Radiochemical and Electrochemical Studies of the Interaction of Zn^{2+}(^{65}Zn) with the Radiosensitizer Misonidazole

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Abstract

Differential pulse polarography (DPP) of solutions containing misonidazole and zinc ions shows the disappearance of the reduction peak of the sensitizer and the appearance of a peak at a more positive potential, attributable to an eventual complex. None of the measurements performed has demonstrated the existence of a complex between zinc ions and the radiosensitizer misonidazole or some of its stable reduction products. To explain the results obtained by DPP, the electrolysis of misonidazole has been performed in the presence of ^{65}Zn oxalate. In this way it has been demonstrated that zinc ions interact with one or more short-lived reduction intermediates. These results suggest that zinc ions can play the role of scavengers of the toxic intermediates of nitro-heterocyclic radiosensitizers produced by metabolic reduction. Experiments performed on Chinese hamster V79 cells, which have shown a decrease in the cytotoxicity of misonidazole and metronidazole in the presence of zinc ions, support this hypothesis.

Introduction

There are many compounds which are capable of differentially sensitizing hypoxic cells to the lethal effects of ionizing radiation while leaving oxic cells unaffected. It is now well established that a wide variety of nitro-aromatic and nitro-heterocyclic compounds, particularly nitroimidazoles, are known to act as radiosensitizers of hypoxic mammalian cells *in vitro* [1]. The sensitizing efficiency of any particular compound *in vitro* has been quantitatively established to be a function of its electron affinity, e.g., the one-electron reduction potential at pH 7 (E_7^1) [2]. Adams *et al.* [3] have demonstrated that both chronic aerobic toxicity and radiosensitization are phenomena which correlate with electron affinity. The correlation of aerobic cytotoxicity with reduction potential obtained for nitro compounds was explained by suggesting that in air the toxic action of the nitro compounds was through interference with electron-transport processes.

The fact that aerobic cytotoxic properties correlate with electron affinity besides lipophilicity, suggests that it might be difficult to improve the therapeutic ratio of any sensitizer. However, chronic aerobic toxicity is not the limiting factor in the use of these compounds as radiosensitizers or as hypoxic cell cytotoxic agents. For metronidazole (METRO), misonidazole (MIS), [1-(2-nitro-1-imidazole)-3-methoxypropan-2-ol], SR-2508, and desmethyl misonidazole, drugs which have already undergone clinical trials, neurotoxicity is the most severe limitation to the drug levels and hence to the degree of radiosensitization which can be achieved [4, 5]. Maximum tolerated doses are far below those which would produce a cytotoxic effect in aerobic cells [6].

The requirement for a replacement of the drugs above cited as radiosensitizers of hypoxic cells in human tumours, particularly of MIS, which has been the nitroimidazole most used for clinical applications, can in principle be met in two different ways. First, the new drug could be a more efficient radiosensitizer and be equally as toxic as MIS. Alternatively, the new compound could be equal to MIS in its radiosensitizing ability but less toxic, thus allowing more drug to be given.

Recently, the radiosensitizing ability of some transition metal–nitroimidazole complexes has been assessed. In particular, attention has been focused on *cis* complexes of Pt(II) containing 2- or 5-nitroimidazoles as ligands [7, 8], Rh(II) carboxylates combined with 2-nitroimidazoles [8], and Ru(II) complexes containing 2-,4- or 5-nitroimidazoles [9]. These complexes exhibit an increase in E_7^1 compared to the free ligand and for this reason they should theoretically have an enhanced radiosensitizing effect. Indeed, Rh and Ru complexes show much greater sensitization than can be obtained with the free ligand and encourage further investigation.

Our interest has turned to biologically relevant metals such as Co(II), Fe(III) and Zn(II). In a previous paper the interaction between these ions and MIS or its demethylated metabolite has been studied

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by means of electrochemical techniques [10]. The results obtained have been explained by hypothesizing the formation of a complex.

The present work has been undertaken to characterize better the interaction Zn(II)–MIS in view of the fact that preliminary clinical tests show a decrease in the neuropathies caused by MIS when administered with zinc sulphate.

Experimental

Chemicals

Analytical and reagent grade chemicals were used throughout. The reagent solutions were prepared in doubly distilled water. Fetal calf serum, growth medium and antibiotics were purchased from Biochrom KG, Beteiligungs Gmbh & Co., Berlin. A solution of carrier free $^{65}ZnCl_2$ in 0.1 M HCl was purchased from Amersham. MIS was supplied by Hoffmann La Roche, Basle, Switzerland. The reduction products of MIS, AZOMIS and AZOXYMIS were synthesized in our laboratory as previously described [11]. Zinc (^{65}Zn) oxalate was prepared from $^{65}ZnCl_2$ and oxalic acid.

