Biochemical Pharmacology, Vol. 38, No. 10, pp. 1667–1671, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

CYTOTOXICITY AND GLUTATHIONE DEPLETION BY 1-METHYL-2-NITROSOIMIDAZOLE IN HUMAN COLON CANCER CELLS

R. TIMOTHY MULCAHY,*† JERRY J. GIPP,* GREGG A. UBLACKER, RICK PANICUCCI‡ and ROBERT A. MCCLELLAND‡

*Department of Human Oncology, University of Wisconsin Clinical Cancer Center, University of Wisconsin, Madison, WI 53792, U.S.A. and ‡Department of Chemistry and Scarborough College, University of Toronto, Toronto, Ontario, M5S 1A1, Canada

(*Received* 6 *June* 1988; *accepted* 7 *October* 1988)

Abstract—The biological effects of 1-methyl-2-nitrosoimidazole (INO), the 2 electron reduction product of biologically active 1-methyl-2-nitroimidazole, were examined in HT-29 human colon cancer cells by clonogenic assay and glutathione (GSH) determination. INO was very toxic towards HT-29 cells and was equally toxic under aerobic and hypoxic conditions. Cytotoxicity was highly dependent on cell concentration, decreasing as cell concentration increased. INO also resulted in an initial dose-dependent depletion of intracellular GSH which plateaued when the GSH content of INO-treated cells reached approximately 8% of control levels. As was the case for cytotoxicity, the magnitude of GSH depletion by any given INO dose was highly dependent on cell concentration, being greatest at low cell densities. Both toxicity and GSH depletion were more pronounced when cells were exposed in culture medium without the reducing agent, ascorbate. HT-29 cells preincubated with the GSH synthesis inhibitor, buthionine sulfoximine (BSO), to reduce GSH levels to approximately 10% of control levels were more sensitive to subsequent INO exposure. These data suggest that the nitroso- reduction product of 2-nitroimidazoles may be responsible for cytotoxicity and glutathione depletion associated with hypoxic exposure to 2-nitroimidazoles.

Various nitroimidazole compounds have been used successfully for the treatment of protozoal diseases and anaerobic bacterial infections [1] and more recently have been evaluated as hypoxic cell cytotoxic agents and radiation-sensitizing drugs [2, 3]. Several nitroimidazoles, particularly 2-nitro compounds, have also been shown to enhance the antitumor effectiveness of certain conventional cancer chemotherapeutic agents [4], a process referred to as chemosensitization. Many important biological activities of N_1-substituted nitroimidazoles, including hypoxic toxicity, thiol depletion, chemosensitization, binding to macromolecules and interactions with DNA, have been shown to be associated with the metabolic nitroreduction of the parental compound to active reduction intermediates under hypoxic conditions [5–12]. Complete reduction of 2-nitroimidazoles to their corresponding, biologically inactive 2-aminoimidazole products (6 electron product) involves the generation of nitroso- (2 electron product) and hydroxylamino- (4 electron product) intermediates, either or both of which may represent active species.

Until recently, attempts to identify the active intermediates have been hampered by the reactive nature of the nitroso- and hydroxylamino-compounds and the lack of stable forms of these agents. Recent evidence suggests that the half-life and biological activity of N_1-substituted 2-(hydroxylamino)-imidazoles are compatible with the possibility that the 4 electron reduction product is the active species [12–16]. Indeed, these compounds are capable of binding macromolecules, forming stable conjugates with glutathione (GSH§) [14, 15], and reacting with guanosine [13]. However, experimental data suggest that the hydroxylamine derivatives of 2-nitroimidazoles may not be potent cytotoxic agents in mammalian cells [17, 18], indirectly implicating other intermediates in this and possibly other effects of reduced 2-nitroimidazoles.

Investigation of the potential role of the nitroso-reduction products in the biological activity of nitroimidazoles has been facilitated by the recent synthesis [18] of 1-methyl-2-nitrosoimidazole (INO), the 2 electron reduction product of the model compound, 1-methyl-2-nitroimidazole (INO_2). Noss *et al.* [18] have reported that this intermediate is highly toxic in CHO cells and mutagenic in *Salmonella typhimurium*. In the present study, we examined the toxicity of INO in the human colon cancer cell line, HT-29, and documented depletion of cellular GSH concentrations following drug exposure. INO toxicity experiments with HT-29 cells pretreated with the GSH synthesis inhibitor, buthionine sulfoximine (BSO), indicated that GSH plays a major role in modulating the toxic effects of INO.

