COMBINATION STUDIES WITH MISONIDAZOLE AND A CIS-PLATINUM COMPLEX: CYTOTOXICITY AND RADIosenSITIZATION IN VITRO

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Summary.—*cis*-Dichlorodiammineplatinum(II) (cis-Pt(II)) can act as a radio-
sensitizer of hypoxic mammalian cells in vitro. Used in combination with misonid-
azole the level of sensitization achieved is greater than that seen with either drug
alone, and it is suggested that these compounds sensitize by independent mechanisms.

For cells held at 37°C, cis-Pt(II) shows much greater toxicity to hypoxic cells than
to aerobic cells. In combination with misonidazole, no additional cytotoxic effect is
shown towards aerobic cells than that seen for cis-Pt(II) alone. However, there is
additional killing of hypoxic cells when they are treated with both drugs.

The use of misonidazole (MISO) as a radiosensitizer of hypoxic cells in tumours
is well established (for review see Adams et al., 1978). In addition, a potential role
for its use in chemotherapy is being considered because of the ability of this drug
to be preferentially cytotoxic towards hypoxic cells (Hall & Roizin-Towle, 1975;
Moore et al., 1976; Sridhar et al., 1976; Stratford & Adams, 1977). If MISO is to
be of value in chemotherapy it is necessary to use it in combination with drug(s)
which can sterilize the aerobic fraction of tumour cells. We are currently studying
the cytotoxic effect of some common anti-neoplastic drugs, including a *cis*-Platinum
complex, *cis*-dichlorodiammineplatinum(II) (cis-Pt(II)). cis-Pt(II) is a proven
 cytotoxic agent (see Roberts & Thomson, 1979, for review) and can also act as a
radiosensitizer of hypoxic cells (Richmond & Powers, 1976; Richmond et al., 1977;
Douple & Richmond, 1978; Nias et al., 1979). Therefore we have examined the
radiosensitizing and cytotoxic properties of MISO and cis-Pt(II) in combination.

MATERIALS AND METHODS

Cells.—Chinese hamster V79-379A cells
used in this work were maintained in spinner
culture in Eagles' Minimal Essential Medium
(MEM) modified for suspension cultures (Flow
Laboratories Ltd.) supplemented with 7.5% foetal calf serum (FCS, Gibco-Biocult Ltd.).
Cells were kept in log phase at concentrations
ranging between 10^5 and 10^6/ml.

Cytotoxicity experiments.—250ml spinner
flasks were fitted with a gas inlet/outlet system
and a sidearm through which samples
could be withdrawn. Asynchronous, log-
offase cells at a concentration of 2x10^5/ml
were suspended in MEM + 7.5% FCS and held
in a water bath at 37°C. The compounds
were dissolved in MEM + 7.5% FCS and added
to the suspension which was buffered with bicarbonate to pH 7.4. When appropriate,
the spinner containing cells was deaerated by
flowing N2 plus 5% CO2 (<10 pts/10^6 O2;
BOC Ltd) at 500 ml/min over the surface of
the stirred suspension throughout the experiment. Samples of cells were withdrawn at
appropriate times, centrifuged, resuspended,
counted, diluted, plated in MEM + 15% FCS
and incubated for 7–10 days at 37°C before
scoring for colony formation. Further details

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of the technique are described elsewhere (Stratford & Adams, 1977).

*Radiation experiments.*—Cells were harvested from log-phase cultures, diluted appropriately and added to glass Petri dishes containing 2.5 ml MEM, supplemented with 15% FCS. Cells were allowed to attach at 37°C for 2-3 h before the medium was removed and replaced with fresh medium containing drug(s). Irradiations with 60Co γ-rays were carried out in “dural” containers which can hold 4 Petri dishes (Cooke et al., 1976). These vessels were made hypoxic by purging with N2 + 5% CO2 (BOC Ltd) for 1 h, after which the vessels were sealed and irradiated at room temperature at a dose rate of 4.2 gray/min. After irradiation, the medium was removed and replaced with fresh MEM + 15% FCS and the cells incubated for 7-9 days before scoring for colony formation.

