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EFFECTS OF SUBSTITUTED 2-NITROIMIDAZOLES AND RELATED COMPOUNDS ON UNSCHEDULED DNA SYNTHESIS IN RAT HEPATOCYTES AND IN NON-TRANSFORMED (BL8) AND TRANSFORMED (JB1) RAT LIVER EPITHELIAL DERIVED CELL LINES

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Abstract—1. Using unscheduled DNA synthesis as an index, the possible interaction of a number of substituted nitroimidazoles, e.g. misonidazole, with cellular DNA has been investigated. Transformed (JB1), non-transformed (BL8) rat liver epithelial derived cell lines and freshly prepared rat hepatocytes have been used.

2. Under anaerobic or aerobic conditions, relative to cells exposed to a nitroquinoline-*N*-oxide standard, misonidazole and related nitroimidazoles were very poor at stimulating unscheduled DNA synthesis in JB1 or BL8 cells or in hepatocytes, even at the highest concentrations tested (10 mM). Under anaerobic conditions, metabolic activation did occur as judged from the time-dependent depletion of cellular reduced glutathione in all three cell types.

3. It was concluded that in hypoxic cells an important mode of action of such nitroimidazoles as chemotherapeutic sensitisers may be by their interaction with cellular thiols rather from their interaction with DNA.

4. Functionalisation of the nitroimidazole ring with a side chain containing an aziridine function, e.g. RSU-1069 (1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol), results in the induction of unscheduled DNA synthesis in cells exposed under both aerobic and anaerobic conditions. On a molar basis, however, this induction was not so great as that caused by the simple monofunctional alkylating agent 1-aziridineethanol itself. Methyl-substitution of the aziridine ring in RSU-1069 reduced the extent of unscheduled DNA synthesis.

5. With all the compounds tested, unscheduled DNA synthesis was greater in JB1 cells than in BL8s or in hepatocytes.

During the past decade, 2-nitroimidazoles such as misonidazole (Fig. 1, **VIII**) have been used as radiosensitisers [1, 2] and more recently as chemopotentiators in the treatment of human cancers (reviewed in refs 3, 4). *In vitro*, such compounds are more effective to cells cultured under hypoxic rather than aerobic conditions [5–7]. Activation through reduction of the nitro-function appears to be involved in the hypoxic cell cytotoxicity of nitro compounds although the precise mechanism of action is not known. There is no known direct involvement of bioreduction in radiosensitisation reactions. Cytotoxicity of misonidazole is associated with a depletion of cellular glutathione [8, 9]. Misonidazole also causes DNA damage to mammalian cells under hypoxic conditions [10, 11]. The extent of DNA stand breakage has been directly correlated with the reduction potential of these compounds [12].

More recently, new ranges of 2-nitroimidazoles containing the aziridine function such as RSU-1069, (1 - 2 - (nitro - 1 - imidazolyl) - 3 - (1 - aziridinyl) - 2 - propanol) (Fig. 1, **I**) have been synthesized with the intention of producing bifunctional alkylating agents [7]. Such aziridine substituted nitroimidazoles will alkylate calf thymus and plasmid DNA under aerobic condition *in vitro* without the need for metabolic activation [13]. However, the very high toxicity of RSU-1069, relative to misonidazole, under anaerobic conditions has been attributed to bifunctional alkylating action [13, 14].

The object of this study was to compare the possible genotoxicity of a number of nitro-compounds in relation to their potential use as chemotherapeutic agents. Unscheduled DNA synthesis (UDS) was used as the index for genotoxicity. Effects on reduced glutathione concentrations were used as a parameter for the assessment of the metabolic activation of the nitro-function. Freshly prepared hepatocytes and two different cell lines were used in this investigation. Hepatocytes which are rich in certain potential drug activating enzymes, e.g. cytochrome P-450, and may give a more realistic picture of the situation *in vivo*, were compared with two different cell lines poor in cytochrome P-450, one derived from a hepatoma (JB1), the other from normal liver cells (BL8) [15].

