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EFFECTS OF GLUTATHIONE DEPLETION USING BUTHIONINE SULPHOXIMINE ON THE CYTOTOXICITY OF NITROAROMATIC COMPOUNDS IN MAMMALIAN CELLS *IN VITRO*

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Abstract—The inhibitor of glutathione biosynthesis, buthionine sulphoximine (BSO) has been used to deplete endogenous thiols in mammalian cells *in vitro*. The effect of such depletion on the toxicity of nitroaromatic compounds has been investigated. Substantial enhancement of both aerobic and hypoxic toxicity of the 2-nitroimidazole, misonidazole is observed in thiol-depleted cells; the hypoxic toxicities of metronidazole, nitrofurantoin and nimorazole are also increased by thiol depletion. These data of significance for the potential combined use of BSO with nitroaromatic radiosensitizers to increase their radiosensitizing efficiency in radiotherapy, and as a potential method for enhancing the efficiency of anti-protozoal nitroaromatic drugs.

The toxicity of nitroaromatic compounds to mammalian cells *in vitro* correlates with one-electron reduction potential under oxic [1, 2] and hypoxic [3] conditions. However, the toxicity of nitroaromatic compounds is much greater to hypoxic compared with aerobic cells, e.g. [4–7]. This selective toxicity may be useful in helping to eliminate hypoxic tumour cells in cancer radiotherapy when nitroaromatic compounds are used as hypoxic cell radiosensitizers, and may play a role in the chemosensitization of other chemotherapeutic agents by e.g. nitroimidazoles, which are also radiation sensitizers [8]. However, using the 2-nitroimidazole, misonidazole in a clinical radiotherapy course of 25 fractions with a total dose of 12 g/m^2 [9] results in plasma and tumour concentrations of the order of 0.1 mmol dm^{-3}; at these concentrations of misonidazole, hypoxic toxicity towards mammalian cells would be expected to be undetectable.

The hypoxic toxicity of nitroaromatic compounds has been shown to be reduced by a range of sulphydryl compounds, e.g. [10–12]. The corollary of these observations is that reduction of intracellular thiols should enhance the hypoxic toxicity of nitroaryl compounds, as indeed has been observed using the agent diethylmaleate [13] and with glutathione-deficient cells [14]. We have previously used the inhibitor of glutathione (GSH)‡ biosynthesis, buthionine sulphoximine (BSO) [15] to deplete GSH levels in hypoxic mammalian cells, resulting in a marked enhancement of misonidazole radiosensitizing efficiency [16].

This paper reports enhancement of hypoxic toxicity of nitroaromatic compounds in mammalian cells *in vitro* by buthionine sulphoximine.

MATERIALS AND METHODS

V79 379A Chinese Hamster cells were maintained in exponentially growing, single cell suspension cultures in aerated, buffered Eagles' Minimum Essential Medium (MEM) with 7.5% foetal calf serum.

For toxicity measurements, cells incubated in growth medium in stirred suspension culture at 5×10^5 cells per ml were gassed at 0.4 L min^{-1} with nitrogen + 5% CO_2 (<10 ppm O_2) obtained from British Oxygen Company Ltd. Gassing began 1 hr before addition of nitro-compounds to reduce the oxygen tension in the culture. Where the volume of drug stock solution to be added to the culture was > 5% of the total volume, the drug solution was also stirred under nitrogen for 1 hr before addition to the cells. Cell samples taken immediately after addition of the nitro compound and at various times afterwards were centrifuged to remove the drug solution and plated on to plastic petri dishes to assess viability in a 7-day colony-forming assay.

Cells were depleted of GSH by treatment with BSO aerobically at 0.05 or 0.1 mmoles dm^{-3} for 16 hr at 37° before the start of degassing. BSO was left in the medium during the course of the toxicity measurements, to avoid regeneration of GSH during the longer experiments. For estimation of non-protein thiol (NPSH) content, a sample containing about 10^7 cells was centrifuged, the pellet resuspended in PBS and recentrifuged. The pellet was then resuspended in 1 ml distilled water, 1 ml 10% trichloroacetic acid added and the cell debris removed by centrifugation. The supernatant was then neutralised with 3 ml 0.5 mol dm^{-3} sodium phosphate (pH 7.5) and the thiol content determined by the method of Ellman [17].

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‡ Abbreviations used: GSH, reduced glutathione; BSO, DL S-*n*-butyl homocysteine sulphoximine, buthionine sulphoximine; MEM, Eagles' minimal essential medium; NPSH, non-protein thiol.