METHODS

Materials. 1-Methyl-2-nitrosoimidazole was synthesized according to the technique published previously [18] employing 2-hydroxylamino-1-

† Correspondence: R. Timothy Mulcahy, Ph.D., 600 Highland Ave., K4/334, Madison, WI 53792, U.S.A.

§ Abbreviations: GSH, glutathione; INO_2, 1-methyl-2-nitroimidazole; INO, 1-methyl-2-nitrosoimidazole; BSO, buthionine sulfoximine; DMEM, Dulbecco's Minimum Essential Medium; and HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid.

methylimidazole hydrochloride generated by radiolytic reduction of 1-methyl-2-nitroimidazole. For all experiments, concentrated stock solutions of INO were prepared in ice-cold distilled water.

L-Buthionine sulfoximine was purchased from the Sigma Chemical Co. (St Louis, MO).

Cell line and drug treatments. The human colon cancer cell line, HT-29, was used for these studies. Cells were maintained in exponential growth in α-MEM medium supplemented with 10% fetal bovine serum, 25 mM HEPES buffer and gentamycin (50 μg/ml). Cultures were incubated at 37° in an atmosphere of 97% air/3% CO_2 and transferred at weekly intervals. All cultures were determined to be Mycoplasma free.

The technique used to treat cells with INO under aerobic or hypoxic conditions has been described in detail previously [19]. For aerobic exposures, exponentially growing HT-29 cells were suspended in 10 ml of either α-MEM or Dulbecco's MEM at a concentration of 1–2 × 10^5 cells per ml and transferred to glass treatment vials. In initial experiments α-MEM was employed, but, as described in the Results, drug exposures were subsequently performed in Dulbecco's MEM lacking ascorbate. For hypoxic exposures, the cells were injected into 10 ml of medium in treatment vials only after the medium had been gassed for 3 hr with a 95% N_2/5% CO_2 gas mixture. Prior to being injected into the treatment vials at the conclusion of the 3-hr gassing phase, the cells were incubated for 10 min at a concentration of 1–2 × 10^7/ml in a Hamilton gas-tight syringe at 37° to deplete oxygen by consumption.

For certain aerobic experiments the cell concentration at the time of treatment was varied in order to examine the effect of cell density on the cytotoxicity of INO.

To deplete cellular levels of GSH, HT-29 cells were incubated in the presence of 1.0 mM BSO for 24 hr prior to INO treatment. This pretreatment reduced GSH levels to 11 ± 4% (mean and SD of six determinations) of control HT-29 cells but did not reduce plating efficiency relative to untreated controls.

To initiate INO exposure, various concentrations of the compound in distilled water were diluted 100-fold by injection into the appropriate treatment vial. Cells were incubated in the presence of INO for 1 hr at 37°. This exposure interval was sufficient to allow >99% decomposition of the parent INO (data not shown; [8]). At the conclusion of the exposure interval the cell suspensions were centrifuged, washed in drug-free medium, and prepared for survival assay.

Cell survival assay. Survival was determined using a standard plating efficiency assay. Colony formation in treated and control groups was enumerated 12–14 days after plating, and survival was calculated as the ratio of plating efficiency of treated groups to that for controls.

Glutathione determinations. Total intracellular glutathione (GSH and GSSG) was determined by the method of Tietze [20] as modified by Bump *et al.* [8]. Cells were exposed to INO as described for cytotoxicity studies and were then immediately prepared for GSH assay at the conclusion of the incubation interval.

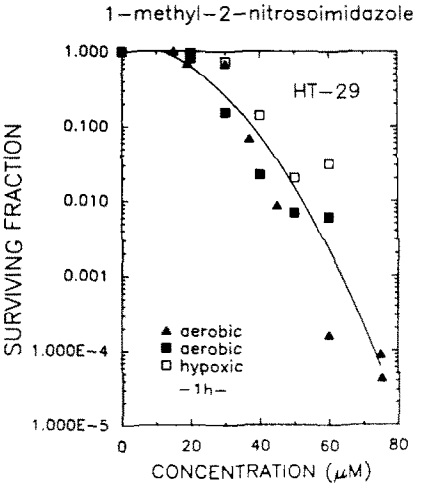

Fig. 1. Dose–response curves for HT-29 cells exposed to INO for 1 hr under aerobic or hypoxic conditions in α-MEM medium. Different symbols represent separate experiments. Cell concentration was 1–2 × 10^5 cells/ml at the time of treatment.

