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In vitro antitrichomonal activity of water-soluble prodrug esters of metronidazole

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Summary

Several water-soluble prodrug esters of metronidazole were tested for their in vitro antitrichomonal activity and their in vitro stability. The antitrichomonal activity seemed to be correlated with the release rate of the parent drug metronidazole and with the hydrophobicity of the spacer. The phenylalanine and leucine prodrugs appeared to present a higher intrinsic antitrichomonal activity.

Introduction

For more than a decade, interest has been increasing concerning the optimization of pharmacological and pharmacokinetic properties of existing drugs through chemical modification of the parent compounds. Work was done on the pharmacokinetics of low molecular weight prodrugs, such as metronidazole phosphate (Cho et al., 1982), metronidazole amino acid esters (Bundgaard et al., 1984a,b; Cho and Haynes, 1985) and metronidazole dicarboxylic acid monoesters (Larsen et al., 1988a) with the intention of developing a water-soluble prodrug for parenteral administration of metronidazole. Macromolecular derivatives of a wide variety of drugs have been prepared as a means of achieving greater pharmacological activity (Trouet et al., 1982), reduced toxicity (Takakura et al., 1987a) or higher cell-selective uptake (Ringsdorf, 1975; Notari, 1981; Duncan, 1984; Kopocek, 1984; Azori, 1987). The kinetics of formation of the active agent from the macromolecular dextran metronidazole dicarboxylic acid monoester prodrugs have also been studied (Larsen, 1986; Larsen et al., 1988b). Concerning the pharmacological properties and body distribution of macromolecular prodrugs, studies were carried out on mitomycin C-dextran conjugates (Kato et al., 1982; Hashida et al., 1984; Takakura et al., 1987a,b). In few cases, however, was the effect of binding a model drug to a polymer backbone on the activity, stability, body distribution, and immunogenicity, etc., evaluated. In this study, metronidazole was chosen as a model drug and bound to dextran as carrier by means of

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M-Gly, metronidazole-glycine; M-Gly-Gly, metronidazole-glycylglycine; M-Leu, metronidazole-leucine; M-Phe, metronidazole-phenylalanine; M-Succ, metronidazole-succinate

a spacer. Dextran was selected as carrier because of its excellent physicochemical properties and pharmacological acceptance (Schacht et al., 1984; Sezaki and Hashida, 1984).

Several amino acids (Phe, Gly, Leu), a dipeptide (Gly-Gly) and an organic acid (succinic acid) were used as spacer arms. In this paper, we report the in vitro stability and antitrichomonal activity of the water-soluble esters of metronidazole with the respective spacers.

Materials and Methods

Metronidazole esters were synthesized as previously described (Permentier et al., submitted). The different compounds used were the metronidazole esters of glycine, leucine and phenylalanine, the dipeptidic ester metronidazole-glycylglycine and the succinic monoester.

Activity tests were performed on *Trichomonas vaginalis*. *T. vaginalis* was grown and maintained in Kupferberg medium at 37 °C (Wéry, 1983). Kupferberg medium is a non-buffered medium consisting of 20 g bacto-tryptone, 1 g bacto-maltose, 1.5 g cysteine HCl, 1 g bacto-agar and 0.003 g bacto-methylene blue. The lyophilized powder (Difco, Detroit, MI) was rehydrated to a 950 ml solution, autoclaved, cooled and 50 ml heat-inactivated sterile fetal calf serum (Gibco, Paisley, U.K.) were aseptically added. The esters of metronidazole were dissolved in water to an equivalent metronidazole concentration of 1 mg/ml and then frozen (−20 °C) until use. Prior to experiments, solutions were thawed at room temperature.

Evaluation of the in vitro sensitivity

Test cultures were prepared as follows: to a culture of approx. 10000 organisms/ml in the log phase growing in 14.00 ml Kupferberg medium, contained in borosilicate screw-capped glass vials, metronidazole or the metronidazole ester stock solution and an appropriate volume of water were added to a final prodrug concentration equivalent to 1, 2.5, 5, 10, 20 or 30 μg/ml metronidazole in a final volume of 14.50 ml. A reference culture was grown simultaneously: to a culture of approx. 10000 organisms/ml in the log phase growing in 14.00 ml Kupferberg medium, 0.50 ml water were added to a final volume of 14.50 ml. At regular time intervals (0, 4, 8, 24, 28, 32, 48, 52, 56 h) the number of protozoans/ml was evaluated in a Bürker counting chamber. The evolution of the numbers of viable organisms in test and reference cultures was compared. Each experiment was performed twice.