Electrochemical Experiments

All electrochemical experiments were performed on solutions deaerated with nitrogen. Differential pulse polarography (DPP) was carried out with an Amel Model 472 multipolarograph (Milan, Italy), using a dropping mercury electrode (DME) as working electrode and a saturated calomel electrode (SCE) as reference on solutions of MIS and/or zinc ions in ethanol/aqueous KCl 0.1 M (50:50, *v/v*).

Controlled potential electrolysis (CPE) was performed on a mercury pool cathode with an Amel Model 550 potentiostat at a potential of –940 mV *vs.* SCE, corresponding to the plateau of the reduction wave of MIS in ethanol/aqueous KCl 0.1 M solution. The reduction of MIS was carried out in the presence of solid ^{65}Zn oxalate.

Spectroscopic Measurements

UV spectra were recorded on water/ethanol (50:50, *v/v*) solutions prepared by mixing appropriate quantities of MIS and metal salt. Raman spectra were obtained in aqueous solutions by using an Ar^+ laser, $\lambda_{exc} = 488$ nm; infrared spectra by the KBr disc technique. The solid examined was obtained by crystallization of solutions of MIS and zinc ions in a 1:2 ratio in water/ethanol (50:50, *v/v*).

Radiolysis

Steady-state radiolysis was carried out with a Gamma cell 220 ^{60}Co source with a dose rate of 12.75 Gy min^{-1} as measured by Fricke dosimetry. Aqueous solutions (2 ml samples) containing MIS (10^{-4} M), GMP (5×10^{-4} M), or zinc ions (2×10^{-4} M) and mixtures of the compounds were saturated with nitrogen and irradiated to a total dose of 500 Gy. The released inorganic phosphate was measured immediately after irradiation by means of a standard method by Merck.

Radiochromatography

Chromatography was performed on 0.25-mm thick silica gel layers (Merck) using acetone or the mixture CH_3OH–CH_2Cl_2 (1:4, *v/v*) as eluent. After development the radioactivity of the spots was monitored either by autoradiography or by a radio TLC scanner (Berthold, Model 2821).

Radiochemical Evaluation of Complex Formation

Solutions containing MIS or AZOMIS or AZOXYMIS at different concentrations were added to zinc (^{65}Zn) oxalate and kept under stirring. After 24 h the suspension was centrifuged and the gamma activity of the supernatant was counted and compared to a blank consisting of a suspension of zinc (^{65}Zn) oxalate. Furthermore, a measurement was made using EDTA as test ligand.

Biological Experiments

Chinese hamster V79 cells were used for the biological tests. They were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, non-essential amino acids, L-glutamine and antibiotics (penicillin 100 units/ml; streptomycin 100 μg/ml) in a humidified atmosphere of 5% CO_2 in 95% air at 37 °C. Cells were removed from stock flasks by trypsinization, counted, diluted in physiological saline (PBS) and prepared for the experiments by seeding on Petri dishes (about 200 cells/dish). After about 24 h the cells were used for testing both chronic and acute cytotoxicity of the compounds of interest, which was based on the determination of cell survival by their colony-forming ability after 10 days of growth at 37 °C. Misonidazole and/or $ZnSO_4$ was dissolved at a concentration of 1 mM in the growth medium and the solutions were filtered through a Millipore filter, pore size 0.2 μm.

For the chronic cytotoxicity tests, the growth medium was removed by aspiration, replaced with the medium containing the drugs, and the cells were incubated for 10 days. For the acute cytotoxicity tests, the growth medium was removed, cells were washed with PBS and exposed to the drugs dissolved in PBS for 2 h at room temperature. The solution containing the drugs was then removed, cells were washed with PBS and incubated with fresh growth medium for 10 days. At the moment of counting the colonies, the growth medium was removed, cells were washed with PBS, fixed at first with a solution of methanol/PBS (50:50 *v/v*) and then with pure methanol and stained with methylene blue. For each

experiment of toxicity, the colony-forming ability of the cells exposed to the drug was compared with appropriate blanks which underwent no treatment.

Results and Discussion

The reduction curves of MIS, obtained by DPP in the absence or presence of zinc ions, are shown in Fig. 1. The more relevant result is the disappearance of the reduction peak of the sensitizer and the appearance of a peak at a more positive potential. This behaviour could be explained by the hypothesis of the formation of a complex between MIS and zinc ion. In order to support this hypothesis, a series of experimental measurements was carried out. Conductance values obtained for solutions of MIS and/or zinc ions in ethanol/water (50:50 *v/v*) are reported in Table I. As can be seen, the presence of MIS does not influence the conductance of zinc ions. This result is in disagreement with the hypothesis of the formation of a complex. The same conclusion can be drawn from the spectroscopic measurements.

In the UV spectrum of a solution of MIS/Zn^{2+} there is neither a shift of the band at 323 nm due to the nitro-aromatic chromophore nor a change in its intensity in comparison to the pure radiosensitizer.