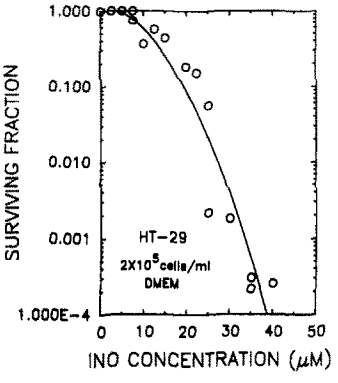

Fig. 2. Dose–response curve for HT-29 cells exposed to INO for 1 hr in DMEM medium without ascorbate. For all subsequent figures, INO exposure was performed in DMEM.

RESULTS

Figure 1 presents the cytotoxic dose–response relationship for HT-29 cells exposed in suspension culture to various doses of INO for 1 hr. In the experiments depicted in Fig. 1, the cells were exposed in α-MEM medium which contained the reducing agent, ascorbate. The survival curve is characterized by a wide shoulder with minimal cell kill at INO doses of less than 20 μM, followed by a rapid decline in survival at progressively larger doses. INO was equitoxic to HT-29 cells exposed under aerobic or hypoxic conditions. As C-nitroso compounds, such as INO, can be reduced by interaction with ascorbate [21], the cytotoxicity experiments were repeated using Dulbecco's MEM (DMEM) without ascorbate, and, as shown in Fig. 2, INO was found to be much more toxic. If ascorbate was added to a solution of INO in DMEM, the activity of the drug

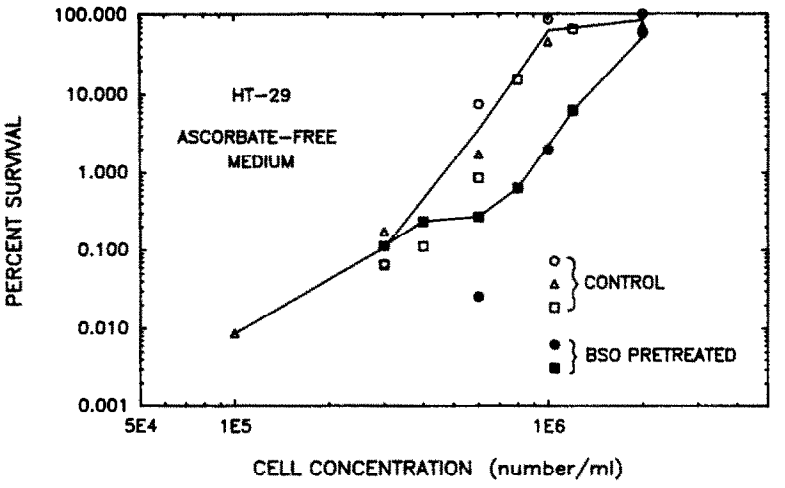

Fig. 3. Effect of cell density on the aerobic toxicity of 40 μM INO in HT-29 cells (open symbols). Cells were exposed for 1 hr at cell concentrations from 10^5 cells/ml to 2×10^6 cells/ml. Solid symbols depict the toxicity of 40 μM INO in HT-29 cells that had been pretreated with 1 mM BSO for 24 hr immediately prior to INO exposure. BSO pretreatment reduced GSH levels to 11% of controls. Different symbols represent independent determinations.

was rapidly reduced or eliminated, depending upon the INO/DMEM ratio (data not shown). Consequently, for all subsequent experiments the cells were exposed to INO in DMEM.

INO toxicity was also influenced markedly by cell concentration at the time of treatment. To demonstrate this effect, the relationship between surviving fraction and cell concentration for HT-29 cells exposed to a 40 μM dose of INO is shown in Fig. 3 (open symbols). At low cell concentrations this dose of INO was extremely toxic and resulted in a four-log reduction in survival. However, toxicity rapidly decreased as cell concentration increased. A 10-fold change in cell concentration (from 10^5 to 10^6 cells/ml) was accompanied by a four-log reduction in cell kill, such that this dose of INO was rendered nontoxic.

Considering the rapid reaction of INO with reducing agents, including GSH, in solution and by analogy to nitrosobenzene, it was reasoned that the cell concentration-dependent toxicity of INO might result from an interaction of INO with intracellular GSH. If operative, such a reaction might be expected to produce a measurable reduction in total cellular GSH. As shown in Fig. 4, a linear decrease in total (GSH and GSSG) intracellular GSH levels did indeed result from exposure to INO concentrations of up to approximately 60 μM. Following a 1-hr exposure at this dose, GSH content of treated cells was reduced to approximately 8% of untreated controls. Treatment with increasing doses of INO did not further reduce the GSH levels. Like cytotoxicity, INO depletion of GSH was also dependent upon the concentration of cells being treated. This relationship is demonstrated in Fig. 5 for HT-29 cells exposed to 40 μM INO for 1 hr at differing cell densities.