Evaluation of the in vitro stability

The metronidazole levels were determined by high-pressure liquid chromatography using the method of Jensen and Gugler (1983) which was slightly modified. At the sampling times as indicated in the previous section, 1.00 ml of homogenised medium was withdrawn and 250 μl of a $ZnSO_4$ (Flandria, Zwijnaarde, Belgium) solution (0.1 M) in acetic acid buffer (pH 3.7; 2.6752 g HOAc + 1.4835 g NaOAc/1000 ml) (UCB, Leuven, Belgium) were added. Samples were taken until no more viable organisms could be detected. The buffer was used to minimize the hydrolysis after the sample was taken. After mixing for 1 min the sample was frozen at −20 °C until analysis. To the sample 250 μl of an internal standard solution, containing 0.01 mg/ml (w/v) tinidazole (Sigma, St. Louis, U.S.A.) for the determination of the lower concentrations (1, 2.5, 5 μg/ml) or 0.1 mg/ml (w/v) tinidazole for the determination of the higher concentrations (10, 20, 30 μg/ml) were added. In the case of the succinic acid ester of metronidazole, a standard solution containing 0.01 mg/ml (w/v) tinidazole was used for the determination of metronidazole in media with a low and a high prodrug concentration. After mixing for 1 min the sample was centrifuged at 4000 rpm for 10 min. Then 50 μl of the supernatant were injected into the chromatograph and eluted with a mobile phase (KH_2PO_4, 0.005 M: tetrahydrofuran, 100 : 1.4; v/v) on a reversed-phase column (5 μm, 12.5 cm × 4 mm, Lichrocart 125-4, Merck, Darmstadt, F.R.G.). The operating flow rate was 1.0 ml/min and the temperature was ambient. Detection was set at 324 nm. Calibration curves were prepared using known concentrations of metronidazole in culture medium. Linear response was obtained within the following range: 0.05–10

μg/ml metronidazole ($y = 0.0854x + 0.0334$; $r^2 = 0.9991$). The detection limit was 0.05 μg/ml. All samples were kept at 4°C. Each experiment was performed twice except for the glycine-metronidazole derivative supplemented to the medium at a concentration equivalent to 2.5 μg/ml metronidazole, this experiment being performed five times as a control for the reproducibility of the method.

The same procedure was followed for the evaluation of the diffusion of metronidazole into the trichomonads. At regular time intervals (0, 4, 8, 24 h) 1.00 ml of homogenised medium was withdrawn and treated as previously mentioned.

Results and Discussion

An inoculum size of 10000 organisms/ml was chosen because of the lower susceptibility of larger inocula for drug testing (Korner and Jensen, 1976).

For the evaluation of the in vitro activity of the different monoesters of metronidazole, the viable trichomonads were counted in a Bürker counting chamber. The motility of the protozoans was regarded as the criterion of viability. The end point of viability was defined as the time at which no motile trichomonads were detected. The results of these experiments are given in Table 1.

The antitrichomonal activity of the different prodrug esters and the parent drug metronidazole must be interpreted by comparison of the end points of viability for the entire range of concentrations. When the end points of trichomonad viability in the presence of the different metronidazole esters were compared with those of pure metronidazole, it appeared that the activity of the phenylalanine and the leucine-metronidazole prodrug closely approximated the antitrichomonal activity of metronidazole. The monopeptide glycine and the dipeptide glycylglycine derivative of metronidazole showed a lower activity in comparison to the parent drug. The two glycine derivatives showed no lethal effect at a concentration equivalent to 1 μg/ml metronidazole. The succinic acid ester of metronidazole showed almost no activity at all except for the highest concentration.

The pH of the aqueous drug solutions ($\pm$4.4) was already close to that of minimal hydrolysis of the investigated prodrugs ($\pm$4.5) (Permentier et al., submitted), so buffering seemed unnecessary. Besides, using a buffer at pH 4.5 could have a negative influence on the growth rate of the cultures.

Several aspects, such as the rate of hydrolysis of the prodrugs as well as the uptake of free metronidazole and prodrugs by the trichomonads, had to be taken into consideration when the meaning of the free metronidazole levels was being interpreted. The prodrugs released metronidazole due to hydrolysis. This regeneration of the parent

TABLE 1

End point of viability (h) of Trichomonas vaginalis in the presence of pure metronidazole and several monoesters of metronidazole (Gly, Phe, Leu, GlyGly, succinic acid) in different concentrations (equivalent to 1, 2.5, 5, 10, 20 or 30 μg/ml metronidazole)

Each experiment was performed twice; no variation was seen between the two observations.