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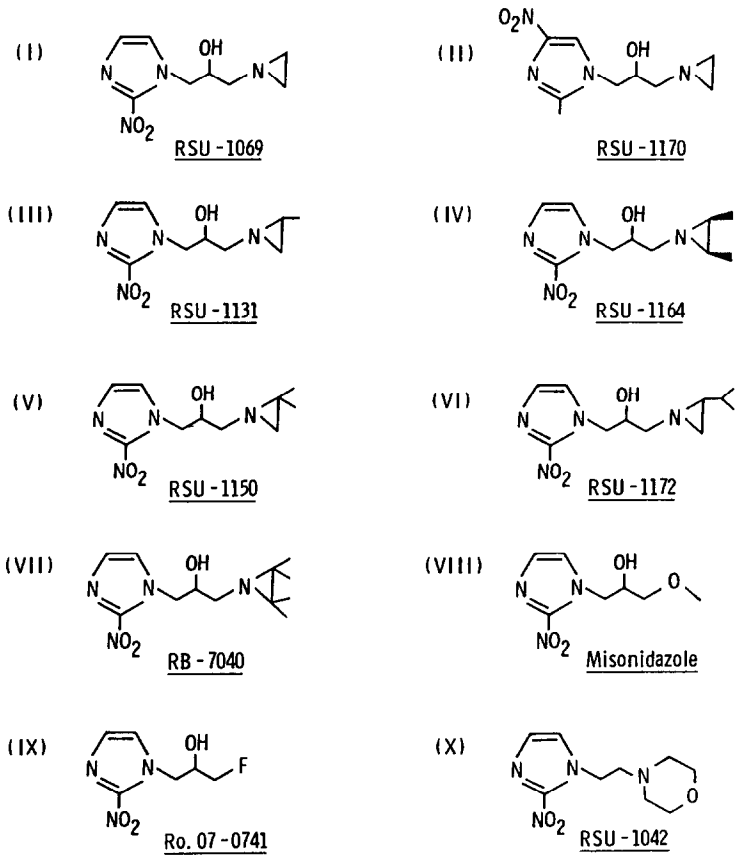

Fig. 1. Chemical structures of nitroimidazoles.

MATERIALS AND METHODS

Animals

Male Fischer F344/N rats weighing 180–220 g were used.

Chemicals

Misonidazole, Ro. 07-0582 and Ro. 07-0741 were a kind gift from Dr C. E. Smithen, Roche Products, Welwyn Garden City, U.K. The RSU-prefixed compounds were synthesised using previously reported methods [16–18]. Other nitro-compounds were from Sigma Chemical Co. [Methyl-^{3}H] thymidine, specific activity 40–52 Ci/mmol was from Amersham International (Amersham, Bucks., U.K.).

Cell lines

Hepatocytes were prepared by collagenase perfusion of rat liver according to the method of Paine and Legg [19]. Cells of viability > 80% as assessed by Trypan blue exclusion test were used. JB1 and BL8 cell lines were obtained from Dr G. E. Neal of the MRC Toxicology Unit.

Unscheduled DNA synthesis (UDS)

(a) *Anaerobic studies.* For UDS studies under anaerobic conditions, freshly prepared hepatocytes were initially suspended in complete Leibovitz L_{15} medium (Leibovitz L_{15} supplemented with 5% v/v foetal calf serum, 1% w/v L-glutamine and 50 μg/ml gentamycin) and left for 2 hr in 15 cm diameter glass or plastic Petri dishes (10^7 cells/dish in 20 ml medium) at 37° in air. BL8 or JB1 cell lines were used 3 days after subculture when the cells were confluent. The old medium was replaced with 20 ml of complete Leibovitz L_{15} and left at 37° in air for 2 hr. At the end of 2 hr, the medium was replaced with fresh Leibovitz (20 ml) containing 10 mM hydroxyurea. Hydroxyurea was included in order to inhibit replicative DNA synthesis [20]. One hour later, the plates were transferred to a chamber gassed with oxygen free nitrogen (British Oxygen Ltd., Brentford, Middlesex) by means of a continuous flow throughout the incubation period. An oxygen electrode was used to monitor oxygen concentration in the chamber. After achieving anoxic conditions, the test compound dissolved in Leibovitz medium (1 ml) was added followed by 15 μCi ^{3}H-thymidine. Cultures were maintained in N_2 atmosphere for the desired period (usually 2 hr for UDS studies). The medium was then aspirated and the cells were washed twice with phosphate buffered saline containing 2 mM unlabelled thymidine. Finally the cells were scraped off the plate in 10 ml phosphate buffered saline + thymidine and centrifuged at 500 *g* for 5 min. In some instances, where indicated, the cell nuclear fraction was isolated by sucrose gradient centrifugation [20]. Elsewhere cell pellets were kept at

−20° overnight before DNA extraction as described by Martin *et al.* [21]. DNA concentrations were estimated by the method of Burton [22] and radioactivity by liquid scintillation counting. Conversion of cpm to dpm was computed using the external standard ratio method.

