CYTOTOXICITY OF METRONIDAZOLE (FLAGYL) AND MISONIDAZOLE (Ro-07-0582): ENHANCEMENT BY LACTATE

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Summary.—The cytotoxic activity of metronidazole (Flagyl) and misonidazole (MISO) to hypoxic Chinese hamster ovary (CHO) cells suspended in standard medium in sealed vials and in gassed spinner flasks has been investigated. Flagyl (5 mM) was only cytotoxic at high initial cell densities. However, when lactate (20 mM) was included in the medium the cytotoxicity of Flagyl at low cell densities was considerable, and similar to that of misonidazole under the same conditions. The relevance of this “lactate effect” to in vivo systems, and the possible mechanisms involved, are discussed.

Metronidazole (Flagyl) and misonidazole (MISO) are currently under clinical investigation as hypoxic radiosensitizers (Urtasun et al., 1975; Dische & Saunders, 1978; Wiltshire et al., 1978). It has long been known that both drugs are appreciably more toxic to anaerobic than to aerobic microorganisms (Prince et al., 1969). The possibility that both drugs may be used as selective cytotoxic agents towards hypoxic mammalian cells is currently attracting considerable attention (Sutherland, 1974; Foster & Willson, 1976; Foster et al., 1976; Sridhar et al., 1976; Mohindra & Rauth, 1976; Hall et al., 1977, 1978; Foster, 1977; Taylor & Rauth, 1978; Adams et al., 1980; Whillans & Rauth, 1980; Pettersen, 1978).

Although in several anaerobic protozoal and bacterial systems Flagyl has been shown to be the more potent drug (Prince et al., 1969; Basaga et al., 1978), studies reported to date indicate that in vitro MISO is the more active cytotoxic agent towards mammalian cells (Adams et al., 1980). During studies into the possible cytogenetic effects of these drugs we have found that the activity of both drugs is strongly dependent on the initial cell density used in the study. Furthermore, the activity of Flagyl is more strongly affected than MISO. We now report studies in which we have investigated this phenomenon further, using different incubation conditions and media supplemented with sodium lactate.

MATERIALS AND METHODS

Chinese hamster ovary (CHO-kl) cells were grown in glass roller bottles in medium (Ham F12, Flow Laboratories, supplemented with 10% foetal calf serum). The same medium was used throughout the study unless otherwise stated.

Cells to be incubated in sealed ampules (Hall et al., 1977) were suspended in medium at an initial density of 5 x 10^6 cells per ml and 1.2ml aliquots placed in glass ampules (Pierce and Warriner (U.K.) Ltd) of nominal 1ml capacity. These were subsequently sealed in a flame after the air above the medium had been replaced by a 95% N_2, 5% CO_2 mixture (BOC Ltd). Ampoules were then incubated at 37°C in a shaking water bath and at various times removed and sterilized by immersion in alcohol before opening. Under these conditions the metabolic activity of the cells is assumed to produce complete oxygen depletion (Hall et al., 1977).

In studies using spinner flasks, cells were suspended in medium (7% serum) and 50ml volume gently stirred at 120 rev/min in 50ml Bellco flasks with 95% N_2, 5% CO_2 (1 L/min) flowing over the surface of the medium.
Total cell numbers were determined with a Coulter counter, and viable cell numbers by colony counting after 7–10 days’ incubation at 37°C. Before appropriate serial dilution and plating on 5cm dishes, cells were re-suspended in equal volumes of fresh medium. Regular microscopical examination showed no indication of cell aggregation.

Flagyl was kindly supplied by May and Baker Ltd and MISO by Professor G. E. Adams and Roche Products Ltd. L(+) lactic acid (Sigma) was titrated to pH=7.4 with NaOH before use.

RESULTS

On incubation of CHO cells at an initial density of $5 \times 10^6$/ml in ampoules, both Flagyl and MISO (5 mM) were cytotoxic (Fig. 1a). In the case of metronidazole the percentage cell viability dropped to 0.3% in 8 h and for MISO to 0.09% over the same period. Since the activity of Flagyl was much greater than previously reported (Adams et al., 1980) experiments were repeated in spinner flasks with an initial cell density of $5 \times 10^4$/ml. Under these conditions Flagyl showed little toxicity, the percentage cell viability dropping to only 65% after 8 h. MISO however again showed greater activity, the cell viability after 8 h dropping to 12.5% of controls, and to 2% by 10 h (Fig. 1b).

