PRELIMINARY OBSERVATIONS ON INTERACTION OF ^{14}C-METRONIDAZOLE WITH MACROMOLECULES *IN VIVO* AND *IN VITRO*

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Summary : Preliminary studies on the *in vivo* and *in vitro* interactions of ^{14}C-metronidazole with macromolecules showed that the agent or its metabolite(s) can interact with nucleic acids and proteins *in vivo*. *In vitro* studies suggest that in absence of DNA synthesis trace amount of metronidazole does bind to DNA/protein and addition of metabolic activation system (from mouse liver) generates more reactive species from metronidazole.

Key words : metronidazole, 5-nitroimidazole, activation, macromolecules, interaction

INTRODUCTION

Several animal and bacterial studies have shown metronidazole, a 5-nitroimidazole to be carcinogenic and mutagenic (3,4,6, 12-15, 18). We found low doses of metronidazole to be carcinogenic in swiss mice and mutagenic in Ames' salmonella/mammalian - microsome assay (5). Several authors (1,10,11) have implied binding of *reduced* metronidazole to the macromolecules, thus explaining its specificity for anaerobic micro-organisms. Although nitro compounds are frequently reduced in mammals, Ings *et al.* (8) and Stambaugh *et al.* (16) did not find any evidence of reduction of the nitro group in metronidazole, thus ruling out the possibility of harmful effects in mammals. However Goldman (7) has reported that one of the metabolic markers indicative of the reduction of metronidazole was found in the urine of patients.

Our aim was to study if intact metronidazole reacted with nucleic acids and proteins *in vitro* and/or whether any active intermediates are formed either *in vivo* or *in vitro* by incubation with mammalian activation system.

MATERIAL AND METHODS

In vivo and In vitro experiments : Inbred, 8-week old swiss virgin female mice and 16 days-pregnant Swiss mice were used. ^{14}C-Metronidazole (16.2 mCi/mmol), May and Baker Ltd., England was used throughout the experiments.

Animals were injected ^{14}C-metronidazole (10 μCi per animal, ip) and sacrificed after 4 hr or 18 hr. The liver, lungs, kidney, thymus and fetus were dissected out (each tissue being pooled from 3-5 animals) and DNA, ribosomal RNA and proteins were isolated by the phenol extraction procedure (9).

In order to see whether it was intact drug itself, or its metabolite(s) which are binding to macromolecules, *in vitro* incubations were carried out using calf thymus DNA or proteins and ^{14}C-metronidazole in presence or absence of metabolic activation system.

In *in vitro* assay, standard reaction mixture contained the following components in a final volume of 3 *ml.*

50 μ*moles* tris-HCl buffer, pH 7.4, 10 μ*moles* EDTA, 0.36 μ*moles* NADPH, 7.5 *mg* Calf thymus DNA, 10 μCi ^{14}C-metronidazole and distilled water. Microsomal protein (2 *mg*) was added in the tubes where metabolic activation was desired, contents were mixed and tubes were incubated at 37°C for 45 min (wherever microsomes were added) or 24 hr (without microsomes). At the end of incubation period, contents were cooled and tubes containing microsomal protein were centrifuged at 120,000 *g* for 1 hr to remove microsomes and then dialysed against distilled water. Contents of tubes to which microsomes were not added, were dialysed without centrifugation.

Microsomal fraction used in these experiments was prepared by differential centrifugation from liver tissues obtained on 5th day from mice treated with 500 *mg/kg* body weight of Aroclor 1254. Whenever protein was required in absence of activation system it was heat inactivated and used.

Radioactivity measurements :

In vivo studies : The DNA or RNA isolated from various tissues were hydrolysed in 0.1 M HCl at 90°C for 30 min and radioactivity was counted in LKB RacBeta Scintillation spectrometer using a quench curve prepared by using various volumes of 0.1 M HCl and known amount of radioactivity.

Proteins isolated from various tissues were dissolved in 0.1 M NaOH, known quan-

tity was added to scintillation cocktail and it was neutralised, slightly acidified and radioactivity was measured using appropriate quence curve.

Since the amount of radioactivity was low, every vial was counted twice for 5 min each and results expressed as *dpm/mg* of DNA or RNA or proteins are mean of two separate determinations.

In vitro assays : DNA was extracted by phenol extraction procedure and precipitated with chilled ethanol. Radioactivity was measured and expressed as described above. Microsomal pellet recovered from reaction mixture at the end of incubation was washed several times, dialysed and dissolved in 0.1 M NaOH. Radioactivity was measured and expressed as described above.