To determine whether intracellular GSH could protect against INO toxicity, HT-29 cells were incubated with the GSH synthesis inhibitor, BSO, at a concentration of 1.0 mM for 24 hr prior to treatment

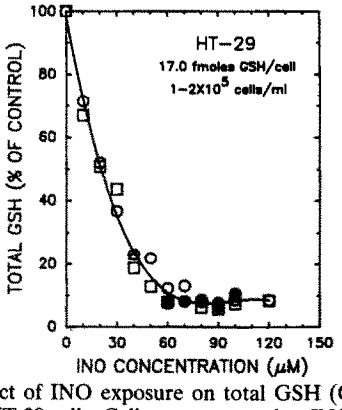

Fig. 4. Effect of INO exposure on total GSH (GSH and GSSG) in HT-29 cells. Cells were exposed to INO for 1 hr at a cell density of 1–2 $\times$ 10^5 cells/ml. Different symbols represent independent determinations.

with INO. This BSO treatment reduced GSH levels to approximately 11% of controls and sensitized the cells to subsequent exposure to INO. When the cell survival/cell concentration relationship for BSO-pretreated cells was compared with that for control cells, as in Fig. 3 (solid symbols), the BSO curve was shifted to the right by a factor of 2 rather than a factor of 10 as might be expected to accompany a 10-fold reduction in GSH. However, at low cell concentrations the two curves converged, indicating that INO was equitoxic to control and BSO-pretreated cells at these low cell concentrations.

DISCUSSION

Many of the biological effects associated with exposure to nitroimidazoles under hypoxic or anaerobic conditions require the nitroreduction of the parent heterocycle. Consequently, the active inter-

mediate(s) is assumed to be one or more of the reductive metabolites. Identification of the active species has not been trivial owing to the reactive nature of these compounds, but substantial evidence from chemical and biological systems suggests that the hydroxylamine derivative may be at least one of the active forms of reduced 2-nitroimidazoles [12–16]. However, until recently it has not been possible to assess the contribution of the nitroso intermediate. Noss *et al.* [18] have reported the synthesis of INO, the nitroso analogue of a model 2-nitroimidazole, and found it to be extremely toxic to CHO cells, implicating this species in the biological activity of these agents as well. We are interested in identifying metabolites of nitroimidazole compounds which are responsible for the ability of these drugs to enhance the cytotoxicity of chemotherapeutic agents and have, therefore, initiated studies designed to examine the effects of INO in HT-29 human colon carcinoma cells.

Exposure to INO at doses in excess of 20 μM resulted in very rapid reduction in the fraction of HT-29 cells surviving drug treatment. At lower doses very little cell kill was evident. These results are compatible with those reported by Noss *et al.* [18] and suggest that a cytotoxic threshold exists for INO exposure. However, the threshold dose is highly dependent upon the concentration of HT-29 cells treated. As illustrated in Fig. 3, INO was markedly more toxic as cell concentration was decreased. Noss *et al.* reported that the rate of decomposition of INO was also related to cell concentration, progressively increasing as the number of cells treated increased. These authors suggested that an interaction with cellular GSH could account for the enhanced loss of INO and the reduced toxicity at high cell densities. Eyer [22] has identified and characterized glutathione conjugates formed upon reaction of the thiol with the model C-nitroso compound, nitrosobenzene. The reaction mechanism described for nitrosobenzene could be characteristic of other C-nitroso compounds and could contribute to the GSH depletion observed in these experiments. To examine this possibility, total cellular GSH was determined for HT-29 cells exposed to increasing concentrations of INO. GSH concentration decreased linearly with INO dose, but plateaued at a level of approximately 8% of untreated controls at INO doses in excess of 60 μM. This stable GSH level could conceivably reflect compartmentalization of the thiol as has been observed following exposure to other thiol depleting agents. At present, the actual localization of this INO-resistant GSH is unknown but may represent nuclear, mitochondrial pools and/or enzyme bound forms.