Buthionine sulphoximine was prepared as described [16, 18, 19]. Misonidazole was obtained from Dr. C. E. Smithen, Roche Products Ltd. (Welwyn Garden City, U.K.). Metronidazole and nitrofurantoin were obtained from Sigma Chemical Co. (St Louis, MO) and nimorazole from Dr. A. P. Launchbury, Montedison Pharmaceuticals Ltd., Barnet, Hertfordshire.

RESULTS

Treatment of cells with 0.1 or 0.05 mmoles dm^{-3} BSO under air or nitrogen reduces NPSH content of cells over a few hours, Fig. 1. A residual level of about 10% of the control NPSH is reached after about 6 hr and is maintained in the presence of 0.1 mmoles dm^{-3} BSO for at least 24 hr, with no significant loss in plating efficiency (>70%). BSO does not cause any significant loss in plating efficiency under hypoxic conditions over the time course of the experiments. A similar reduction in endogenous thiols has been achieved with 1 mmole dm^{-3} BSO in human cells [20].

A large enhancement of the hypoxic toxicity of misonidazole is seen in cells pretreated with BSO for 16 hr, compared with that in untreated cells, Fig. 2a. The toxicity of 5 mmoles dm^{-3} misonidazole in untreated cells is similar to that of 0.5 mmoles dm^{-3} misonidazole in cells pretreated with BSO for 16 hr, suggesting a 10-fold increase in toxicity on a concentration basis. The aerobic toxicity of misonidazole is also increased by BSO pretreatment, Fig. 2b. However, over the concentration ranges examined a large differential is still observed between oxic and hypoxic misonidazole toxicity in both untreated and thiol-depleted cells; *misonidazole is more toxic to undepleted hypoxic cells than to thiol-depleted oxic cells*. Smaller increases upon GSH depletion in the hypoxic toxicity of nitrofurantoin and metronidazole are shown in Figs. 3–4 respectively, with an approximately 2-fold enhancement of the toxicity of nitrofurantoin by pretreatment of cells with BSO for 16 hr. The hypoxic toxicity of nimorazole is also increased in thiol depleted cells, Fig. 5, although the low toxicity to hypoxic control cells precludes an estimate of the increase in toxicity on a concentration basis.

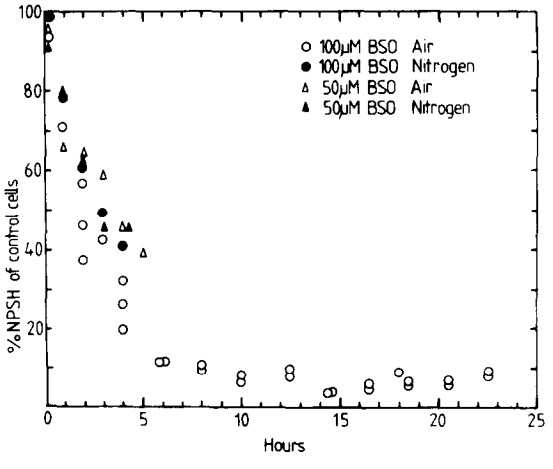

Fig. 1. NPSH content of V79 379A cells treated with BSO, as a percentage of NPSH content of untreated controls.

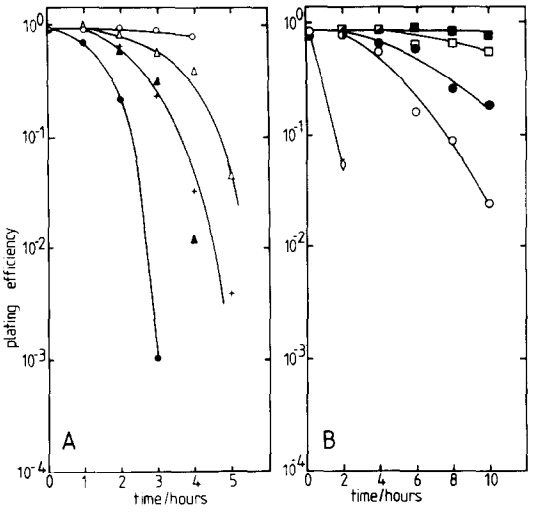

Fig. 2. Plating efficiency of V79 379A cells incubated at 37°. (A) Hypoxic conditions with: (○) 1.0 mmole dm^{-3} or (+) 5.0 mmoles dm^{-3} misonidazole, or after 16 hr treatment with 0.1 mmole dm^{-3} BSO: (△) 0.25 mmole dm^{-3} (▲) 0.5 mmole dm^{-3} or (●) 1.0 mmole dm^{-3} misonidazole. (B) Oxic conditions with: (■) 10 mmole dm^{-3} or (□) 50 mmoles dm^{-3} misonidazole, or after 16 hr treatment with 0.1 mmoles dm^{-3} BSO: (●) 5 mmoles dm^{-3} (○) 10 mmoles dm^{-3} or (◇) 20 mmoles dm^{-3} misonidazole.