Metronidazole equivalent concentration (μg/ml)	Prodrugs					Metronidazole
	M-Gly	M-Phe	M-Leu	M-GlyGly	M-Succ	
1	/	24	24	/	/	24
2.5	24	8	24	24	/	8
5	24	4	8	24	/	4
10	8	4	4	8	/	4
20	8	4	4	8	/	4
30	4	4	4	4	48	4

(/) Viable trichomonads are still present after 56 h.

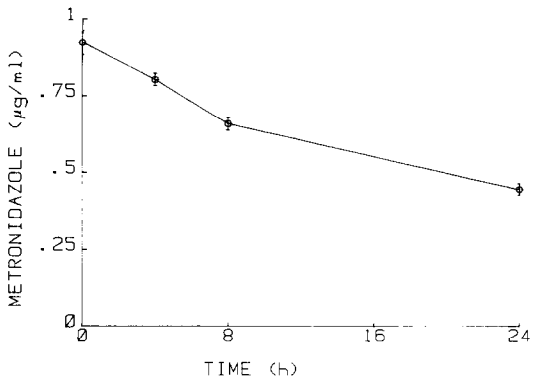

Fig. 1. Metronidazole levels in the test medium as a function of time (h). Each value is the mean of three measurements ± S.D.

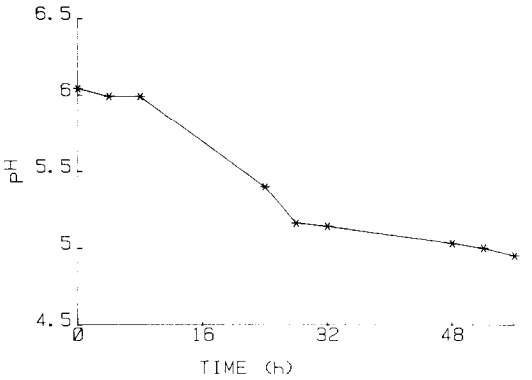

Fig. 2. The change of pH as a function of time (h) in the reference culture.

drug caused an increase in free metronidazole levels while the uptake of metronidazole by the trichomonads caused a decrease. This diffusion process into the trichomonads is clearly demonstrated in Fig. 1. After 24 h, 52% of the metronidazole, added in a concentration of ±1 µg/ml, was taken up by the trichomonads. Due to the growth of the trichomonads the pH of the Kupferberg medium changed. In Fig. 2 the pH change in the reference culture over a period of 56 h is shown. The decrease in pH due to the release of acid metabolites (Honigberg, 1978) was most obvious in the late log phase between 8 and 24 h. This decrease in pH from 6.1 to 5.0 towards the pH of minimal hydrolysis rate (Permentier et al, submitted) had an important influence on the amount of metronidazole released from the prodrug ester as the hydrolysis rate changed with the pH. Another important but rather unknown parameter was the uptake of the prodrug ester by the trichomonads. The influence of those parameters on the growth cycle of *T. vaginalis* and on the amount of metronidazole detected in the test medium was clearly demonstrated when a prodrug was supplemented to the medium at a sublethal concentration, as in the case of metronidazole-

TABLE 2

Concentration of free metronidazole (µg/ml) in the test medium at different time intervals (h) determined by high pressure liquid chromatography

The prodrugs were supplemented to the medium at a concentration equivalent to 1 µg/ml metronidazole. Each experiment was performed twice.

Type of prodrug	Sampling time (h)								
	0	4	8	24	28	32	48	52	56
M-Phe	0.11	0.28	0.33	0.30					
	0.12	0.27	0.29	0.27					
M-Leu	0.17	0.24	0.29	0.38					
	0.17	0.29	0.40	0.42					
M-Gly	0.18	0.27	0.29	0.25	0.16	0.10	/	/	/
	0.16	0.25	0.27	0.27	0.20	0.16	0.10	/	/
M-Gly-Gly	0.06	0.17	0.33	0.27	0.24	0.10	0.12	0.10	0.05
	0.07	0.18	0.26	0.16	0.13	0.10	0.10	0.12	0.10
M-Succ	/	/	/	/	/	/	/	/	/
	/	/	/	/	/	/	/	/	/

(/) No free metronidazole detected.

