Efficacy of New 5-Nitroimidazoles against Metronidazole-Susceptible and -Resistant Giardia, Trichomonas, and Entamoeba spp.

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The efficacies of 12 5-nitroimidazole compounds and 1 previously described lactam-substituted nitroimidazole with antiparasitic activity, synthesized via SN1 and subsequent reactions, were assayed against the protozoan parasites Giardia duodenalis, Trichomonas vaginalis, and Entamoeba histolytica. Two metronidazole-sensitive lines and two metronidazole-resistant lines of Giardia and one line each of metronidazole-sensitive and -resistant Trichomonas were tested. All except one of the compounds were as effective or more effective than metronidazole against Giardia and Trichomonas, but none was as effective overall as the previously described 2-lactam-substituted 5-nitroimidazole. None of the compounds was markedly more effective than metronidazole against Entamoeba. Significant cross-resistance between most of the drugs tested and metronidazole was evident among metronidazole-resistant lines of Giardia and Trichomonas. However, some drugs were lethal to metronidazole-resistant Giardia and had minimum lethal concentrations similar to that of metronidazole for drug-susceptible parasites. This study emphasizes the potential in developing new nitroimidazole drugs which are more effective than metronidazole and which may prove to be useful clinical alternatives to metronidazole.

The introduction of nitroheterocyclic drugs in the late 1950s and the 1960s heralded a new era in the treatment of infections caused by gram-negative and -positive bacteria and a range of pathogenic protozoan parasites. The antibiotic azomycin (a 2-nitroimidazole), isolated in Japan from a streptomycete, was the first active nitroimidazole to be discovered (15) and acted as the main impetus for the systematic search for drugs with activity against anaerobic protozoa. This led to the synthesis of the 5-nitroimidazole metronidazole (1β-hydroxyethyl-2-methyl-5-nitroimidazole) and the demonstration of its activity against Trichomonas vaginalis by Cosar and Julou (6). Subsequently, metronidazole was shown to cure giardiasis (21), amoebiasis (18), and Balantidium infections (7). Metronidazole is the drug now most widely used in the treatment of anaerobic protozoan parasitic infections caused by T. vaginalis, Giardia duodenalis, and Entamoeba histolytica (22). It is remarkably safe compared with the toxic amoebicide emetine (12) and is safe compared with the toxic amoebicide emetine (12) and is especially valuable for the synthesis of a large number of complex and highly branched compounds. Recently, 5-nitroimidazole derivatives including the lactam-substituted nitroimidazole have been shown to be significantly more effective antiprotozoal agents than metronidazole (8, 30). In the study described here we examined the activities of other new 5-nitroimidazole compounds against metronidazole-sensitive and -resistant G. duodenalis (synonymous with Giardia lamblia and Giardia intestinalis) and T. vaginalis and against E. histolytica, the three most medically important anaerobic protozoa.

MATERIALS AND METHODS

Drugs. All compounds used in this study were identified by spectral data, purified by chromatography on silica gel columns, and recrystallized from appropriate solvents. Their purity was checked with appropriate controls by thin-layer chromatography and elemental analysis (C, H, N). The purity was always over 99.6%. The synthesis, structural identification, and purity of compounds 1, 2, 3, 4, 5, 6, 7, 8, and 13 have been reported previously (8, 27, 28). Data for products 9, 10, 11, and 12 were presented at 33rd International Meeting on Medicinal Chemistry (28a).

Briefly, the 3-chloro-2-chloromethyl-1-(1-methyl-5-nitroimidazol-2-yl)prop-1-ene compound (compound 1) reacted with the 2-nitropropane anion and led to products 2, 3, and 4 formed by an initial SN1 mechanism followed by an SN2 or SN12 and Michael reactions or another SN1 reaction, respectively (28) (Fig. 1). The extension of the bis-SN1 reaction to 2,2-dimethyl-5-nitro-1,3-dioxane salt led to compound 5 (27). Base-promoted nitrous acid elimination from bis-C-alkylation products gave mono- or diunsaturated compounds 6, 7, and 8 (Fig. 1).

Compounds 9 and 10 were synthesized by the reaction of the bis-chloride (compound 1) with p-toluenesulfonic acid in dimethyl sulfoxide. The E and Z isomers (compounds 11 and 12, respectively) were obtained by the LD-SN1 mechanism after subjecting compound 9 to 2-nitropropane anion (Fig. 1).