DISCUSSION

The protective action of several thiols against the hypoxic toxicity of misonidazole has been docu-

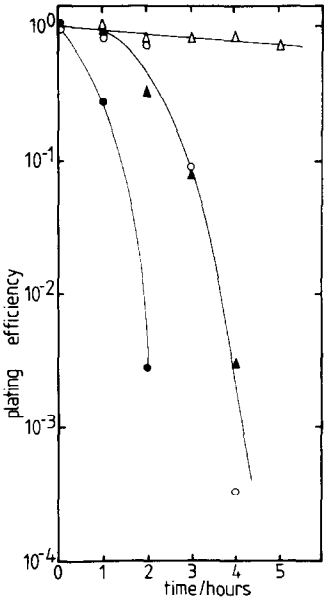

Fig. 3. Plating efficiency of hypoxic V79 379A cells incubated at 37° with: (△) 0.05 mmole dm^{-3} or (○) 0.1 mmole dm^{-3} nitrofurantoin, or after 16 hr treatment with 0.1 mmole dm^{-3} BSO: (▲) 0.05 mmole dm^{-3} or (●) 0.1 mmole dm^{-3} nitrofurantoin.

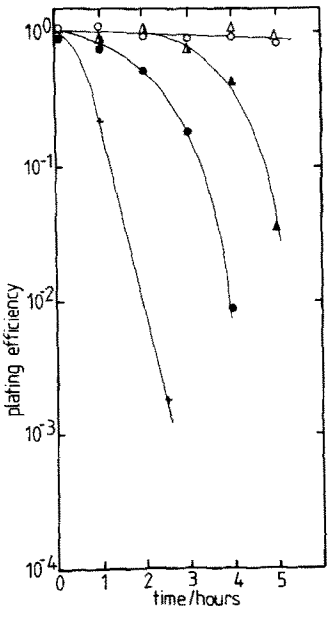

Fig. 4. Plating efficiency of hypoxic V79 379A cells incubated at 37° with: (○) 5 mmoles dm^{-3} (△) 7.5 mmoles dm^{-3} or (+) 75 mmoles dm^{-3} metronidazole, or after incubation for 16 hr with 0.1 mmole dm^{-3} BSO: (▲) 5 mmoles dm^{-3} or (●) 7.5 mmoles dm^{-3} metronidazole.

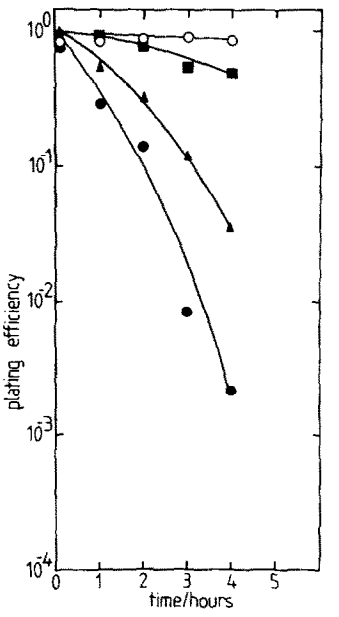

Fig. 5. Plating efficiency of hypoxic V79 379A cells incubated at 37° with: (○) 10 mmoles dm^{-3} nimorazole, or after incubation for 16 hr with 0.1 mmole dm^{-3} BSO: (■) 2.5 mmoles dm^{-3} (▲) 5 mmoles dm^{-3} or (●) 10 mmoles dm^{-3} nimorazole. (Control [undepleted] cells exposed to 2.5 mmoles dm^{-3} nimorazole gave no detectable reduction in plating efficiency over this time period).

