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METRONIDAZOLE (FLAGYL), A RADIOSENSITISER OF POSSIBLE CLINICAL USE IN CANCER CHEMOTHERAPY: SOME BIOCHEMICAL AND PHARMACOLOGICAL CONSIDERATIONS

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Abstract—Pharmacokinetic experiments have shown that radiobiologically significant levels of metronidazole (Flagyl) can be achieved in the serum, brain, liver and kidneys of rats following oral administration of the compound. Drug doses of 1 g/kg body wt in rats bearing Walker 256 sarcoma resulted in tumour levels of approx 1 mM (170 μg/ml) 1 hr post-treatment. No change occurred in the weights of liver, kidneys, spleen, lungs, heart and adrenals or in the erythrocyte count following administration of metronidazole (0·2 g/kg body wt) over a period of 6 weeks compared to the control group. The body weight gain of the metronidazole-treated animals in the 6-week period showed a small but significant decrease; a slight leucocytosis was observed but this was not statistically significant. No significant change in liver cytochrome P-450, NADPH-cytochrome *c* reductase, aminopyrene demethylation or glucose-6-phosphatase levels were observed in rats given 1 g/kg body wt metronidazole daily for 4 days compared to water treated control animals. However, a small but significant decrease in NADPH-linked lipid peroxidation and NADPH-neotetrazolium reductase activity was observed. Several enzymatic parameters in serum remained unchanged. A 2-fold increase in the weight of the caeca of drug treated animals was associated with a change in the bacterial flora of their intestinal contents. Whereas the contents of the caeca of the control group rapidly degraded metronidazole, those of the drug-treated group did not. The significance of these results to the use of metronidazole in cancer radiotherapy is discussed.

It is believed that the failure to cure a primary tumour by radiotherapy is due in some instances to the presence of malignant cells that are relatively distant from a capillary vessel at the time of treatment; in consequence, oxygen diffusing from the capillaries may be utilized prior to reaching these cells. At the time of radiation treatment a proportion of these cells may be viable but extremely hypoxic. Unfortunately hypoxic cells are much more resistant to radiation killing than normal cells and following radiation exposure may remain undamaged. On subsequent shrinkage of the tumour the oxygen supply to the previously hypoxic zones may be improved and rapid regrowth of the remaining tumour cells may occur.

Attempts to overcome this hypoxic cell problem have included the use of hyperbaric oxygen [1]. Clinical trials to date, however, have yielded equivocal data [2]. Following the discovery that several organic nitroxyl derivatives selectively sensitized hypoxic cells to radiation damage, attempts have been made to discover other drugs with more favourable pharmacological properties [3, 4]. If such drugs are not metabolized as rapidly as oxygen they may diffuse further within the tumour mass and consequently lead to a generally increased sensitivity of the tumour to radiotherapeutic treatments.

Recently several organic nitro compounds used in the treatment of urinary tract infections, have shown considerable promise in radiosensitising applications [5–17]. In particular, studies with metronidazole ('Flagyl', May and Baker Ltd.) showed that radiobiologically significant concentrations of the nitroimidazole could be obtained in the serum of mice following injection i.p. of one-twentieth of the LD_{50} [10]. The radiation dose which cured 50 per cent of a group of mouse mammary tumours was subsequently reduced by a factor of 1·2–1·3 when the drug was administered orally 30 min before radiation treatment [8, 9].

Whilst these results were encouraging, the drug doses administered to mice were much higher than those normally used clinically for anaerobic infestations (200–400 mg t.i.d.). For a detectable radiotherapeutic gain, doses of the order of 0·2 g/kg (i.e. 14 g/70 kg) will probably be required. Although recent preliminary clinical studies [15–17] suggest that such high levels of metronidazole may be tolerated three times per week for two weeks, it was considered that further pharmacological and toxicological studies in animals would be advisable before any long-term clinical usage of such high doses.

In this paper experiments with rats are described: additional information on the pharmokinetics of the drug administered in high doses and its effect on various liver parameters have been obtained.