Compound 13 was prepared by reacting 1-methyl-2-chloromethyl-5-nitroimidazole with 1-methyl-3-nitro-2-pyrrolidinone anion under phase-transfer catalysis (8) (Fig. 1).
Metronidazole (Fig. 1) was from Sigma. All drugs were dissolved in chromatography-grade dimethylformamide (DMF) (Sigma) at 100 mM and were diluted into medium as required.

Cultures. All strains used in this work are described in Table 1.

Growth Conditions. *Giardia* and *Trichomonas* parasites were grown in TYI-S-33 medium supplemented with bile (1 mg/ml; Sigma) (17). The same medium without bile was used for *Entamoeba*. Parasites were grown at 37°C (35.5°C for *Entamoeba*) in filled 5-ml plastic tubes and were maintained upright. *Giardia* and *Trichomonas* trophozoites were subcultured every 2 to 3 days, and *Entamoeba* trophozoites were subcultured every 3 to 4 days.

Metronidazole-resistant lines of protozoa were maintained in culture in the presence of metronidazole (Table 1).

| N° | Formula | MW  | N° | Formula | MW  |
|----|---------|-----|----|---------|-----|
| 1  | ![Structure 1](image1) | 250.08 | 2  | ![Structure 2](image2) | 282.25 |
| 3  | ![Structure 3](image3) | 371.35 | 4  | ![Structure 4](image4) | 355.35 |
| 5  | ![Structure 5](image5) | 499.47 | 6  | ![Structure 6](image6) | 308.33 |
| 7  | ![Structure 7](image7) | 261.32 | 8  | ![Structure 8](image8) | 341.45 |
| 9  | ![Structure 9](image9) | 369.82 | 10 | ![Structure 10](image10) | 489.56 |
| 11 | ![Structure 11](image11) | 422.45 | 12 | ![Structure 12](image12) | 422.45 |
| 13 | ![Structure 13](image13) | 236.23 | MZ | ![Structure MZ](imageMZ) | 171.13 |

**FIG. 1.** Structures of new 5-nitroimidazoles. The formula and molecular weight (MW) of the compounds used in this study are presented. MZ, metronidazole.
The aim of this study was to identify those compounds which were consistently and significantly more effective than metronidazole against *Giardia, Trichomonas*, and *Entamoeba*. All compounds except compound 3 were as effective or more effective than metronidazole, and none was as effective as compound 13 against all three parasite species tested. Compounds 4, 6, 7, 10, 11, 12, and 13 demonstrated increased activity over that of metronidazole against *Giardia* and *Trichomonas*. Compounds 1, 2, and 13 maintained similar levels of activity against metronidazole-sensitive and -resistant *Giardia* parasites. When we assayed parasites of *Giardia* sp. strain BRIS/83/HEPU/106 against several of the most effective drugs (compounds 4, 6, 10, 12, and 13) on three occasions, the MLCs ranged between <1 and 5 μM on all occasions.

Metronidazole-resistant *Trichomonas* was generally resistant to the same concentrations of all of the nitroimidazole compounds tested.

**DISCUSSION**

The observations that important antibacterial and antiprotozoal activities of nitroimidazoles are associated with reductive metabolism have led to considerable interest in nitroimidazole reduction chemistry and the synthesis of new, highly effective drugs. Recently, we demonstrated that 1-methyl-2-chloromethyl-5-nitroimidazole reacted by the SRN1 mechanism with various aliphatic, cyclic, or heterocyclic nitrate anions led to a new class of 5-nitroimidazoles bearing a trisubstituted double bond at the 2 position (8, 29). The subsequent structure-activity relationships revealed that the most antimicrobial and antiparasitic compounds showed a greater resonance conjugation in the molecular structure. In order to increase the conjugate system and in connection with mechanistic studies, we have synthesized new mono- and bis-alkylating agents and explored their reactivities with nitrate anions. The structures and antiprotozoal activities of these compounds are described here.

A great deal of variation in the antiprotozoal efficacies of the 13 compounds tested was revealed. Only one compound was less effective than metronidazole against all three species of protozoa examined. All other compounds were as effective as those of previous reports (10, 16) but higher than the previously reported doses of metronidazole inhibitory for *Giardia* which have relied on a variety of criteria (26). The MLC of about 10 μM metronidazole for *Entamoeba* and the MLC of 100 μM (16 μg/ml) metronidazole for *Trichomonas* grown under anaerobic conditions are similar to previously reported MLCs (see reference 20 and references therein; 14). The high levels of metronidazole resistance that developed in *Giardia* lines WB1B-M3 and BRIS/83/HEPU/106-2ID10 and the *Trichomonas* line BRIS/92/STD1/F1623-M1 are described in Table 1.