mented (e.g. [10, 12, 13, 21]). It is therefore not unexpected that depletion of endogenous thiols by BSO pretreatment should enhance the hypoxic toxicity of nitroaromatic compounds, as found in this work. Hall *et al.* [22, 23] plotted the logarithm of the misonidazole concentration against the logarithm of the time required to reduce survival to 0.1; from the slope of a straight line fit to the data they deduced that for a constant level of survival, the drug concentration required is inversely proportional to the square of the exposure time. A similar plot of our data for toxicity of misonidazole in control and thiol-depleted cells at constant levels of survival, Fig. 6A, gives slopes of -0.53 ± 0.05 and -0.61 ± 0.08 respectively suggesting that this empirical time squared dependence is not thiol dependent. The more electron-affinic nitrofurantoin gives a slope of -0.92 ± 0.05, Fig. 6B, in thiol depleted cells. The data summarised in Fig. 6B for the 5-nitroimidazoles, metronidazole and nimorazole exhibit both an increased scatter and shallower slopes of -0.14 ± 0.09 and -0.18 ± 0.17 respectively for their concentration/time dependence. We stress, however, the difficulty of reliable measurements of hypoxic toxicity with 5-nitroimidazoles because of the marked susceptibility of their toxicity to very low oxygen tensions [24]. The relative toxicity of the different drugs in thiol-depleted cells shows a similar dependence on one-electron reduction potential to that observed by Adams *et al.* [1, 2] although thiol depletion increases the absolute toxicity.

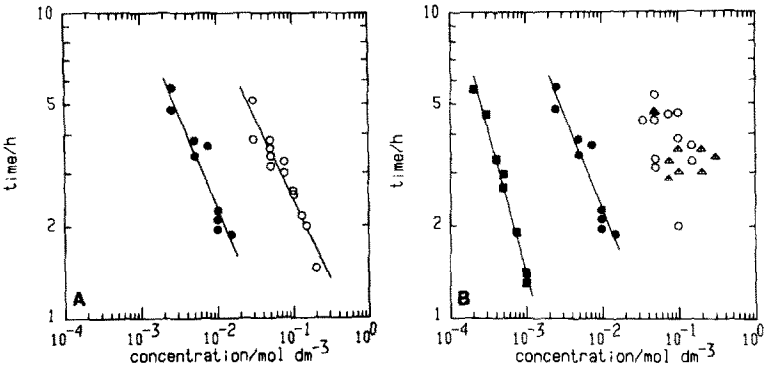

Fig. 6. Concentration–time relationships for hypoxic cytotoxicity of nitroaryl compounds at 37° at a constant survival level of 0.1. (A) (○), misonidazole in control cells; (●), misonidazole in thiol depleted cells. (B) In thiol depleted cells (■), nitrofurantoin; (●), misonidazole; (△), metronidazole and (○), nimorazole. Points plotted represent values interpolated from data similar to that shown in Figs. 2–5. The function fitted is log(concentration) = $m \times$ log(time/h) + constant, yielding slopes, m.

Although the relationship between the hypoxic toxicity of nitroaromatic compounds *in vitro* and *in vivo* is complicated by pharmacological considerations, increased misonidazole toxicity *in vivo* would reduce the anticipated therapeutic gain from the enhanced radiosensitizing efficiency of misonidazole in cells depleted of glutathione by BSO [16]. Some increase in misonidazole toxicity has been reported for combined BSO/misonidazole treatments *in vivo* [25, 26].

Several nitrofurans and nitroimidazoles are routinely administered to man as antibacterial, antifungal and antiprotozoal agents, e.g. [27–29]. BSO has also been shown to increase the survival time of mice infected with trypanosomes by enhancing the oxidative damage of peroxide formed as normal products of oxygen metabolism [30]. Under oxic conditions nitrofurantoin and many other nitro compounds undergo reduction to a radical which is then auto-oxidised to the parent compound, generating superoxide and eventually hydrogen peroxide as a consequence [31, 32]. Thus in catalase-deficient trypanosomes such as *T. brucei* used by Arrick *et al.* [30], combined chemotherapy by BSO and nitrofurantoin should be much more effective than the individual drugs.

Although additional work is clearly needed to assess the purely chemotherapeutic use of BSO in combination with nitroaromatic drugs *in vivo*, the enhanced toxicity of nitro compounds in cells depleted of glutathione could be of benefit in combating anaerobic and parasitic infections. It is possible that the increased toxicity towards aerobic normal tissues caused by thiol depletion will be less of a problem with the relatively small doses of nitroaryl compounds used in chemotherapy than when the dose regime is at the maximum tolerated dose, as is necessary in the applications of these compounds in radiotherapy [9].

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