METHODS

Except in the case of tumour-bearing animals where male rats were used, studies were undertaken using female albino Wistars fed diet 41B modified

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(Oxoid Ltd., London, S.E.1) and water *ad lib*. The dose of metronidazole was in the range of 0·2 to 1 g kg body wt and was usually administered orally as a suspension.

To examine the chronic effect of metronidazole, 0·2 g kg body wt was given three times a week for six weeks including the final day of the experiment. The growth rates, organ weights and their histological appearance were compared with a control group of rats which had been given an equal volume of water. Before sacrifice, a blood sample was removed from the tail vein for a blood cell count which was performed using a Coulter Counter (Coulter Electronics Ltd., Harpenden, Herts). Protein was precipitated from a serum sample (0·2 ml) by the addition of 4 ml of ethanol. Following centrifugation, the metronidazole was estimated spectrophotometrically [15] at 316 nm taking $\epsilon = 9310\ M^{-1}\ cm^{-1}$. For histological examination, tissue samples were fixed in formal saline and sections cut and stained with haematoxylin and eosin.

To examine the short term effects of metronidazole on induction of endoplasmic reticulum, the microsomal fraction was prepared by differential centrifugation in 0·25 M sucrose from the livers of rats which had received 1 g kg body wt of metronidazole daily for the 4 days before sacrifice. The microsomal pellet was resuspended in 0·15 M KCl to give the equivalent of 1 g wet wt liver ml. Protein concentration was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard. The methods of determination of NADPH-cytochrome *c* oxidoreductase, NADPH-neotetrazolium oxidoreductase activities and aminopyrene demethylation have been described previously [19, 20]. Glucose-6-phosphatase was assayed by the method described by Delaney and Slater [21] and NADPH-dependent lipid peroxidation by the system given by Jose *et al.* [22]. Blood samples were removed from these rats under pentobarbital anaesthesia and the serum levels of lactate dehydrogenase, sorbitol dehydrogenase and glutamate pyruvate transaminase were determined by the methods of the Boehringer Corporation (London) Ltd.

Walker 256 Sarcomas (Chester Beatty Cancer Research Institute, London) were induced in male Wistars by subcutaneous inguinal injection of 2×10^5 cells. After 8 days metronidazole was administered *per os* in a range from 0·25 to 1 g kg body wt. Blood samples were taken under ether anaesthesia. Tumours and brains were excised and homogenized in ice-cold borate buffer and the tissue drug concentration estimated using a polarograph (Shandon Southern Instruments), calibrated by adding known aliquots of metronidazole to tissue suspension under identical conditions. The level of metronidazole in the liver and kidney in non-tumour bearing rats was also estimated by this procedure.

In the investigation of the metabolism of metronidazole by anaerobic bacteria, a suspension of the caecal contents from both normal and metronidazole-treated rats (1 g kg body wt for 4 days) was prepared. Following cervical dislocation, the caecum was removed from the animal inside a glove box that had been flushed with oxygen-free nitrogen and the caecal contents suspended in nutrient broth containing 100 mM succinate. The suspension was filtered through gauze and an aliquot transferred to a nitrogen-flushed glass cell metabolism vessel. Metronidazole was added, and the suspension was incubated at 37° while being continually bubbled with nitrogen. At timed intervals 0·5 ml of suspension was drawn off into 1·5 ml of ethyl acetate and 0·2 ml 100 mM KOH added. After centrifugation, the metronidazole was extracted from 0·5 ml of the organic layer by the addition of 2 ml of 100 mM HCl. 1·5 ml of the aqueous layer was removed, 2 ml of 1 M KOH added and the absorbance read at 320 nm taking $\epsilon = 9310\ M^{-1}cm^{-1}$.