(b) *Aerobic studies.* In aerobic experiments, similar procedures were used, except complete William's medium E (William's E supplemented with 5% v/v foetal calf serum, 1% w/v L-glutamine and 50 μg/ml gentamycin) replaced Leibovitz medium and all incubation steps were carried out at 37° in an atmosphere of 95% air, 5% CO_2 (v/v). William's medium E was used instead of Leibovitz medium to maintain physiological pH values when cells were incubated under aerobic conditions.

Glutathione depletion in JB1, BL8 cells and hepatocytes

Cells were incubated anaerobically as described above except hydroxyurea was omitted. Following harvesting and centrifugation, the cell pellets were homogenised in 2.5 ml ice-cold buffer comprising KH_2PO_4 (24 mM), EDTA (0.1 mM) mixed in the ratio 3:1 (v/v) with ethanol and centrifuged at 3000 *g* for 15 min at 4°. Reduced and oxidized glutathione were estimated in the supernatants as 2,4-dinitrophenyl derivatives using the HPLC procedure of Reed *et al.* [23].

Determination of DT-diaphorase (EC 1.6.99.2)

DT-diaphorase was estimated in cell homogenates using 2,6-dichlorophenol-indophenol as substrate [24]. Protein concentrations were determined by the Lowry method [25] using bovine serum albumin as standard.

RESULTS

Dose-response relationship of certain nitro-compounds and 1-aziridineethanol on unscheduled DNA synthesis

In the present study three cell lines were used: rat hepatocytes which possess the cytochrome P-450 dependent mixed function oxidases, non-transformed (BL8) and transformed (JB1) rat liver derived epithelial cell lines lacking detectable levels of this cytochrome. Both JB1 and BL8 cells were used since the precise identity of the enzyme(s) involved in nitro-activation are not known. JB1 cells possess higher activities of certain enzymes (15) including DT-diaphorase than do BL8 cells (10.8 ± 0.6 versus 5.8 ± 0.4 μmoles/min/mg protein, mean ± SE, for 4 experiments). DT-diaphorase has been implicated in the metabolic activation of nitroquinoline-*N*-oxide [26].

Figure 2a shows the effects on hypoxic JB1 cells of different concentration of certain nitro-substituted compounds. Misonidazole was poor at stimulating UDS even at the highest concentrations tested (10 mM). Similar results were obtained using DNA extracted from isolated nuclei according to the method of Althaus [20]. The presence of an aziridine function, e.g. in RSU-1069, greatly increased UDS