RESULTS AND DISCUSSION

Among various tissues studied, liver showed relatively greater radioactivity associated with its macromolecules. Since the amount of radioactivity associated with other tissues was very low or undetectable, comparison has been made between values for liver of virgin mice and of pregnant mice 4 hr and 18 hr after exposure to ^{14}C-metronidazole. When results are expressed in *dpm/mg* DNA or RNA or protein the values are relatively low and hence total number of dpm observed, amount of sample added and *dpm/mg* have been presented in Table I.

TABLE I : *In vivo* interaction of ^{14}C-Metronidazole with macromolecules in mice.

			DNA			*RNA*			*Protein*		
Group	*Tissue*	*Time*	*dpm observed*	*Amount used mg*	*dpm mg*	*dpm observed*	*Amount used*	*dpm mg*	*dpm observed*	*Amount used mg*	*dpm mg*
Virgin	Liver	4	424	5.5	77	759	14.6	52	2610	10	261
		18	105	6.2	17	ND	ND	ND	240	12	20
Pregnant	Liver	4	117	6.5	18	320	10	32	5835	15	389
		18	56	7	8	192	12	16	2781	9	309
	Fetus	4	94	16	4	64	16	4	506	11.5	44
		18	189	14.5	13	230	16.4	14	212	11.2	19

ND — Not done.

These results show that there was detectable amount of radioactivity in RNA, DNA and proteins obtained from the liver or virgin female and pregnant Swiss mice. It

also appears that in most of the cases, with an increase in the time after exposure, there was a decrease in magnitude of radioactivity. The nucleic acids in liver of virgin mice had relatively more radioactivity than that of liver of pregnant mice.

If the radioactivity detected were due to incorporation of one carbon fragments, there would have been more radioactivity with passage of time and that would have remained constant for several weeks. Thus it is likely that most of the radioactivity associated with macromolecules may be due to interaction of the drug and/or its metabolite(s) with macromolecules and redioactivity decreased at later time possibly because of removal of DNA adducts.

Radioactivity in the macromolecules from fetus at 4 hr was very low or undetectable and it was relatively more at 18 hr. This may be due to time taken for access of marker to fetus and due to incorporation of one carbon fragment in growing tissue wherein DNA synthesis is expected to be brisk.

Results of *in vitro* experiments are recorded in Table II. Little radioactivity was still associated with DNA and inactivated microsomal protein even after repated washings and extensive dialysis. The amount of radioactivity associated after 24 hr incubation was relatively higer than the amount present after 45 min of incubation. Thus these results though preliminary, suggest that trace amount of intact drug may bind to DNA and protein. This is in contrast to results (11) that unreduced metronidazole bind to protein but not to DNA. The difference may be due to different experimental protocols. Using NMR technique, Sukkowska *et al.* (17) showed that unreduced metronidazole does bind to plasma proteins.

TABLE II : *In vitro* interaction of ^{14}C-Metronidazole with macromolecules.

Macromolecules	*DPM/mg*		
	45 min		*24 hr*
	+Microsomes	*−Microsomes*	*−Microsomes*
DNA	419	35	169
Microsomal protein	522	61[a]	256[a]

\+ In presence of micorsomes.
− In absence of microsomes.
a − Heat inactivated microsomes used.

Radioactivity associated with calf thymus DNA in presence of activation system was much higher when compared with values obtained in absence of metabolic activation. This suggests that mouse liver enzymes activate metronidazole to generate reactive species. It is interesting to note that *S. typhimurium* TA 100 FRI which is known to be deficient in nitro reductase, did mutate with metronidazole in presence of rat liver microsomes suggesting generation of mutagenic metabolites by mammalian microsomes (12). Whether this is by oxidation or by reduction is not clear. There have been reports on the binding of the reduced from of metronidazole to DNA (10,11) So far, it was believed that reduction of its nitro group does not occur in normal mammalian cells but recent reports (1,19) suggest that reduction does occur when mammalian metabolic activation system is used. Thus it is probable that reduced product reacts with nucleic acids and proteins.

There is solitary report by Bradley *et al.* (2) showing the association of radioactivity with DNA and RNA of brain, spinal cord and dorsal root ganglia from a rat injected with 500 μCi of ^{14}C - metronidazole (27 μCi/g).

There are no other reports on *in vivo* or *in vitro* experiments in which metabolic activation system was used. Our results suggest that metronidazole and/or its metabolite(s) interact with macromolecules *in vivo* and these modifications are possibly repaired or removed. *In vitro* studies show that in absenca of DNA synthesis (absence of incorporation of one carbon fragment) trace amount of metronidazole does bind to DNA and protein and addition of metabolic activation system generated more reactive species. Because of difficulties in getting labelled DNA it has not been possible to identify the site of interaction in DNA; however, as reported in case of reduced product (11), modifications may be occurring in guanine and/or cytosine residues.

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