RESULTS

There was no change in the weights of liver, kidneys spleen, lungs, heart and adrenals or in the

Table 1. The effect of metronidazole or organ weights, blood cell count and growth rate in female rats

	Metronidazole (g)	Control (g)
Liver	9·36 ± 0·30	9·50 ± 0·24
Kidneys	1·60 ± 0·03	1·77 ± 0·04
Spleen	0·45 ± 0·02	0·56 ± 0·03
Lungs	1·23 ± 0·04	1·29 ± 0·03
Heart	0·81 ± 0·02	0·88 ± 0·03
Adrenals	0·11 ± 0·01	0·12 ± 0·01
Original body wt	157 ± 2	154 ± 2
Gain in body wt over 6 weeks	74 ± 4*	87 ± 3
Red blood cell count, million per mm^3	7·81 ± 0·13	7·98 ± 0·14
White blood cell count per mm^3	12,700 ± 1104	11,200 ± 627

* $P = > 0{\cdot}02$.

Metronidazole was given 0·2 g kg body wt 3 times a week for 6 weeks, *per os*. The control group was given an equal volume of water.

Results are expressed as means ± S.E.M. of the 12 rats in each group.

Table 2. The effect of metronidazole on caecal weight

Dose (g/kg body wt)	Route	Duration	Caecal metronidazole	Weight control
0·2 (12)	p.o.	18 doses in 6 weeks	5·7 ± 0·3*	4·1 ± 0·2
1·0 (8)	i.p.	4 doses in 4 days	7·9 ± 0·3*	3·6 ± 0·1
1·0 (12)	p.o.	6 doses in 5 days	9·9 ± 0·3*	4·2 ± 0·2

* $P > 0.001$.

The results are expressed as means ± S.E.M. with the number of animals shown in parentheses. The control group received an equal volume of water.

erythrocyte count following the administration of metronidazole (0·2 g/kg body wt) over a period of 6 weeks when compared with the control group (Table 1). There was a slight leucocytosis but this was not statistically significant. The body weight gained in the 6-week period showed a small but significant decrease in the metronidazole-treated groups (Table 1). The most striking effect of metronidazole administration was on caecal weight together with an alteration in the appearance from grey to orange–brown. The caecal weight increased with increasing doses of metronidazole (Table 2).

There was little change in cytochrome P_{450}, NADPH-cytochrome *c* reductase, aminopyrene demethylation or glucose-6-phosphatase levels in the liver microsomes prepared from rats given 1 g/kg body wt for 4 days (Table 3). However, a small but significant decrease in NAPDH-linked lipid peroxidation and NADPH–neotetrazolium reductase activity was observed. The serum sorbitol dehydrogenase, glutamate–pyruvate transaminase and lactate dehydrogenase activities remained unchanged (Table 3).

The level of metronidazole in serum, liver and kidney following oral administration of 1 g/kg body wt is shown in Figure 1. A serum concentration of over 1 mM was attained by 60 min with only a further small rise in the next hour. While the level of the drug in the liver closely followed that of serum, the values obtained for the kidney were somewhat higher (Fig. 1).

In tumour-bearing rats the serum levels were followed after a dose of 1 g/kg. Here a rapid increase in serum concentration was observed followed by a partial decrease and a late rise reaching a maximum by 60 min (Fig. 2). The level of metronidazole in brain and the Walker 256 sarcomas also gradually increased to a maximum after 1 hr but was at a lower concentration than serum (Fig. 2).

Studies with lower doses of metronidazole showed that the serum or tissue level after 60 min varied approximately linearly with dose over the range 0·2–1·0 g/kg (Fig. 3).

In view of the changes in the appearance and weight of the caeca from metronidazole treated rats and previous reports that nitrofuran derivatives [24], metronidazole [25] and chloramphenicol [26] are degraded by bacterial suspensions, the activity of the caecal contents was investigated. It was found that

Table 3. The effect of metronidazole on enzyme activities in the liver and serum of female rats