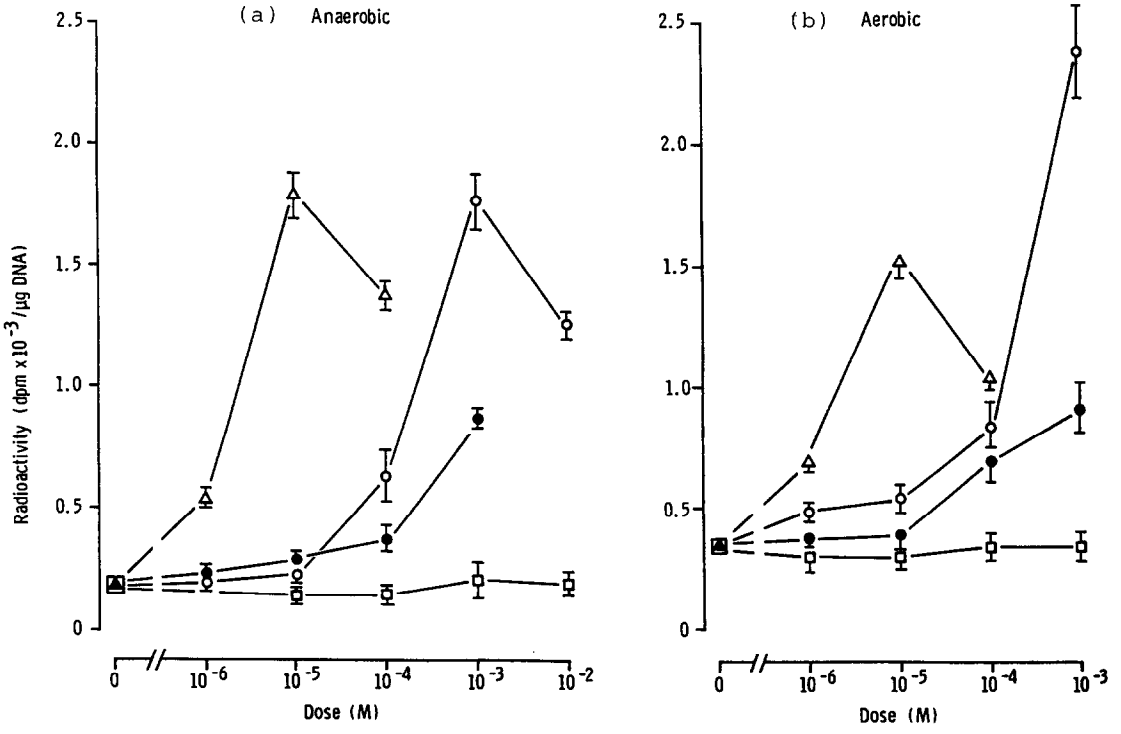

Fig. 2. Dose response of nitroquinoline-*N*-oxide, aziridineethanol, RSU-1069 and misonidazole on UDS of JB1 cells. (a) Cells incubated under anoxic conditions in an atmosphere of N_2. (b) Cells incubated in 95% air, 5% CO_2. Confluent cultures were exposed to drugs for 2 hr at 37°. △—△, Nitroquinoline-*N*-oxide; ○—○, aziridineethanol; ●—●, RSU 1069; □—□, misonidazole.

Table 1. Unscheduled DNA synthesis caused by various nitro-compounds in JB1 and BL8 cell lines and in freshly prepared hepatocytes during 2 hr anaerobic incubation

Compounds	Dose (M)	Unscheduled DNA synthesis† (dpm/μg DNA) JB1	BL8	Hepatocytes
Nitroquinoline-*N*-oxide	1×10^{-5}	1786 ± 170***	787 ± 32***	144 ± 19**
Nitropyridine-*N*-oxide	1×10^{-4}	261 ± 22**	ND	69 ± 4*
Ro. 07-0741	1×10^{-3}	217 ± 11*	187 ± 14	54 ± 6
Misonidazole	1×10^{-3}	205 ± 13*	195 ± 18	43 ± 5
RSU-1042	1×10^{-3}	186 ± 22	163 ± 12	ND
Metronidazole	1×10^{-3}	153 ± 8	ND	ND
Control		158 ± 14	179 ± 15	54 ± 4

† Results represent the mean ± SE for four experiments.
ND = Not determined.
Probability of significance of difference between treated cells and control: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

relative to misonidazole but not to the extent caused by aziridineethanol itself. On a molar basis, both aziridineethanol and RSU-1069 were considerably less effective than nitroquinoline-*N*-oxide at all concentrations $< 10^{-3}$ M. In JB1 cells incubated aerobically, misonidazole caused no significant increase in UDS (Fig. 2b). Similarly aerobic conditions did not affect UDS induced by RSU-1069, aziridineethanol or nitroquinoline-*N*-oxide.

Table 1 compares the UDS caused by nitro-substituted compounds in JB1 and in freshly prepared hepatocytes incubated anaerobically. All compounds tested were more effective in JB1 cells than in hepatocytes. Nitroquinoline-*N*-oxide was the most effective compound in both cell types at causing UDS.

The role of the aziridine function in stimulating unscheduled DNA synthesis

In order to investigate the role of the aziridine function in stimulating UDS, the effect of ring substitution was investigated. Table 2 shows that the introduction of one or more methyl groups into the aziridine ring generally reduced the level of UDS caused by these compounds. With the exception of RSU-1172 there was a good correlation between the pK_a of the aziridine nitrogen atom and the level of UDS (Table 2). None of the nitroimidazole-aziridines were, however, as effective as aziridineethanol. The 2-nitroimidazole-aziridine RSU-1069 was more effective than the 4-nitro-compound RSU-1170 at inducing UDS in both JB1 and BL8 cell lines. With all compounds tested, JB1 cells were more responsive than the BL8 cell line.

