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THE *IN VITRO* EFFECT OF SOME NITROIMIDAZOLES ON MICROTUBULE FORMATION

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Abstract—The *in vitro* effects of some nitroimidazoles (metronidazole, ornidazole) and their metabolites on microtubule formation have been tested. Cyclic metabolites are without effect. Metabolites proceeding from cleavage of the imidazole ring inhibit microtubule formation and reduce the polymerization rate of tubulin. This inhibitory effect might be correlated to some of the side-effects of these drugs. Isaxonine phosphate corrects this effect.

The nitroimidazole series supplies therapeutics with several anti-protozoal drugs [1–3], which are, in addition, active against anaerobic bacteria [4–6]. In this paper we consider two molecules of this series: metronidazole and ornidazole (Fig. 1). These compounds show similar metabolisms and it is possible to separate two types of metabolites: on the one hand, cyclic metabolites [3, 7, 8] which proceed either from methyl-2 oxidation or from N-1 side-chain oxidation; on the other hand, acyclic metabolites proceeding from cleavage of the imidazole ring [3, 9–11]. Among these, it must be stressed that acetamide is a metabolite common to both ornidazole and metronidazole.

For a better knowledge of some of the side effects observed [12–15] in patients treated by nitroimidazoles, we studied the *in vitro* action of nitroimidazoles and their metabolites on the formation of microtubules, which are an important structural component of axons [16].

Isaxonine phosphate (*N*-isopropyl-amino-2-pyrimidine phosphate) is a neurotropic agent which is able to promote nerve growth in rats [17] and to inhibit partially the effects of vinblastine both on microtubule disruption and tubulin aggregation [18].

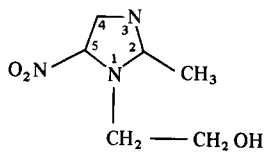
Moreover, it was proved that this molecule may correct *in vitro* the inhibitory effect of some uremic toxins on tubulin [19]. Therefore when the studied molecules caused inhibition of microtubule formation, we tested the corrective effect of isaxonine phosphate.

MATERIALS AND METHODS

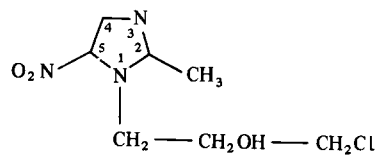
Reagents. Microtubular protein (MTP), i.e. tubulin with microtubule-associated proteins (MAPs), was obtained from fresh pig brains using the method of Shelanski *et al.* [20], with three cycles of *in vitro* assembly (37°) and disassembly (4°). We used two buffers: buffer A (for disassembly), pH 6.5, was: 0.1 M MES*, 1 mM EGTA and 0.5 mM $MgCl_2$; buffer B (for assembly), pH 6.5, was: 0.1 M MES, 1 mM EGTA and 0.5 mM $MgCl_2$, 1 mM GTP and 8 M glycerol. Each preparation was controlled by polyacrylamide gel electrophoresis [21]. Protein concentration was measured by Lowry's method using bovine serum albumin as a standard [22].

1-(2-Methyl-5-nitro-imidazolyl)-acetic acid and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole were supplied by Specia (France). We prepared *N*-

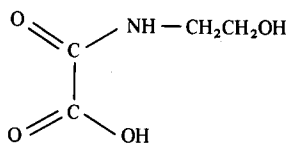
* Abbreviations: MES, 2-*N*-morpholino-ethanesulfonic acid; EGTA, ethylene glycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid.



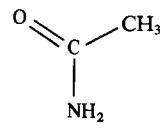
Métronidazole



Ornidazole



N – (2 Hydroxyethyl) oxamic acid



Acetamide

Fig. 1. Formulae of metronidazole, ornidazole and some of their metabolites.

(2-hydroxyethyl)-oxamic acid according to the method of Koch and Goldman [9] and identified it by ^{13}C NMR and ^{1}H NMR spectroscopy. Acetamide puriss. was purchased from Fluka. We only used ornidazole (Hoffman La Roche, France) and one of its metabolites, acetamide [3]. Isaxonine phosphate (IAPP) was supplied by I.P.S.E.N. (Paris, France).