If an interaction between INO and cellular GSH accounted for the cell concentration-dependent lethality of INO exposure, then the extent of GSH depletion following drug treatment should be expected to demonstrate a similar cell density dependence. As shown in Fig. 5, when cell number, and consequently total GSH levels, were increased, the extent of cellular GSH depletion induced by exposure to a 40 μM dose of INO decreased, consistent with this possibility. Collectively, the data suggest that INO may be inactivated by interaction

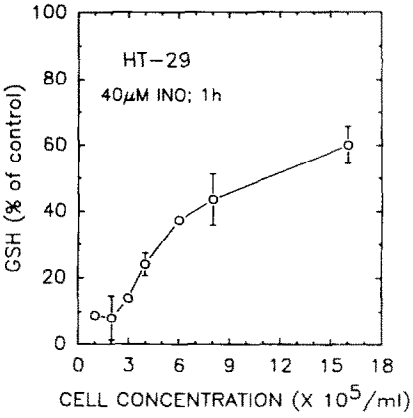

Fig. 5. GSH depletion by exposure to INO (40 μM) as a function of cell concentration. Values are the means ± SD of three to four determinations. (GSH concentration in controls = 17.0 fmol/cell.)

with cellular GSH, resulting in consumption of the thiol as opposed to its conversion to the oxidized (GSSG) form. As cell density is increased, the amount of GSH available for reaction with INO increases and consequently cytotoxicity decreases. Although stable glutathione conjugates have been isolated from cells exposed to the hydroxylamino derivative of 2-nitroimidazoles, similar products have not as yet been identified in INO-treated cells. However, by analogy to the reactions between nitrosoarenes and thiols described by Eyer [22] and Klehr *et al.* [23], such adduct formation seems likely.

A protective effect of GSH was further demonstrated by the increased sensitivity of GSH-depleted HT-29 cells to INO exposure. Glutathione depletion has also been shown to sensitize cells to subsequent exposure to the 2-nitroimidazole, misonidazole [8]. In our current experiments, a 10-fold reduction in cellular GSH only shifted the curve relating toxicity of 40 μM INO and cell concentration to the right by a factor of 2–3, as opposed to an expected factor of 10. This apparent anomaly may be related to the large INO dose used in these experiments which is itself capable of substantially reducing GSH levels. Indeed, the coincidence of the two curves at low cell concentrations may be attributable to efficient thiol depletion accompanying exposure to 40 μM INO at the lower cell densities. Alternatively, the displacement of the curve for BSO-treated cells could reflect less than a 10-fold GSH reduction in some critical cellular compartment, such as the nucleus. Edgren and Revesz [24] have reported that the rate of GSH depletion secondary to BSO treatment differs in the nucleus and cytoplasm of V79-379A cells, with significantly less depletion occurring in the nucleus. It is conceivable therefore that the shift in the curve for BSO-pretreated HT-29 cells reflects the level of GSH depletion in the nucleus as opposed to that determined by assaying total cellular GSH. This possibility is currently being investigated.

The data presented in this report indicate that the nitroso- intermediate of a model 2-nitroimidazole displays biological activity, including thiol depletion and cytotoxicity, compatible with its being an active

metabolite of the reduced parent compound. The work described does not eliminate the possibility that the hydroxylamino- or other intermediates also contribute to the biological activity of these compounds. Preliminary studies indicate that neither the amino- nor the hydroxylamino- compounds are toxic to HT-29 cells at millimolar concentrations (Mulcahy, unpublished). While these findings are in agreement with those reported by Noss *et al.* for CHO cells [18], studies examining the activity of exogenously added hydroxylaminoimidazoles must be interpreted cautiously as these compounds may not accumulate intracellularly by virtue of being protonated at neutral pH. Clearly, the identification of active species responsible for the biological properties of nitroimidazole compounds, a long espoused objective, will require considerably more investigation. Nevertheless, the data presented indicate that the nitroso- reduction product should be considered a viable candidate.

Acknowledgements—The authors are grateful to Peggy Shager for assistance in the preparation of the manuscript. This work was supported by NIH Grant CA42325 (R. T. M.) and a grant from NCI-Canada (R. A. M.).