Effects of nitroimidazoles on cellular reduced glutathione concentrations

As an independent assessment of the possible metabolic activation of misonidazole and related compounds, their effects on the intracellular concentration of reduced glutathione were investigated under anaerobic conditions. Figure 3 shows that in hepatocytes, misonidazole (1 mM) caused a rapid

Table 2. Unscheduled DNA synthesis caused by various aziridine-containing compounds in JB1 and BL8 cell lines during 2 hr anaerobic incubation

Compounds (1×10^{-3} M)	pK_a‡	Unscheduled DNA synthesis† JB1 cells dpm/μg DNA	% of control	BL8 cells dpm/μg DNA	% of control
Aziridine-ethanol		1778 ± 75***	1125	ND	ND†
RSU-1069	6.04	1117 ± 36***	707	777 ± 55***	434
RSU-1170	6.04	840 ± 78***	532	692 ± 28***	386
RSU-1131	6.49	811 ± 42***	513	579 ± 52***	323
RSU-1164	6.82	619 ± 75***	392	592 ± 39***	331
RSU-1150	7.47	541 ± 61***	342	455 ± 42***	254
RSU-1172	6.17	299 ± 38*	189	295 ± 23***	165
RB-7040	8.45	237 ± 31	150	222 ± 20	124
Control		158 ± 14		179 ± 15	

† Results represent the mean ± SE for 4 experiments.
ND not determined.
‡ pK_a values obtained at 25° in 0.05 M ionic strength (from refs. 18, 41).
Probability of significance between treated cells and controls: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

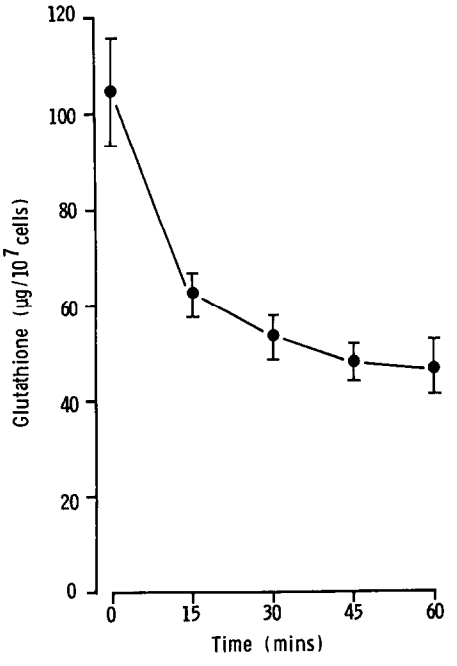

Fig. 3. Effect of misonidazole on depletion of glutathione of freshly prepared hepatocytes. Cells (10^7) were exposed to misonidazole (1 mM) for various times under anoxic conditions in an atmosphere of nitrogen. Reduced glutathione was estimated in cell supernatants as the 2,4-dinitrophenyl derivative using HPLC as described in the Methods section.

loss of reduced glutathione with time reaching about 45% of control values after 1 hr. Over this time there was no corresponding increase in the concentration of oxidised glutathione which remained below the limits of detection ($< 1\ \mu g/10^7$ cells). The marked depletion of glutathione by only 15 min anaerobic exposure to 1 mM nitroimidazoles in hepatocytes and the cell lines appears considerably greater than that observed after much longer exposure times in other cell lines [27, 28]. In hepatocytes incubated aerobically for 1 hr misonidazole caused no significant reduction in the concentrations of reduced glutathione.

Table 3 shows that the depletion of reduced glutathione concentrations in cells incubated anaerobically was not confined to misonidazole but was a general property of such nitroimidazoles. The presence of an aziridine function, e.g. RSU-1069, did not significantly enhance glutathione depletion. Similar reductions in reduced glutathione concentrations were observed in hepatocytes, JB1 and BL8 cells (Table 3).