Turbidimetry. Microtubule formation was monitored by turbidimetry at 335 nm using an Uvikon LS Printer 48 with a thermostated 1 cm light path cell, at 37°. The MTP concentrations were *ca* 1 mg/ml in the cell. The plateau value of the optical density allowed us to measure the degree of polymerization. For the determination of the inhibitory effect, all the tested substances were dissolved in buffer A. Then 10–50 μl of these solutions was added to 0.5 ml of MTP in buffer A (1 mg of MTP). After a 30 min contact time at 4°, polymerization was initiated by adding 0.5 ml of assembly buffer (buffer A, 1 mM GTP) and was triggered by a temperature of 37°. In all cases, controls were performed with MTP alone under the same conditions of dilution, temperature and contact time. The degree of polymerization in the presence of inhibitors was expressed as a percentage of the control value obtained with MTP alone. In order to study the corrective effect of isaxonine phosphate, it was dissolved in buffer A and added to the MTP +inhibitor solutions immediately before the beginning of polymerization. We used several concentrations of IAPP.

Electron microscopy. Electron microscopy was performed in a Philips EM 400 T Instrument. Samples were taken 20 min after the addition of 'assembly' buffer, and then negatively stained with 2% uranyl acetate on carbon-coated girds.

The lengths of 300 microtubules were measured from electron micrographs and the data were reported in a histogram of frequency vs length.

RESULTS

Turbidimetry

Metronidazole, ornidazole and cyclic metabolites were without effect on *in vitro* MTP polymerization whatever the concentrations and contact times used. On the contrary, acyclic metabolites showed a significant inhibitory effect on this polymerization.

The MTP polymerization curves for several acetamide concentrations are shown in Fig. 2. It can be seen that acetamide induced a strong inhibition of MTP polymerization. Indeed, at 10^{-5} M concentration, the inhibitory effect reached 20% and at 5×10^{-3} M it exceeded 50%. The results obtained with *N*-(2-hydroxyethyl)-oxamic acid are reported in Table 1. These results are the mean of three measurements. Once again inhibition percentages are very important since a 2×10^{-7} M concentration induced a 22% inhibition. Moreover, the curves of Fig. 2 show that the inhibitor acted not only on the degree of polymerization but also on the microtubule formation rate. Therefore in Table 1 we indicate the percentage decrease of the MTP polymerization rate: 2×10^{-7} M induced a 20% decrease in the rate.

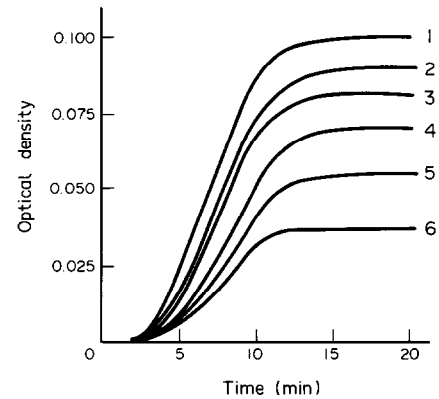


Fig. 2. *In vitro* acetamide effect on tubulin polymerization. (1) MTP alone; (2) acetamide (1×10^{-6} M); (3) acetamide (1×10^{-5} M); (4) acetamide (1×10^{-4} M); (5) acetamide (5×10^{-4} M); (6) acetamide (5×10^{-3} M).

Finally, the *in vitro* corrective effect of isaxonine phosphate was proved by the results of Table 2 (a and b). These results are expressed as a percentage of restitution of the polymerization degree; 100% corresponds to total restitution.

Electron microscopy

Figure 3 shows the histograms of frequency lengths obtained with and without acetamide. The results obtained in the presence of IAPP under the same conditions are also shown.

A 10^{-4} M concentration of acetamide induced a decrease in average length from 3.2 to 1.9 μm. Isaxonine phosphate corrected this inhibitory effect. Some measurements with a 10^{-4} M concentration of *N*-(2-hydroxyethyl) oxamic acid showed a decrease in the average length from 3.4 ± 0.1 to 2.0 ± 0.1 μm. With the addition of IAPP, the average length reached 3.6 ± 0.2 μm.

Table 1. Inhibition percentage of MTP polymerization degree and of MTP polymerization rate with increasing concentrations of *N*-(2-hydroxyethyl) oxamic acid

Concentration of *N*-(2-Hydroxyethyl)-oxamic acid (M)	Inhibition percentage of polymerization degree (%)	Inhibition percentage of polymerization rate (%)
2×10^{-7}	22	20
2×10^{-5}	42	42
1×10^{-4}	48	48
2×10^{-4}	60	71

Table 2. Corrective effect of isaxonine phosphate

Concentration of isaxonine phosphate (M)	% Restitution (a)	(b)
2.1×10^{-3}	30	15
4.2×10^{-3}	64	55
5.3×10^{-3}	89	85
6.3×10^{-3}	100	100

(a) 42% inhibition induced by 2×10^{-5} M *N*-(2-Hydroxyethyl)-oxamic acid.
(b) 30% inhibition induced by 1×10^{-4} M acetamide.