*Compounds.*—Misonidazole was supplied by Dr C. Smithen of Roche Products Ltd, Welwyn Garden City, Herts, and cis-Pt(II) by Dr K. Harrap, Institute of Cancer Research, Sutton, England.

**RESULTS**

*Cytotoxicity studies*

The effect of 5 mM MISO (1 mg/ml) on Chinese hamster V79-379A cells held under aerobic or hypoxic conditions is shown in Fig. 1. At this concentration MISO has no cytotoxic effect on cells in air over the duration of the experiments (5 h). In contrast, MISO under hypoxic conditions reduces the surviving fraction to $5 \times 10^{-3}$. In the absence of MISO, hypoxic cells show no loss of viability.

A similar series of experiments were done with 10 μM cis-Pt(II) (3 μg/ml) under aerobic and hypoxic conditions at 37°C. These results are shown in Fig. 2. When cells are held in air 10 μM cis-Pt(II) reduces survival in an exponential manner and after 5 h the surviving fraction of cells is $10^{-2}$. However, under hypoxic conditions cells are very much more sensitive to treatment with cis-Pt(II), and after 5 h exposure survival is reduced to $10^{-5}$.

The results of cytotoxicity experiments when 5 mM MISO and 10 μM cis-Pt(II) are used in combination are illustrated in

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**Fig. 1.**—Toxicity of 5 mM MISO to V79-379A cells in vitro. ○, 5 mM MISO under hypoxic conditions; □, 5 mM MISO in air; X, cells under hypoxia alone. Data points are means from 7 replicate experiments.

**Fig. 2.**—Toxicity of 10 μM cis-Pt(II) to V79-379A cells in vitro. ○, cells under hypoxic conditions (4 replicate experiments); ●, cells in air (2 replicate experiments).
COMBINATION OF MISO AND CIS-Pt (II)

1. Sensitization experiments

Following the protocol of previous studies (Adams et al., 1976, 1979) we determined enhancement ratios for radiosensitization of hypoxic Chinese hamster cells in vitro by MISO and cis-Pt(II). At the concentrations tested, both drugs were non-toxic to non-irradiated cells over a 2h

Fig. 3.—Toxicity of the drug combination 10µM cis-Pt(II) + 5mM MISO to V79-379A cells in vitro. □, cells under hypoxic conditions (4 replicate experiments); ●, cells in air (2 replicate experiments). The dashed lines are the responses for cells treated with 10µM cis-Pt(II) (Fig. 2). The dotted line is the response for hypoxic cells treated with 5mM MISO (Fig. 1).

Fig. 4.—Survival curves for V79-379A cells irradiated under hypoxic conditions. +, hypoxia alone; ○, 5µM cis-Pt(II); △, 1mM MISO; ●, 5µM cis-Pt(II) + 0.2mM MISO.

Fig. 5.—Dependence of enhancement ratio for irradiated hypoxic V79-379A cells on MISO concentration. Dashed line, MISO alone (Adams et al., 1976); ○, MISO + 5µM cis-Pt(II); the dotted line is the enhancement ratio for 5µM cis-Pt(II) alone.
contact time in hypoxia at room temperature. Full survival curves, examples of which are shown in Fig. 4, were obtained for each compound at one or two concentrations in N₂. Assuming an unchanged extrapolation number, enhancement ratios for other concentrations were calculated from a single survival point (usually between $2 \times 10^{-2}$ and $10^{-1}$) which was obtained by appropriate choice of radiation dose.