DISCUSSION

Metabolic activation of the nitro function: effects on unscheduled DNA synthesis

Nitroimidazoles such as misonidazole or metronidazole react poorly with cellular DNA in rat hepatocytes, JB1 or BL8 cells under hypoxic conditions, as judged by the lack of induction of unscheduled DNA synthesis (Fig. 2, Table 1). Stimulation of UDS by misonidazole could not be detected using autoradiographic techniques (M. Suzangar and I. N. H. White, unpublished results). This finding is difficult to reconcile with previous studies which report that radiolabelled misonidazole binds covalently to liver DNA when administered to mice *in vivo* [29] and causes DNA strand breaks in hypoxic Chinese hamster cell lines [30]. In the present study, misonidazole underwent metabolic activation in hypoxic hepatocytes, JB1 and BL8 cells, as seen from the depletion of cellular glutathione (Fig. 3, Table 3). This process seems not to be dependent on cytochrome P-450 as it occurs in JB1 cells which lack detectable levels of this cytochrome (< 0.01 nmoles/10^6 cells, Table 1). Activation of misonidazole by nitro-reduction is believed to take place mainly via the NADPH-dependent cytochrome *c* reductases [31, 32]. This may result in the formation of a hydroxylamine derivative which could react with glutathione non-enzymically [33], although we cannot exclude that alternate reactions with for example a nitroso precursor might occur more rapidly [34]. Depletion of glutathione may impair some DNA repair processes [35]. However, nitroimidazoles containing the direct acting aziridine function, e.g. RSU-1069, caused significant increases in UDS at the same time as causing depletion of cellular glutathione (Fig. 2, Table 3).

Certain other compounds containing a nitro func-

Table 3. Glutathione depletion in hepatocytes. JB1 and BL8 cell line caused by various nitro-compounds (1×10^{-3} M) during 15 min anaerobic incubation

Treatment	(μg GSH/10^7 Cells) Hepatocytes	JB1 cell line	BL8 cell line
Control	104 ± 9.3	17 ± 2.3	2.1 ± 0.25
Misonidazole	62 ± 4.00**	12 ± 2.0	1.8 ± 0.30
Benznidazole	67 ± 7.00**	10 ± 1.4*	1.0 ± 0.10*
RSU-1069	83 ± 15.0	11 ± 2.2*	1.5 ± 0.10
RSU-1131	44 ± 7.8**	10 ± 1.8*	ND†
RSU-1172	44 ± 6.6**	10 ± 1.4*	1.5 ± 0.04
RB-0704	52 ± 8.2**	10 ± 1.9*	ND†

† ND not determined.

Results represent the mean ± SE for 4 experiments. Probability of significance of difference between treated cells and controls: **$P < 0.01$; *$P < 0.05$

tion which require metabolic activation were also tested for their ability to cause UDS (Table 1). In the JB1 cell line, on a molar basis, nitroquinoline-*N*-oxide was by far the most effective. Surprisingly, nitropyridine-*N*-oxide which might have been expected to have been activated by a similar mechanism to nitroquinoline-*N*-oxide, [26, 36] caused a much smaller increase in UDS. With all of these compounds, JB1 cells were more responsive than either BL8 cells or rat hepatocytes. In hepatocytes alternative metabolic pathways may cause degradation of the parent compounds.

Unscheduled DNA synthesis caused by aziridine substituted nitroimidazoles

Compounds containing the monofunctional alkylating aziridine function covalently bind and cause strand breakage in DNA without the need for metabolic activation [13, 18, 37, 38, 39]. Protonation of the nitrogen atom is, however, required in order for aziridines to function as effective alkylating agents at neutral pH values [40]. Since the alkyl-substituted aziridines have higher pK_a values [18, 41], the tetramethyl derivative RB-7040 would be expected to be more reactive at pH 7.4 than for example the parent unsubstituted RSU-1069. This was not found experimentally with regard to the induction of unscheduled DNA synthesis. Compounds with low pK_a values proved to be more effective at inducing UDS (Table 2). In order to react with the DNA target, the chemical must be able to penetrate the cell and reach the nucleus. This may not occur with aziridine-compounds of high chemical reactivity and short half-lives [18]. Increasing methyl-substitution on the aziridine ring may also sterically hinder alkylation of DNA and thus reduce the level of unscheduled DNA synthesis observed.

CONCLUSIONS

Most agents currently used in cancer chemotherapy are bifunctional alkylating agents with regard to their reaction with DNA. Present results suggest that nitroimidazoles such as misonidazole or benznidazole, which are currently undergoing clinical trials do not substantially alkylate DNA in hypoxic cells. However, after metabolic activation, they cause the depletion of cellular thiols such as glutathione. We conclude on the basis of our results that this may be an important mechanism in acting as sensitisers in chemotherapy. A similar sensitisation to melphalan cytotoxicity has recently been reported for the inhibitor of glutathione synthesis, buthionine sulfoximine [42].

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