DISCUSSION

First, it must be stressed that in biological fluids tubuline 6S is associated with MAPs. Therefore we performed the first set of experiments with microtubular protein in order to be as close as possible to biological conditions.

Our results show that acyclic metabolites inhibit *in vitro* microtubule formation. Even with low doses, the inhibitory effect is noteworthy (a 2×10^{-7} M inhibitor concentration gives 22% inhibition). Moreover, the microtubule formation rate decreases considerably.

On the whole, this metabolite effect leads to slower microtubule formation, and these microtubules are shorter and less numerous. On the other hand, isaxonine phosphate corrects *in vitro* this inhibitor effect.

Besides, the metronidazole plasmatic concentration is *ca* 10^{-4} M during classical treatment [23]. Koch and Goldman [9] proved that approximately 2% of metronidazole is converted to *N*-(2-hydroxyethyl)-oxamic acid, i.e. 10^{-6} M. It is impossible to know the intracellular concentration but it seems reasonable to suppose that it is in the concentration range that induces an inhibitory effect greater than 20% (Table 1). Therefore if the inhibitory effect of metronidazole metabolites occurred *in vivo* as well as *in vitro*, it could lead to a reduction in the formation of microtubules *in vivo*. So the inhibitory effect might be correlated with peripheric neuropathy with axonal degeneration sometimes observed during long clinical use of high doses of nitroimidazoles [7–10]. However, it is always very difficult to correlate *in vitro* action with *in vivo* effect, although it is possible to establish such correlations in certain cases [24].

Therefore without drawing any final conclusion, our results might be taken into account in explaining the occurrence of this peripheric neuropathy.

Further experiments should be performed in order

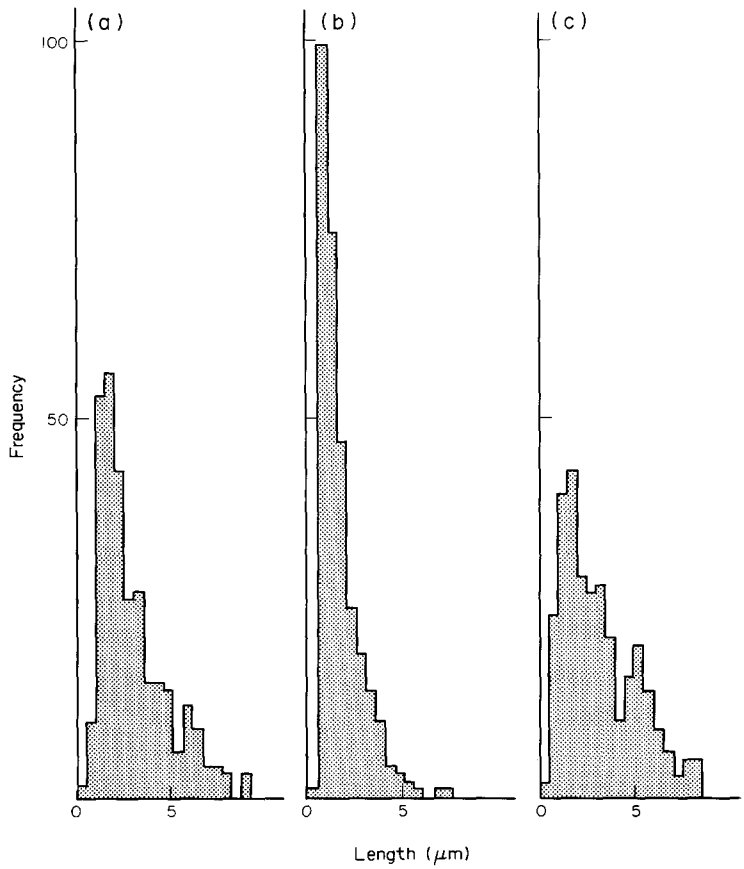


Fig. 3. Histograms of frequency lengths. (A) MTP alone (1 mg/ml): average length 3.2 ± 0.1 μ; (B) MTP (1 mg/ml) + acetamide (1×10^{-4} M): average length 1.9 ± 0.1 μm; (C) MTP (1 mg/ml) + acetamide (1×10^{-4} M) + isaxonine phosphate (6.3×10^{-3} M): average length 3.4 ± 0.2 μm. Histogram interval corresponds to 0.5 μm in length.

to test the activity of the second metabolite resulting from ornidazole-ring cleavage. This work, which is only a qualitative step, allowed us to prove the activity of some nitroimidazole metabolites on *in vitro* microtubule formation. In a second step we will study the mechanism of this effect, particularly the metabolite interactions with tubulin 6S using microcalorimetry and NMR spectroscopy.

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