In the light of these results and the differential cell densities used in the experiments, the effect of initial cell density on the activity of Flagyl on cells in spinner flasks was undertaken. Up to an initial cell density of $5 \times 10^5$/ml Flagyl again showed little activity, but at a density of $5 \times 10^6$/ml considerable toxicity was apparent (Fig. 2). Initially it was thought that the difference in activity might be due to O$_2$ contamination, the toxic effect of Flagyl is considerably reduced if not eliminated by O$_2$ (Foster et al., 1976; Sridhar et al., 1976; Basaga et al., 1978; Stratford, 1978; Whillans & Rauth, 1980; Willson, 1977).

On re-examination of the experimental results and procedure, however, this was considered unlikely, and the alternate possibilities that the effect could be associated with a lowering of pH at the highest cell density or to the build-up of a metabolic product was considered. Although in the studies in sealed ampoules the internal pH indicator did change from the normal

![Fig. 1](image-url)

Fig. 1.—Percentage viability of hypoxic CHO cells incubated with 5 mM metronidazole (Flagyl) or misonidazole (MISO) in (a) sealed ampoules (initial cell density $5 \times 10^6$/ml) and (b) spinner flasks (initial cell density $5 \times 10^4$/ml). ● Control, ■ Flagyl, ▲ MISO.
red to yellow, indicating a drop from pH 7.4 to ~6.8, no change was apparent in spinner flasks, and in view of the buffering capacity of the system the possibility that pH lowering was the principal reason for the observed differences was thought slight. Prompted by recent work (Mother-
sill et al., personal communication) showing that lactate has a pronounced effect on the radiosensitivity of CHO cells and that lactate concentration approaching 20 mm can build up in suspensions, it was thought useful to examine the effect of this metabolite on drug activity.

The effect of various drug–lactate combinations on the viability of hypoxic cells (initial density $2 \times 10^4$/ml) incubated in spinner flasks is shown in Fig. 3. Lactate at a concentration of 2 mm had little effect on Flagyl activity, but at 20 mm appreciable cell killing was observed. In the absence of lactate MISO showed little toxicity up to 6 h, but significant killing then followed. In the presence of 20 mm lactate, the marked drug resistance at shorter times was absent. After 10 h both drugs showed an increased toxicity, the cell viability falling from 55-4 to 0.16% for Flagyl and from 7-5 to 0.09% with MISO. Sodium lactate (20 mm) had little effect on cell viability over 10 h incubation.

In order to examine whether the effect of lactate on Flagyl toxicity is confined to hypoxic cells, a preliminary experiment under euoxic conditions was undertaken. In the absence of lactate, 5 mm Flagyl
cytotoxicity has been shown for 5 and 10 mM Flagyl to Ehrlich ascites carcinoma cells (Foster et al., 1976). It is interesting that appreciable concentrations of lactic acid have been found in the medium in which these cells are incubated (Shapot, 1972). As to the explanation of this “lactate effect” we are currently investigating possibilities associated with the enzyme lactate dehydrogenase (LDH), which facilitates the equilibrium reaction:

\[
\text{lactate} + \text{NAD}^+ \xrightleftharpoons{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+.
\]

High lactate concentrations may be associated with:

(a) An increased activation of the drugs through the formation of NADH and a general raising of the reduction potential of the cell.

(b) A decrease in the cells’ ability to dispose of reducing equivalents to the surrounding medium.

(c) An increase in the levels of the LDH–NADH or related enzyme complex, sensitive to attack by drug-free radicals.

(d) A decrease in the intracellular pH at critical sites.

(e) An increased uptake of the drugs.

It remains possible that high lactate concentrations somehow lead to a decrease in the level of undetected traces of O₂ which, if present, could offer some degree of protection (Willson et al., 1974; Foster & Willson, 1976). A recent report (Sridhar & Koch, 1978) that sodium oxamate, a lactate dehydrogenase inhibitor, is toxic to Chinese hamster V79 cells under hypoxic but not aerobic conditions, does however lend support to a direct involvement of a lactate-related enzyme.

Meanwhile, whatever the mechanism, the dramatic effect of lactate demonstrated here clearly should be taken into account when considering the relevance of in vitro studies to in vivo tumours. Furthermore, the metabolism of lactate in skin, a tissue used by several groups to investigate normal tissue reactions (Stone...
& Withers, 1974; Denekamp et al., 1974) Johnson et al., 1976; Dische et al., 1976; is known to differ from that in other tissues such as muscle or liver (Meir & Cotton, 1976). With CHO cells in vitro in the presence of lactate, Flagyl is as effective as MISO at the same concentration used. The fact that Flagyl is better tolerated in animals and in the clinic, and that higher serum levels of the drug can be achieved, seems to us to point to the need for a reappraisal of its usefulness as an adjunct in cancer therapy.

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