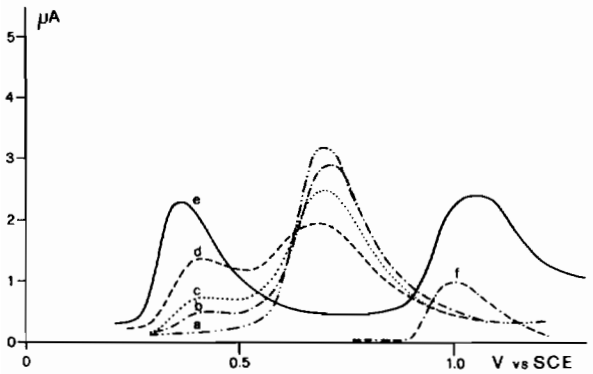

Fig. 1. Differential pulse polarography of: MIS 1.8×10^{-4} (a), MIS + Zn^{2+}, molar ratios 4:1 (b), 2:1 (c), 1:1 (d), 1:4 (e), Zn^{2+} 10^{-4} M (f), in KCl 0.1 M/ethanol (50:50 *v/v*) solutions.

TABLE I. Conductance Values of Ethanol/Water Solutions of MIS/Zn^{2+}

Sample	Conductance (μS)
Ethanol/water	3.8
MIS 10^{-3} M	2.4
Zn^{2+} 2×10^{-3} M	145.0
MIS 10^{-3} M + Zn^{2+} 2×10^{-3} M	143.0

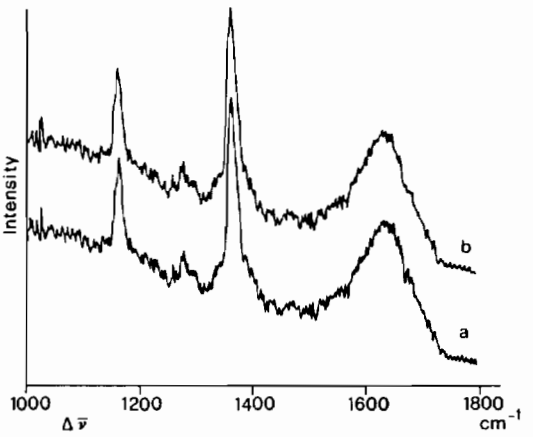

Fig. 2. Raman spectrum of MIS (a) and MIS + Zn^{2+}, molar ratio 1:2 (b), in aqueous solution.

Analogously, the IR and Raman spectra show that the presence of zinc ions do not produce any change in the characteristic bands of MIS. The IR spectrum has been reported elsewhere [11] and the Raman spectrum is shown in Fig. 2.

Further indirect evidence that Zn^{2+} does not interact directly with MIS is given by the radiolytic experiments performed in aqueous solutions of GMP in the presence of MIS and zinc ions. It is well known that hydroxyl radicals produced by gamma radiolysis of a water solution are the major species responsible for radiation-induced inorganic phosphate release from this kind of phosphate ester [12]. Raleigh *et al.* [12] have demonstrated that Pi release from GMP is enhanced by the presence of radiosensitizers such as MIS, and that this enhancement is related to the reduction potential of the radiosensitizer. If the peak at −370 mV shown in Fig. 1 were attributable to a stable complex between Zn^{2+} and MIS, an increased yield in Pi should be expected when GMP is irradiated in the presence of MIS and zinc ions. In the following scheme are reported the results obtained by gamma radiolysis.

H_2O ∿∿→	·OH + . . .	
GMP	Pi	G(Pi) = 0.22
GMP + MIS	Pi	G′(Pi) = 0.26
GMP + MIS + Zn^{2+}	Pi	G″(Pi) = 0.26

As can be seen, the zinc ions do not affect the yield in Pi, as shown by G(Pi) values.

Also the radiochromatography of a solution of MIS/$^{65}Zn^{2+}$ (molar ratio 1:2) revealed a single peak corresponding to the free $^{65}Zn^{2+}$.

To explain the electrochemical results the possibility of the formation of complexes with some final reduction products of MIS has been considered, exploiting the solubility equilibrium of ^{65}Zn oxalate, according to the scheme:

$$^{65}ZnOx \rightleftharpoons {}^{65}Zn^{2+} + Ox^{--}$$

$$^{65}Zn^{2+} + nL \rightleftharpoons {}^{65}ZnL_n{}^{2+}$$

If any interaction between zinc ions and the compounds examined occurs, an increase in radioactivity of the supernatant must result. Figure 3 shows the radioactivity of the supernatant of a suspension of $^{65}ZnOx$ added alternatively with different amounts of MIS, AZOMIS and AZOXYMIS. The effect of EDTA, which is known to complex Zn^{2+}, on the solubility equilibrium of $^{65}ZnOx$ is also reported. It is evident that neither MIS nor AZOMIS nor AZOXYMIS is able to form stable complexes with zinc ions.

