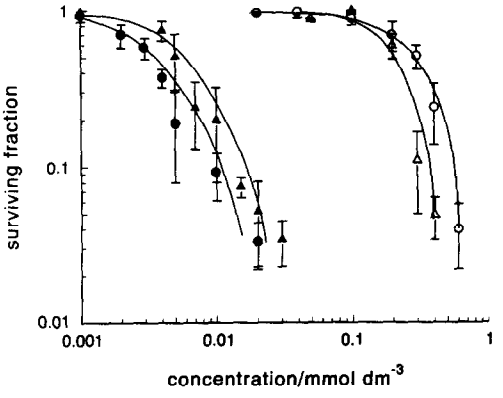

Fig. 1. The concentration dependence for *in vitro* cytotoxicity of RSU-1069 (●, ○) and RSU-1131 (▲, △) towards BSO-treated V79-4 cells following a 2 hr incubation at 310°K under hypoxic (closed symbols) and aerobic (open symbols) conditions.

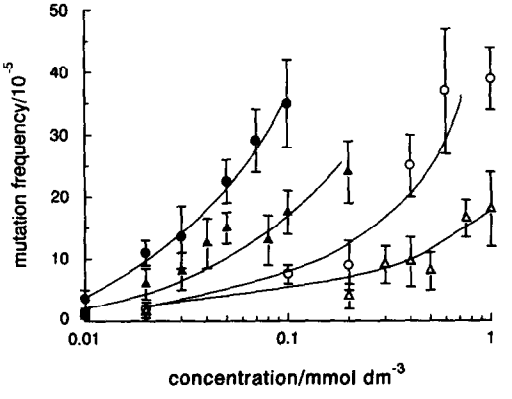

Fig. 2. The concentration dependence for induction of mutations in V79-4 cells by RSU-1069 (●, ○) and RSU-1131 (▲, △) following 2 hr incubation at 310°K under hypoxic (closed symbols) and aerobic (open symbols) conditions.

Table 2. The relative effect on cytotoxicity of and mutation frequency yielded by RSU-1069 and RSU-1131 for thiol-depleted compared with normal V79-4 cells

Compound	Cytotoxicity Aerobic	Hypoxic	Mutation frequency Aerobic	Hypoxic
RSU-1069	2.9	5.2	0.8	3.3
RSU-1131	5.6	37.5	2.3	8.3

both conditions investigated, the agents become more cytotoxic upon cellular thiol depletion with the greater effect being observed under hypoxic conditions. A conventional 2-nitroimidazole, misonidazole, is non-toxic in the concentration range used in these studies for RSU-1069 and RSU-1131. With BSO-treated cells, a slight increase in toxicity (~20%) was observed at the highest concentrations of misonidazole used of 3 mmol/dm^3. Previous studies, using higher concentrations of misonidazole, have shown that it is more cytotoxic with thiol-depleted cells [31].

Induction of mutations in V79-4 cells by RSU-1069 and RSU-1131

The dependency of the mutation frequency per survivor on the concentration of RSU-1069 and RSU-1131 is shown in Fig. 2 for a 2 hr incubation with V79-4 cells at 310°K under either aerobic or hypoxic conditions. The mutation frequency increases with increasing concentration of the agents. With both agents, the mutation frequency is greater under hypoxic conditions as shown in Fig. 2 and Table 3. The differential aerobic to hypoxic effect of RSU-1069 for mutations is less than that determined for cytotoxicity, in contrast to the observations with RSU-1131, where the differential aerobic–hypoxic effect for mutation induction is larger (Table 3). Pretreatment of the cells with BSO results in an increase in the mutation frequency of these agents under both gassing conditions over that of the untreated cells as shown in Fig. 3. The greatest effect of BSO treatment on the induction of mutations was observed for RSU-1131 under hypoxic conditions (Table 2) although the effect is less dramatic than that observed for cytotoxicity. Up to a concentration of 3 mmol/dm^3, misonidazole does not induce significant levels of mutation above the background [$(1.1 \pm 0.6) \times 10^{-5}$ mutations/survivor]

Table 3. The effect of BSO upon the mutation frequency yielded by RSU-1069 and RSU-1131 after a 2 hr incubation with V79-4 mammalian cells under hypoxic and aerobic condition

Compound	Mutation frequency* ($mmol/dm^3$) Aerobic	Hypoxic	Differential Aerobic–Hypoxic
RSU-1069	0.25	0.02	12.5
RSU-1069 + BSO	0.3	0.006	50.0
RSU-1131	0.7	0.05	14.0
RSU-1131 + BSO	0.3	0.006	50

* Concentration of agent to yield a mutation frequency of 10^{-4} per survivor.