Enhancement ratios (ER) obtained for hypoxic cells irradiated in the presence of MISO alone were similar to those reported previously for this cell line (Adams et al., 1976) and this dependence of ER on MISO concentration is shown by the dashed line in Fig. 5. The maximum concentration of cis-Pt(II) tested was 5 μM, and this gave routinely an ER of 1.12 (see Fig. 4). For combination studies, the radiation response of hypoxic cells was determined for 5μM cis-Pt(II) with concentrations of MISO varying from 10 μM up to 2 mM. Fig. 4 shows a survival curve for hypoxic cells irradiated in the presence of 0-2 mM MISO plus 5μM cis-Pt(II). This combination results in a survival curve which is identical to that obtained for 1 mM MISO alone. Values of ER for 5μM cis-Pt(II) with a range of MISO concentrations are shown as the open circles in Fig. 5. Clearly, the ERs for 5μM cis-Pt(II) plus MISO are greater than those for MISO alone. In addition, the response curves (Fig. 5) appear parallel, which suggests that the two compounds show a degree of additivity that cannot be explained if they sensitize wholly by the same mechanism(s).

**DISCUSSION**

cis-Platinum complexes can increase the radiation sensitivity of cells in vitro under both aerobic and hypoxic conditions (Richmond & Powers, 1976; Szumiel & Nias, 1976; Nias & Szumiel, 1977; Richmond et al., 1977; Douple & Richmond, 1978) with the greatest effect apparently on the hypoxic cells (Richmond & Powers, 1976; Richmond et al., 1977; Douple & Richmond, 1978; Nias et al., 1979). In the present work 5μM cis-Pt(II) increases the radiation sensitivity of hypoxic mammalian cells, giving an ER of 1.12. The combination of cis-Pt(II) and MISO gives at least an additive response for radiation sensitization of hypoxic cells. This can be rationalized if we consider that the ER of 5μM cis-Pt(II) (1.12) is equivalent to 20μM MISO. On the basis of simple equivalence, if the two compounds are acting similarly, the combination of 5μM cis-Pt(II) and 20μM MISO should give an ER similar to that seen for 40μM MISO, viz. 1.20. In fact the observed ER is about 1.40, a value that could only be expected if the compounds are operating on different lesions and/or by different mechanism(s). If this effect were to hold for other cis-Platinum complexes, and if such complexes in combination with MISO produced no increased toxicity to man, this type of drug combination could substantially increase ERs obtainable in radiotherapy.

We have demonstrated that, as a cytotoxic agent, cis-Pt(II) is considerably more toxic to hypoxic cells than to aerobic cells. The difference in toxicity is seen after only 2h exposure to the drug, which is likely to preclude the effect being due to any cell-cycle redistribution induced by hypoxia (Roberts & Fraval, 1978). It is possible that the anaerobic environment allows biochemical reduction of cis-Pt(II) to a platinum (I) intermediate. This is a highly reactive species (Richmond & Simic, 1978) which could interact with vital cellular macromolecules. Alternatively, hypoxia may inhibit repair of DNA damage caused by cis-Pt(II), and this may also modify radiation sensitivity.

The differential cytotoxicity of cis-Pt(II) may be important in influencing the efficacy of the cis-platinum complexes used clinically, since there is evidence that hypoxic cells may be resistant to some chemotherapeutic drugs. There are two possible reasons for this hypoxic resist-
rance. Firstly, hypoxic cells tend to be located near necrotic or poorly vascularized regions in tumours, and therefore these cells are probably less accessible to some chemotherapeutic agents. Secondly, clonogenic cells temporarily rendered hypoxic may become non-cycling, or have their progression through the cell cycle slowed down.

Previous work has shown that bleomycin, actinomycin D and Adriamycin are less effective in killing hypoxic than aerobic cells (Roizin-Towle & Hall, 1978; Adams et al., 1979; Sutherland et al., 1979; Smith et al., unpublished data). It has been suggested that drugs that are specifically toxic to hypoxic cells may be useful in improving combination chemotherapy. Some nitro-aromatic and nitro-heterocyclic compounds show this property (Adams et al., 1980) and one of them, MISO, has suitable pharmacological characteristics of bioavailability and tumour penetration (Dische et al., 1977; Ash et al., 1979).

The combination of MISO and cis-Pt(II) kills hypoxic cells much more effectively than either drug alone. Therefore, if potentially clonogenic hypoxic cells are important in tumour response to chemotherapy, a combination of MISO and cis-Pt(II) may be useful, particularly since the in vitro data reported here show no additional killing of aerobic cells.

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