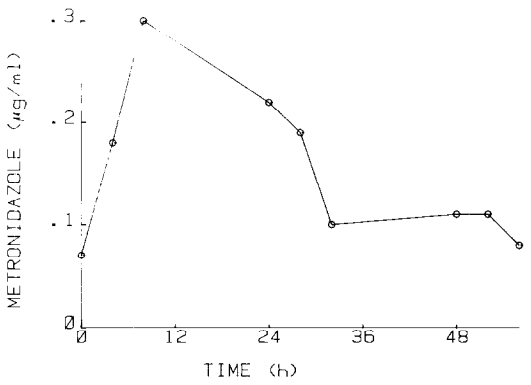

Fig. 3. The time (h) vs concentration (µg/ml) profile of free metronidazole in the case of metronidazole-glycylglycine supplemented to the medium at a concentration equivalent to 1 µg/ml metronidazole.

glycine and metronidazole-glycylglycine supplemented to the medium at a concentration equivalent to 1 µg/ml metronidazole (Table 2). Fig. 3 shows the time-concentration profile of free metronidazole and Fig. 4 the growth curve in the case of the prodrug ester metronidazole-glycylglycine supplemented at a concentration equivalent to 1 µg/ml metronidazole. When the growth curves of the test culture and of an inoculated medium without drug were compared, it appeared that between 4 and 32 h, the test culture showed a longer exponential growth and a lower

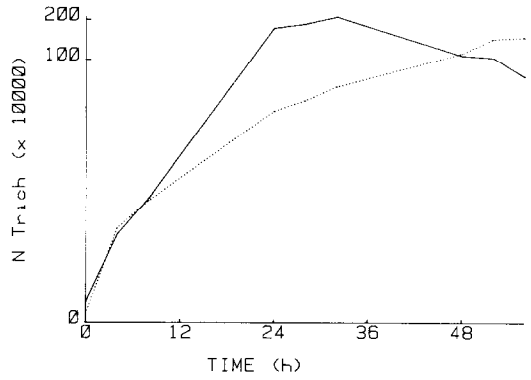

Fig. 4. The growth curve of a reference culture without drug (———) and of a test culture with metronidazole-glycylglycine supplemented at a concentration equivalent to 1 µg/ml metronidazole (· · · · · ·). Growth is expressed as the number of trichomonads (×10000) per ml as a function of time (h).

growth rate, in comparison to the reference culture. The exponential growth of both cultures was proved by the linearity in the linear-log figure (correlation factor between 0 and 24 h for the blank culture: 0.9964; correlation factor between 4 and 32 h for the test culture: 0.9950). In the log phase the growth rate of the reference culture was double that of the test culture. The growth rate could be expressed as the doubling time for the trichomonads, being 3.6 h for the reference culture and 7.7 h for the test culture. As a consequence of the lower growth rate in the test culture, the stationary phase was delayed (Fig. 4). There was a clear correlation between the growth curve of the test culture and the time-concentration profile of free metronidazole which was the result of the hydrolysis of the ester minus the diffusion of the drug into the trichomonads. During the first 4 h, the influence of the relatively low amount of regenerated metronidazole was negligible, the growth rate therefore being equal to that of the inoculated medium without drug. After the first 4 h, the influence of free metronidazole became more marked and the growth rate decreased. The influence of the unchanged prodrug ester on the growth rate was difficult to evaluate because little was known about the uptake of the prodrug. The possible influence of the ester on the viability of the organisms should diminish because of its hydrolysis. Due to an important increase in number of protozoans during the log phase, the diffusion of metronidazole into the trichomonads became greater in extent, explaining the decrease in free metronidazole levels after 8 h. A second factor enhancing the relative importance of the diffusion of metronidazole in the trichomonads was the lower rate of hydrolysis due to a shift of the pH of the medium towards the pH of a minimal hydrolysis rate.

In the case of the highest prodrug concentration (equivalent to 30 µg/ml metronidazole, Table 4) the pH remained almost constant during the 4 h of the experiment. The analytical data were treated as first-order kinetics and the resultant rate constants k are summarized in Table 5. (Cho and Haynes, 1985). The observed rate constant k allowed the interpretation of the difference in degradation rate between the different prodrug es-

TABLE 3

Concentration of free metronidazole (μg/ml) in the test medium at different time intervals (h) determined by high pressure liquid chromatography

The prodrugs were supplemented to the medium at a concentration equivalent to 2.5 μg/ml metronidazole. Each experiment was performed twice. The experiment with the glycine-metronidazole derivative was performed five times as a control for the reproducibility of the method. Values are given as means ± S.D. (in parenthesis).