Enzyme		Metronidazole	Control
NADPH-cytochrome *c* oxidoreductase (per min 25°)	(4)	119 ± 3	108 ± 8
NADPH-neotetrazolium oxidoreductase (per 10 min 37°)	(4)	53·4 ± 5·5*	70·0 ± 4·3
Aminopyrene demethylation (HCHO per 10 min 37°)	(4)	37·5 ± 1·6*	42·8 ± 1·3
NADPH-linked lipid peroxidation (per 15 min 37°)	(4)	1·44 ± 0·12†	2·25 ± 0·12
Cytochrome P_{450}	(5)	0·35 ± 0·01	0·41 ± 0·01
Glucose-6-phosphatase (μg Pi per 10 min 37°)	(4)	17·2 ± 1·7	20·7 ± 2·0
Sorbitol dehydrogenase (mU/ml)	(6)	2·0 ± 0·4	1·8 ± 0·2
Glutamate pyruvate transaminase (mU/ml)	(6)	8 ± 1	8 ± 1
Lactate dehydrogenase (mU/ml)	(6)	117 ± 28	107 ± 21

* $P = > 0.05$

† $P = > 0.001$

Metronidazole was administered as a single daily dose at 1 g/kg body wt i.p. for 4 days. The liver enzyme activities are shown as nmoles product per mg protein. The results are expressed as means ± S.E.M. with the number of animals given in parentheses.

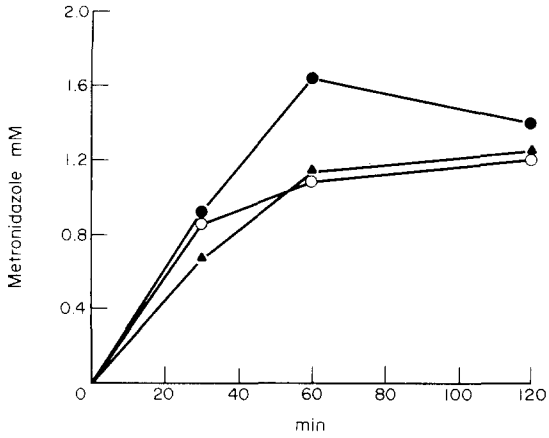

Fig. 1. The concentration of metronidazole in serum (▲ ▲), liver (○ ○) and kidney (●—●) following oral administration of 1 g/kg body wt. The results are the means of 3 animals in each group.

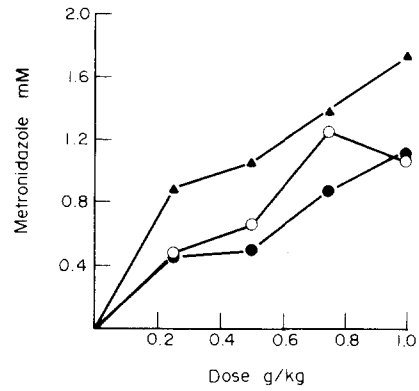

Fig. 3. The level of metronidazole in serum (▲ ▲) tumour (●—●) and brain (○ ○) 60 min after oral administration of metronidazole ranging from 0·2 to 1 g/kg body wt. The results shown are the means of 3 animals in each group.

an anaerobic caecal suspension from untreated rats rapidly metabolized metronidazole which was added to give an initial concentration of 1 mM. The fall in drug concentration was exponential with a half-life of 8·5 min. In contrast a caecal suspension from rats given metronidazole for the 4 previous days (1 g/kg body wt) failed to metabolize metronidazole (Fig. 4).

DISCUSSION

There are several criteria to be attained if a chemical is to be an effective radiosensitiser of hypoxic cells when used in conjunction with radiotherapy. Of these a maintenance of a high serum concentration for a reasonable period of time coupled with the ability to diffuse into all parts of the tumour in a sufficient concentration are of paramount importance. Up to recent times metronidazole has been given mainly to combat parasitic protozoal infections especially *Trichomonas vaginalis*, the therapeutic dose (200–400 mg t.i.d.) giving a serum level of 4–8 μg/ml [27]. It has been estimated that the serum concentration of metronidazole required during the application of each dose of radiation in a functional course of radiotherapy is in the region of 170 μg/ml (1 mM) [10].

The highest human serum level measured up to 1973 was 70 μg/ml after a 4 g oral dose [28]. Since the commencement of this study preliminary clinical trials have been undertaken at two centres [15, 17]; the results have shown that serum level increases linearly with the oral dose of metronidazole administered and produce a maximum concentration in the range of 200–300 μg/ml following a dose of 0·2 g/kg body wt [17].