The activities of the 12 new 5-nitroimidazole compounds tested and 1 previously tested compound were compared with that of metronidazole against the test organisms (Table 2). The aim of this study was to identify those compounds which were

### RESULTS

**Drug assays.** In the assays described here the MLCs of metronidazole for susceptible strains were 50 to 100 μM, which are consistent with those in previous reports (10, 16) but higher than the previously reported doses of metronidazole inhibitory for *Giardia* which have relied on a variety of criteria (26). The MLC of about 10 μM metronidazole for *Entamoeba* and the MLC of 100 μM (16 μg/ml) metronidazole for *Trichomonas* grown under anaerobic conditions are similar to previously reported MLCs (see reference 20 and references therein; 14). The high levels of metronidazole resistance that developed in *Giardia* lines WB1B-M3 and BRIS/83/HEPU/106-2ID10 and the *Trichomonas* line BRIS/92/STD1/F1623-M1 are described in Table 1.

The activities of the 12 new 5-nitroimidazole compounds tested and 1 previously tested compound were compared with that of metronidazole against the test organisms (Table 2). The aim of this study was to identify those compounds which were
or more effective than metronidazole against some or all organisms tested. The lactam-substituted compound (compound 13) was significantly more effective than metronidazole against *Giardia* (50 to 100 times more effective) and *Trichomonas* (50 times more effective), but against the *Entamoeba* strain that we used the compound was not as effective as it was previously (30). Compound 3 was uniformly less effective than metronidazole in all organisms that we studied, and the lactam-substituted compound was essentially inactive against *E. histolytica* (4, 13, 15, 28).

The mechanism of action of metronidazole in anaerobes requires reduction of the critical nitro group to toxic radicals by the enzyme pyruvate:ferredoxin oxidoreductase (PFOR) (22). In highly metronidazole-resistant *Trichomonas*, which we have used in these studies, there is no PFOR or ferredoxin, which itself is reduced by the membrane-localized enzyme pyruvate:ferredoxin oxidoreductase (PFOR) (22). In highly metronidazole-resistant *Trichomonas*, which we have used in these studies, there is no PFOR or ferredoxin (4, 5, 6), and metronidazole and other 5-nitroimidazoles are not activated in these organisms. In metronidazole-resistant *Giardia*, although PFOR activity is decreased (24), it is still detectable and is thus able to activate 5-nitroimidazole drugs, as we have seen in this study. In both these parasites we have reported alternative oxoacid oxidoreductases which do not apparently reduce the characterized reductases and which are at least as active or more active in metronidazole-resistant lines than in their drug-sensitive parent strain (4, 24). These alternative pathways in the anaerobic protozoa are poorly understood and may well be the targets of highly active 5-nitroimidazoles or related compounds. For this reason and with the encouraging results obtained with compounds such as the lactam-substituted nitroimidazole (compound 13), continued assessment of new 5-nitroimidazole drugs is extremely worthwhile.