REFERENCES

1. Brogden RN, Heel RC, Speight TM and Avery GS, Metronidazole in anaerobic infections: A review of its activity, pharmacokinetics and therapeutic use. *Drugs* **16**: 387–417, 1978.
2. Adams GE, Hypoxia-mediated drugs for radiation and chemotherapy. *Cancer* **48**: 696–707, 1981.
3. Narayanan VL and Lee WW, Development of radiosensitizers: A medicinal chemistry perspective. *Adv Pharmacol Chemother* **19**: 155–205, 1982.
4. Siemann DW, Potentiation of chemotherapy by hypoxic cell radiation sensitizers—A review. *Int J Radiat Oncol Biol Phys* **8**: 1029–1034, 1982.
5. Taylor YV and Rauth AM, Differences in the toxicity and metabolism of the 2-nitroimidazole misonidazole (Ro-07-0582) in HeLa and Chinese hamster ovary cells. *Cancer Res* **38**: 2745–2752, 1978.
6. Hall EJ, Miller R, Astor M and Rini F, The nitroimidazoles as radiosensitizers and cytotoxic agents. *Br J Cancer* (Suppl) **37**: 120–123, 1978.
7. Adams GE, Stratford IJ, Wallace RG, Wardman P and Watts ME, Toxicity of nitro compounds towards hypoxic mammalian cells *in vitro*: Dependence on reduction potential. *JNCI* **64**: 555–560, 1980.
8. Bump EA, Taylor YC and Brown JM, Role of glutathione in the hypoxic cell cytotoxicity of misonidazole. *Cancer Res* **43**: 997–1002, 1983.
9. Siemann DW and Mulcahy RT, Sensitization of cancer chemotherapeutic agents by nitroheterocyclics. *Biochem Pharmacol* **35**: 111–115, 1986.
10. Varghese AJ and Whitmore GF, Binding to cellular macromolecules as a possible mechanism for the cytotoxicity of misonidazole. *Cancer Res* **40**: 2165–2169, 1980.
11. Edwards DI, Reduction of nitroimidazoles *in vitro* and DNA damage. *Biochem Pharmacol* **35**: 53–58, 1986.
12. Varghese AJ and Whitmore GF, Modification of guanine derivatives by reduced 2-nitroimidazoles. *Cancer Res* **43**: 78–82, 1983.
13. Varghese AJ and Whitmore GF, Cellular and chemical reduction products of misonidazole. *Chem Biol Interact* **36**: 141–151, 1981.
14. Varghese AJ and Whitmore GF, Properties of 2-hydroxylaminoimidazoles and their implications for the biological effects of 2-nitroimidazoles. *Chem Biol Interact* **56**: 269–287, 1985.
15. Varghese AJ and Whitmore GF, Misonidazole-glutathione conjugates in CHO cells. *Int J Radiat Oncol Biol Phys* **10**: 1341–1345, 1984.
16. Laderoute KR, Eryavec E, McClelland RA and Rauth AM, The production of strand breaks in DNA in the presence of the hydroxylamine of SR-2508 (1-[*N*-(2-hydroxyethyl)acetamido]-2-nitroimidazole) at neutral pH. *Int J Radiat Oncol Biol Phys* **12**: 1215–1218, 1986.
17. Middlestadt MV and Rauth AM, The effects of reduction products of misonidazole on Chinese hamster ovary cells. *Int J Radiat Oncol Biol Phys* **8**: 709–712, 1982.
18. Noss MB, Panicucci R, McClelland RA and Rauth AM, Preparation, toxicity and mutagenicity of 1-methyl-2-nitrosoimidazole. A toxic 2-nitroimidazole reduction product. *Biochem Pharmacol* **37**: 2585–2593, 1988.
19. Mulcahy RT, Misonidazole-induced chemopotentiation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea toxicity in O^6-methylguanine-DNA methyltransferase proficient (Mer^+) and deficient (Mer^-) cell lines. *Cancer Res* **46**: 2892–2897, 1986.
20. Tietze F, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem* **27**: 502–522, 1969.
21. Mulder GJ, Unruh LE, Evans FE, Ketterer B and Kadlubar FF, Formation and identification of glutathione conjugates from 2-nitrosofluorene and *N*-hydroxy-2-aminofluorene. *Chem Biol Interact* **39**: 111–127, 1982.
22. Eyer P, Reactions of nitrosobenzene with reduced glutathione. *Chem Biol Interact* **24**: 227–239, 1979.
23. Klehr H, Eyer P and Schafer W, On the mechanism of reactions of nitrosoarenes with thiols. Formation of a common intermediate "semimercaptal". *Biol Chem Hoppe Seyler* **366**: 755–760, 1985.
24. Edgren M and Revesz L, Compartmentalised depletion of glutathione in cells treated with buthionine sulphoximine. *Br J Radiol* **60**: 723–724, 1987.