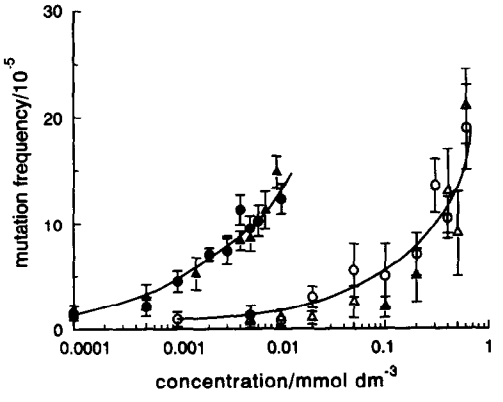

Fig. 3. The concentration dependence for induction of mutations in BSO-treated V79-4 cells by RSU-1069 (●, ○) and RSU-1131 (▲, △) following a 2 hr incubation period at 310°K under hypoxic (closed symbols) and aerobic (open symbols) conditions.

for BSO-treated or untreated V79-4 cells under aerobic or hypoxic conditions.

DISCUSSION

RSU-1069 and RSU-1131 induce mutations in V79-4 cells under both aerobic and hypoxic conditions, whereas a conventional 2-nitroimidazole, misonidazole, does not induce mutations up to a concentration of 3 $mmol/dm^3$. RSU-1069 and RSU-1131 are more mutagenic under hypoxic conditions and are also more effective cytotoxins under hypoxia as shown previously [3–5]. Under both aerobic and hypoxic conditions, the cytotoxic effect of these agents is increased for BSO-treated cells. With the exception of RSU-1069 under aerobic conditions, BSO treatment also increases the mutation frequency of these agents. This treatment does not significantly affect the ability of misonidazole to induce mutations up to 3 $mmol/dm^3$. With RSU-1069, the mutation frequency is almost independent of the thiol status of the cell. BSO treatment of the cells increases to a greater extent the cytotoxicity and mutagenicity of RSU-1131 (see Table 2) compared with those of RSU-1069 under all conditions. The relative effects of RSU-1069 compared with RSU-1131 are shown

Table 4. The relative effects of RSU-1069 compared with RSU-1131 using different end points

Effect	RSU-1069/RSU-1131 Aerobic	Hypoxic
Cytotoxicity	1.9	17.3
Cytotoxicity + BSO	1.0	2.4
Mutations	2.8	2.5
Mutations + BSO	1.0	1.0
Single strand breaks*	3.2	2.4
Double strand breaks*	1.6	5.1
Crosslinks†	—	~15–20

* [20], † [18].

in Table 4 for a variety of different end points. From a comparison of the cytotoxicity and mutation data in Table 4, the greater effectiveness of RSU-1069 compared to RSU-1131 is reduced upon cellular thiol depletion so that the two agents have similar potencies for BSO-treated cells. The only exception relates to the cytotoxicity of RSU-1069 under hypoxic conditions whereby the effectiveness of RSU-1069 is twice that of RSU-1131, although greatly reduced compared to the corresponding value for normal cells.

From these observations on thiol-depleted cells, it is inferred that thiols are involved in the expression of both the cytotoxicity and mutability of these agents. Previous studies [4] and those reported here have shown that RSU-1069 up to 1 $mmol/dm^3$ does not result in an overall depletion of the cellular levels of thiols even though RSU-1069 is known to interact with GSH [3]. Due to the large excess of cytotoxic agent extracellularly, the levels of GSH would be expected to be reduced if the detoxification involved formation of GSH conjugates with these agents. Therefore, the increased effectiveness of these agents in BSO-treated cells is thought not to reflect the reduced ability of the cell to remove the agents through their interaction with thiols. Reduced thiol levels would lead to an increase in the effectiveness of the agents to interact with the DNA. If the agents interact significantly with GSH, it would be fortuitous if the levels of cellular GSH were unaffected [4] due to a corresponding stimulation of GSH production to compensate for losses through drug removal. Based upon the rate constant for

interaction of RSU-1069 with GSH [3], the time required to halve the thiol concentration is estimated to be ~50 hr for 1 mmol/dm^3 RSU-1069. Therefore, insignificant reduction of the thiol levels is estimated through non-enzymatic reactions with the agent for a 2 hr incubation period. However, involvement of GSH-requiring enzymes, such as GSH transferase, may enhance the removal of GSH through its conjugation with RSU-1069, although one *iso* enzyme of GSH transferase has been shown [3] not to catalyse this interaction.

Alternatively, it is suggested that GSH acts as a detoxifying agent under aerobic conditions through removal of some of the drug–DNA adducts. This detoxification may also be catalysed by GSH-dependent enzymes. The cellular studies on DNA damage have indicated formation of DNA adducts with these agents and that several of these adducts are converted into strand breaks under alkaline conditions [22–24]. Previous studies, both molecular and cellular, have shown that these agents act as monofunctional alkylating agents which are converted to bifunctional agents upon bioreduction [3, 4, 9, 16–20]. Under aerobic conditions, it is suggested therefore that mutations result from monofunctional action of the aziridine moiety. Indeed, the relative effect for induction of mutations of 2.8 under aerobic conditions by RSU-1069 and RSU-1131, as shown in Table 4, is consistent with those of the other end points indicative of monofunctional action of these agents. Further, the reactivity of the aziridine moiety of RSU-1069 with the nucleobases has been shown to be about twice that of the methyl-substituted aziridine of RSU-1131 [18]. It is therefore suggested that GSH interacts with monofunctional adducts produced upon interaction of the aziridine moiety with DNA [17, 18, 25, 26]. These reactions may involve GSH-dependent enzymes such as GSH transferase.