The results reported here have shown that serum concentration of the order of 200 μg/ml may be achieved and maintained for at least an hour in rats provided a considerably higher oral dose is given (1 g/kg body wt). In the present studies although the measurements observed have been equated with intact metronidazole, this may not entirely be accurate since the analytical method, i.e. spectrophotometry or polarography, detects the unchanged nitro group [29]. However, since the radiosensitising effect of the

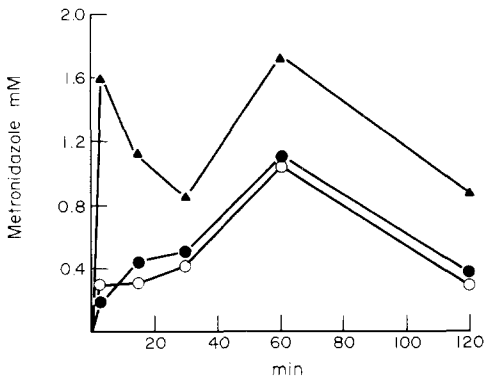

Fig. 2. The concentration of metronidazole in serum (▲ ▲), tumour (●—●) and brain (○—○) following oral administration of 1 g/kg body wt to rats with a Walker 256 sarcoma implanted 8 days before dosing. The results are the means of 3 animals in each group.

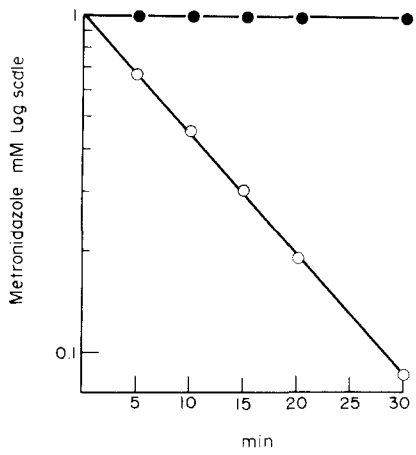

Fig. 4. The removal of metronidazole by an anaerobic caecal suspension prepared from 4 control rats (○ ○) and from 4 rats which had received 1 g/kg body wt of metronidazole orally for 4 days, the final dose being given 24 hr before sacrifice (●—●). For details of the caecal preparation see the text.

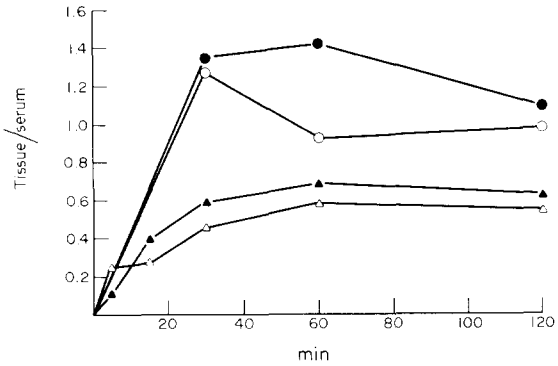

Fig. 5. The ratios of metronidazole in liver (○ ○) and kidney (● ●) to serum in female rats and in Walker 256 sarcoma (▲ ▲) and brain (△ △) to serum in male rats following oral administration of 1 g/kg body wt.

drug is due to the presence of the nitro group the levels quoted here are directly relevant to the sensitising activity.

It can be seen that there is some difference in the pharmokinetic properties of the drug in the two groups studied. Although the groups differ in sex and by the fact that one group was tumour-bearing, it is believed that the differences observed reflect variations in gastric emptying and intestinal motility. These differences in the pharmokinetics of metronidazole have been observed previously both in rats (R. M. J. Ings, private communication) and in humans [30].