Type of prodrug	Sampling time (h)								
	0	4	8	24	28	32	48	52	56
M-Phe	0.20	0.46	0.68						
	0.25	0.48	0.71						
M-Leu	0.27	0.48	0.64	0.67					
	0.24	0.47	0.54	0.68					
M-Gly	0.15	0.46	0.55	0.51					
	(0.01)	(0.02)	(0.02)	(0.04)					
M-Gly-Gly	0.13	0.42	0.61	0.83					
	0.22	0.47	0.71	0.87					
M-Succ	/	/	/	/	/	/	/	/	/
	/	/	/	/	/	/	/	/	/

(/) No free metronidazole detected.

ters. The very low rate of hydrolysis for the succinic ester and the simultaneous diffusion of regenerated metronidazole in the protozoals did not allow the calculations of k_{obs}.

Considering the resulting rate constant, the phenylalanine, leucine and glycine esters showed a similar release rate for metronidazole. In the case of the dipeptide ester M-GlyGly, the release rate was substantially higher, due to an intramolecular reaction with the formation of a diketopiperazine, (Permentier et al., submitted).

The time-concentration profile of metronidazole for the different amino acid prodrug esters was very much the same for concentrations equivalent to 20, 10 and 5 μg/ml metronidazole.

In the case of the lowest trichomonacidal concentrations (equivalent to 1 and 2.5 μg/ml metronidazole, Tables 2 and 3), the uptake of

TABLE 4

Concentration of free metronidazole (μg/ml) in the test medium at different time intervals (h) determined by high pressure liquid chromatography

The prodrugs were supplemented to the medium at a concentration equivalent to 30 μg/ml metronidazole. Each experiment was performed twice.

Type of prodrug	Sampling time (h)						
	0	4	8	24	28	32	48
M-Phe	2.58	5.85					
	2.72	6.15					
M-Leu	2.35	4.88					
	2.42	5.06					
M-Gly	1.92	4.61					
	1.16	4.76					
M-Gly-Gly	0.96	5.82					
	0.97	6.22					
M-Succ	0.16	0.22	0.22	0.42	0.40	0.45	0.47
	0.23	0.27	0.31	0.37	0.41	0.44	0.49

TABLE 5

The observed rate constant k (h^{-1}) of hydrolysis and the half-life (h) for the phenylalanine, glycine, leucine and glycylglycine ester of metronidazole in the test medium (pH ± 6)

Prodrug	k_{obs} (h^{-1})	$t_{1/2}$ (h)
M-Phe	3.3×10^{-2}	21.2
M-Leu	2.5×10^{-2}	28.3
M-Gly	3.0×10^{-2}	23.7
M-Gly-Gly	4.8×10^{-2}	14.5

metronidazole by the trichomonads may explain the slower increase in free metronidazole levels in the medium than could be expected from the observed rate constant of hydrolysis. When the end points of viability of *T. vaginalis* in the presence of metronidazole and of the different prodrugs were compared (Table 1), it appeared that the phenylalanine ester of metronidazole showed the same activity as metronidazole while the activity of the leucine ester was somewhat lower. The glycine ester, having a similar observed rate constant of hydrolysis to those of the former two, and the dipeptide glycylglycine ester, showing an even higher rate of hydrolysis, were markedly less efficient against *T. vaginalis*. The difference in antitrichomonal activity of these four prodrugs could be explained by the difference in hydrophobicity of the three amino acids and the dipeptide. Using the 'consensus' hydrophobicity scale, the values are as follows: glycine, 0.16; leucine, 0.53; phenylalanine, 0.61 (Eisenberg et al., 1982). This scale is the mean of a number of scales whereby different methods were used to determine the hydrophobicity. This hydrophobicity scale indicated that phenylalanine and leucine were more hydrophobic than glycine. The difference in hydrophobicity could be responsible for a difference in the diffusion of the respective prodrug esters through the lipid membrane of the trichomonads (Goldman, 1982). Once the prodrug had entered the cells, it was difficult to predict whether the intact ester was active. The activity of metronidazole is mainly due to a reduction of the nitro group whereby low-redox-potential proteins serve as electron donor. The most important requirement for antitrichomonal activity seems to be the accessibility of the nitro group for the electron-transport proteins. Taking this working mechanism into consideration, it was not impossible that the prodrug esters were active as such. Another possibility may be that metronidazole had to be released intracellularly to obtain an antitrichomonal activity (Ings et al. 1974; Müller, 1981). Considering the results of the HPLC analysis for the monosuccinic acid ester of metronidazole, it appeared that the release rate of metronidazole from this prodrug ester was very low. This explained the long viability of *T. vaginalis* in the presence of the monosuccinic prodrug ester.

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