Under hypoxic conditions, the increased effects of these agents observed for thiol-depleted cells is suggested to be related to their greater effectiveness as bioreductive agents since the differential aerobic–hypoxic effects are increased upon BSO treatment. Therefore, the bifunctional action of these agents becomes more important. If only the monofunctional action of these agents is modified by thiols, the monofunctional effect should increase relative to that of the bifunctional effect under hypoxia for BSO-treated cells. However, this does not appear to be the case. The role of thiols could involve (i) interaction with the reduced metabolite(s) and in particular the nitro-reduced product, and (ii) removal of DNA adducts. One of the products of bioreduction of nitroimidazoles is thought to be the corresponding nitrosoimidazole which is known to be cytotoxic even at μmol/dm^3 concentrations, interacts with GSH and significantly depletes the intracellular concentration of GSH [32–34]. The properties of the nitrosoimidazole are consistent with the suggested detoxification by GSH involving its interaction with a reduced nitro-metabolite formed upon bioreduction of RSU-1069 or RSU-1131. From the similarities in their redox properties [9], the rate of reduction of these agents is expected to be similar so that the steady-state concentration of reduced metabolites should be equivalent. It is therefore suggested that the enhanced effects on both cytotoxicity and mutagenicity observed with RSU-1131 for BSO-treated cells under hypoxia reflects an increase in its ability to produce DNA damage such as crosslinks [19] and dsb [22], reflecting its bifunctional action. Therefore, by decreasing the ability of the cells to remove the reduced metabolites of the agents and/or DNA adducts through depletion of GSH, the effectiveness, particularly of RSU-1131, to induce bifunctional damage is increased under hypoxia.

Table 5. The effect of BSO upon the yield of mutations per lethal event induced by RSU-1069 and RSU-1131 in V79-4 cells

Compound	Number of mutations/lethal event* Aerobic	Hypoxic
RSU-1069	30×10^{-5}	7.5×10^{-5}
RSU-1069 + BSO	10×10^{-5}	7.5×10^{-5}
RSU-1131	27×10^{-5}	23×10^{-5}
RSU-1131 + BSO	10×10^{-5}	11×10^{-5}

* For X-rays under aerobic conditions, 8.0×10^{-5}.

Even under hypoxic conditions, the level of thiols is not significantly modified by these agents consistent with the ability of thiols to remove reactive metabolites or DNA adduct. As the concentration of DNA adducts or cytotoxic metabolites is low compared to the concentration of thiols, their removal by thiols would not lead to a detectable change in the cellular levels of thiols. Therefore, it is concluded that the role of thiols is to detoxify through removal of RSU-1069 and RSU-1131 adducts and/or reactive metabolites under both aerobic and hypoxic conditions. The mutations under aerobic conditions reflect the alkylating effect of the aziridine moiety of these agents [9, 21]. Upon bioreduction, the bifunctional action of these agents is emphasized for BSO-treated cells and, as seen in Table 4, the effectiveness of RSU-1069 and RSU-1131 is similar for thiol-depleted cells.

The effects of these agents expressed as number of mutations per lethal event are shown in Table 5. It has been assumed that on average one lethal event corresponds to a concentration of the agent required to reduce survival to 37%. The number of mutations per lethal event is significantly greater with RSU-1131 under both aerobic and hypoxic conditions, and RSU-1069 under aerobic conditions, for cells untreated with BSO than for cells treated with BSO. In all cases, BSO treatment reduces the number of mutations per lethal event to a value similar to that for X-rays only. Treatment with BSO, therefore, increases the toxic effect of these agents relative to their ability to induce mutations. Since X-rays are generally considered to be weak mutagens; these agents with BSO-treated cells and RSU-1069 under hypoxia may also be considered to be weak mutagens. Even for cells untreated with BSO the number of mutations per lethal event is significantly less than with other alkylating agents [35] based upon the same mutation assay. From transformation studies

with RSU-1069 [12, 13], the effectiveness of RSU-1069 to induce transformants in C3H $10T_{\frac{1}{2}}$ cells at an equivalent toxic level is similar to that for ionizing radiation and is approximately equivalent to the value of 30×10^{-5} mutants/lethal event in air as shown in Table 5.

It is concluded that both RSU-1069 and RSU-1131 induce mutations in V79-4 cells and are cytotoxic under both aerobic and hypoxic conditions. These effects are dependent upon the thiol status of the cell. The role of thiols involves detoxification through removal of drug–DNA adducts and, under hypoxia, the removal of reactive metabolites. From a comparison with other alkylating agents, RSU-1069 and RSU-1131 are weaker mutagens towards V79-4 cells under aerobic conditions. The weak mutagenicity of RSU-1069 especially under aerobic conditions is considered favourable for its development, through the pro-drug RB-6145, as a bioreductive agent of potential clinical use.

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