Therefore, the results obtained by DPP can be explained by assuming that Zn^{2+} can interact with one or more short-lived intermediates generated during reduction of MIS, such as radical anions, nitroso and hydroxylamine derivatives. With this aim the electrolysis of MIS was performed in the presence of $^{65}ZnOx$, and the gamma activity of ^{65}Zn was measured in the supernatant during the course of reduction. Figure 4 shows the results obtained. It is evident that Zn^{2+} interacts with at least one of the reduction products. As a consequence, it can be argued that zinc ions can act as scavengers of the reduction intermediates of MIS which might be responsible for cellular toxicity [13]. This hypothesis is supported by the results obtained for both chronic and acute toxicity of MIS towards Chinese hamster V79 cells. In fact, a decrease in toxicity of MIS is found when cells are incubated in the presence of zinc ions. A similar result has been obtained for another radiosensitizer, metronidazole (Fig. 5).

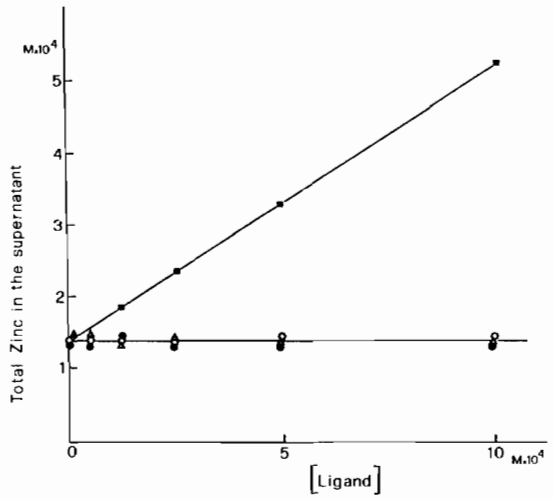

Fig. 3. Concentration of ^{65}Zn in the supernatant *vs.* the concentration of MIS, AZOMIS, AZOXYMIS and EDTA as hypothetical ligands.

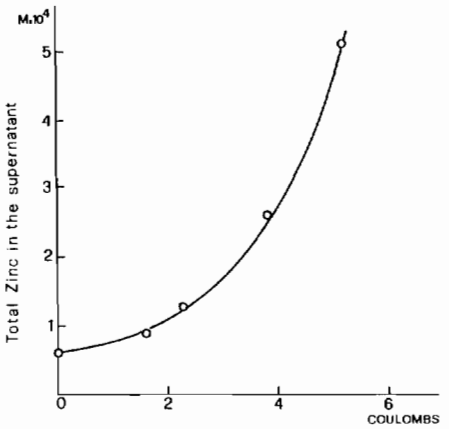

Fig. 4. Concentration of ^{65}Zn in the supernatant *vs.* the charge supplied during the electrolysis of MIS 10^{-3} M in KCl 0.1 M/ethanol performed at −940 mV (*vs.* SCE) in the presence of $^{65}ZnOx$.

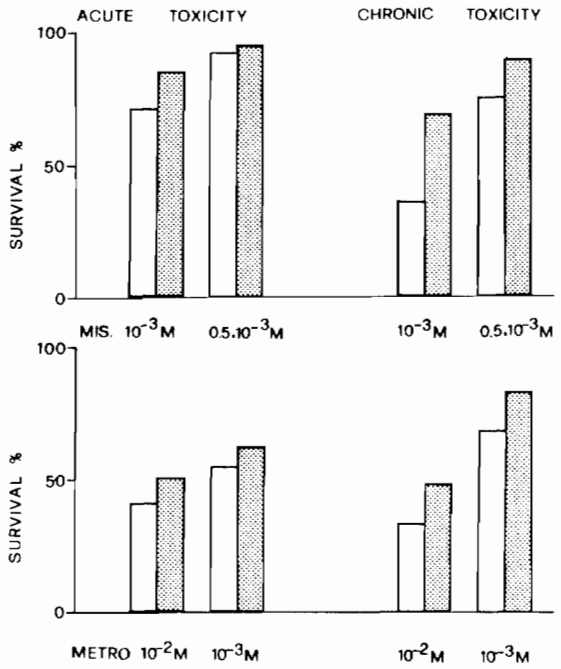

Fig. 5. Acute and chronic toxicity of MIS and METRO towards Chinese hamster V79 cells. Empty boxes refer to the drug alone, full boxes to the drug in combination with $ZnSO_4$.

These biochemical experiments are in agreement with preliminary clinical trials on patients affected by bladder tumours to whom MIS was administered in combination with $ZnSO_4$. In the cases examined a drastic decrease in neuropathies induced by MIS was observed.

Acknowledgements

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