Since the concentration of metronidazole in tissues will be directly related to the serum level, it is useful to examine the tissue to serum ratio as a function of time. This is shown in Fig. 5. Metronidazole can be seen to penetrate the liver to give an equal concentration whilst reaching an elevated level in the kidney. The latter result may be accounted for by the report that the major route of excretion for metronidazole is in the urine and at least 15 per cent of the dose is excreted unchanged [31]. Although a lower ratio of metronidazole was observed between the Walker 256 sarcoma and serum, a concentration of 1 mM was attained within the tumour. Similar results were obtained for brain. The ability to cross the blood-brain barrier [32] and to produce a high concentration in tumour tissue which in general is poorly vascularized reflects the physico-chemical nature of metronidazole. The drug is a relatively small molecule with a lipid/water partition coefficient of 1:1 and is not bound to protein [10].

A further criterion to be considered is whether the drug is toxic at the concentration that will be required for radiotherapy. The observed absence of a rise in the serum enzymes on GPT, SDH and LDH is an indication of no significant major organ injury following 1 g/kg for 4 days. Furthermore, metronidazole did not inhibit the drug metabolizing enzymes NADPH-cytochrome *c* reductase and cytochrome P_{450} or the rate of aminopyrene demethylation. This is in contrast to the 1-alkylimidazoles many of which are powerful inhibitors of the liver microsomal drug oxidations [33]. The lower rate of NADPH-linked lipid peroxidation may reflect a competition by metronidazole for electron flow along the cytochrome P-450 chain.

Although metronidazole has been reported to cause marked degeneration of the Purkinje cells in dogs such alterations were not observed in other species including rat [34]. In the present studies the chronic dose of metronidazole caused few alterations in the parameters measured. The drug has been reported to produce a leucopaenia when administered over a long period [35]; however, in the present studies no significant difference in the mean white cell count was found.

The observed change in the caecal weights and colouring in rats may be due to a disturbance in the balance of the bacterial flora present. Metronidazole is well known for its selective action against anaerobic rather than aerobic bacteria. Since over 90 per cent of the flora of the small intestine of rats and man belong to the group of strict anaerobes, e.g. *Bacteroides* [36], the removal of these species would permit other organisms to flourish.

It has been proposed [37] that the toxic action of metronidazole is due to a reduced product of the drug which interacts with some essential cellular constituent. This implies that the degradation of the drug may well occur at the same time as it exerts its toxic action. A wide variety of other electrophilic compounds including azodyes are metabolized by intestinal microflora [38, 39]. The actual mechanism of degradation remains uncertain, however results of studies with a ferrous–cysteine system [40] suggests interaction with iron–sulphur compounds may be involved. This is supported by previous observations of the rapid degradation of trinitrotoluene [41], nitrofuran derivatives [42], and niridazole [43], by xanthine oxidase, an enzyme containing an iron sulphur moiety and of metronidazole and chloromphenicol by ferredoxin [25, 44 46]. The lack of toxicity of metronidazole towards well oxygenated tissues may similarly be accounted for by the failure to transform the drug to its active principle by a reductive mechanism.

Finally there are reports of possible carcinogenic and mutagenic actions of metronidazole which require comment. The increase of tumours in mice was found to be increased if the animals were fed on a diet containing 0·15 per cent metronidazole [47]. However, there was a high incidence of spontaneous tumours in the control group and other studies in rats no carcinogenic activity was observed [48]. In both studies on mutagenicity, bacteria were incubated with the drug under conditions in which there was a high probability that they were seriously deprived of oxygen. As described above the cytocidal action of metronidazole is probably due to its conversion by reduction to a toxic intermediate which can be rendered harmless in the presence of oxygen by reoxidation. Thus any mutagenic action of metronidazole is more likely to manifest itself under anaerobic conditions and is unlikely in normally aerated cells *in vivo*. Indeed this selective cytocidal action of metronidazole on anaerobes rather than aerobes may also have an analogous conterpart in mammalian cellular systems. Recent results with cells in spheroid culture [49] and also with ascites cells *in vitro* [50] suggest that metronidazole may also selectively kill

hypoxic mammalian cells leaving normal well-oxygenated cells unaffected [51]. Thus metronidazole may provide a two-pronged attack on the hypoxic cell problem in radiotherapy: a sensitising effect at the time and a chemotherapeutic effect before, during and after, radiation treatment.

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