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**REFERENCES**

1. Boreham, P. F. L., R. E. Phillips, and R. W. Shepherd. 1988. Altered uptake of metronidazole in vitro by stocks of *Giardia intestinalis* with different drug sensitivities. Trans. R. Soc. Trop. Med. Hyg. 82:104–106.
2. Boreham, P. F. L., N. C. Smith, and R. W. Shepherd. 1988. Drug resistance and the treatment of giardiasis, p. 3–7. In P. M. Wallis and B. R. Hammond (ed.), Advances in *Giardia* research. University of Calgary Press, Calgary, Alberta, Canada.
3. Bowman, W. R. 1988. Photoinitiated nucleophilic substitution at sp3 carbon, p. 431–486. In M. A. Fox and M. Channon (ed.), Photoinitiated electron transfer, Part C. Elsevier, Amsterdam, The Netherlands.
4. Brown, D. M., J. A. Upcroft, and P. Upcroft. An alternative keto acid oxidoreductase in *Trichomonas vaginalis*. Mol. Biochem. Parasitol., in press.
5. Casar, C., and L. Julio. 1989. Activity of 1-(hydroxy-2-ethyl)-1-methyl-2-nitroimidazole (6,823 R P) vis-a-vis des infections expériementales *Tricho-
monas vaginalis*. Ann. Inst. Pasteur 96:238–241.
6. Garcia-Leverade, A., and L. de Bonilla. 1975. Clinical trials with metronidazole in human balanitis. Am. J. Trop. Med. Hyg. 24:737–738.
7. Jentzer, O., P. Vanelle, M. P. Crozet, J. Maldonado, and M. Barreau. 1991. Nouveaux 5-nitroimidazoles à noyau lactam haute activité. Rev. Fr. Méd. Chir. 26:687–697.
8. Johnson, P. J. 1993. Metronidazole and drug resistance. Parasitol. Today 9:183–186.
9. Jokipii, L., and A. M. Jokipii. 1980. In vitro susceptibility of *Giardia lamblia* trophozoites to metronidazole and tinidazol. J. Infect. Dis. 141:317–325.
10. Kim, K. J., and F. J. Bunnett. 1970. Evidence for a radical mechanism of aromatic “nucleophilic” substitution. J. Am. Chem. Soc. 92:7463–7464.
11. Knight, R. 1980. The chemotherapy of amoebiosis. J. Antimicrob. Chemo-
ther. 6:577–593.
12. Kornblum, N., R. E. Michel, and R. C. Kerber. 1986. Radical anions as intermediates in substitution reactions. J. Am. Chem. Soc. 88:5660–5662.
13. Kornblum, N., R. E. Michel, and R. C. Kerber. 1986. Chain reactions in substitution processes which provide radical-anion intermediates. J. Am. Soc. Chem. 88:5662–5663.
14. Lossick, J. G. 1990. Therapie de urogenital trichomoniase, p. 324–431. In B. M. Honigberg (ed.), Trichomoniasis parasitique dans le human. Springer-Verlag, New York, N.Y.
15. Maeda, K., T. Osata, and H. Umezawa. 1953. A new antibiotic, azomycin. J. Antibiot. 6(suppl. A):182.
16. Majewska, A. C., W. Kasprzak, J. F. De Jongheeree, and E. Kaczmarek. 1991. Heterogeneity in the sensitivity of stocks and clones of *Giardia* to metronidazole and ornidazole. Trans. R. Soc. Trop. Med. Hyg. 85:67–69.
17. Phillips, R. E. P., F. L. Boreham, and R. W. Shepherd. 1984. Cryopreservation of viable *Giardia intestinalis* trophozoites. Trans. R. Soc. Trop. Med. Hyg. 78:604–606.
18. Powell, S. J., L. MacLeod, A. J. Wilmut, and R. Elsdon-Dew. 1966. Metronidazole in amoebic dysentery and amoebic liver abscess. Lancet ii:1329–1331.
19. Russell, G. A., and W. C. Danen. 1966. Coupling reactions of the 2-azido-2-propyl anion. J. Am. Chem. Soc. 88:5653–5665.
20. Samawickrema, N. A., J. A. Upcroft, D. M. Brown, N. Thammapalerd, and R. W. Shepherd. 1997. Superoxide dismutase and pyruvate:ferredoxin oxidore-
ductase involvement in mechanisms of metronidazole resistance in *Entamoeba histolytica*. J. Antimicrob. Chemother. 40:833–840.
21. Scheider, J. 1961. Traitement de la giardiase (lambliase) par le métronida-
ze. Bull. Soc. Pathol. Exot. 54:694–95.
22. Townson, S. M., P. F. L. Boreham, P. Upcroft, and J. A. Upcroft. 1994. Resistance to the nitroheterocyclic drugs. Acta Trop. 56:173–194.
23. Townson, S. M., H. Laqua, P. Upcroft, P. F. L. Boreham, and J. A. Upcroft. 1992. Induction of metronidazole and furazolidone resistance in *Giardia*. Trans. R. Soc. Trop. Med. Hyg. 86:521–522.
24. Townson, S. M., J. A. Upcroft, and P. Upcroft. 1996. Characterisation and purification of pyrurate:ferredoxin oxidoreductase from *Giardia duodenalis*. Mol. Biochem. Parasitol. 79:183–193.
25. Upcroft, J. A., A. Healey, R. W. Mitchell, and P. Upcroft. 1994. A new rDNA repeat unit in human *Giardia*. J. Eukaryot. Microbiol. 41:639–642.
26. Upcroft, J. A., and P. Upcroft. 1993. Drug resistance and *Giardia*. Parasitol. Today 9:187–190.
27. Vanelle, P., K. Benakli, J. Maldonado, and M. P. Crozet. 1998. Synthesis of new 2-highly branched 5-nitroimidazoles by bis-Sn1 methodology. Hetero-
cycles 48:181–185.
28. Vanelle, P., K. Benakli, J. Maldonado, C. Roubaud, and M. P. Crozet. 1996. Cascade Sn1 reactions in 5-nitroimidazole series. Heterocycles 43:731–735.
29. Vanelle, P., K. Benakli, M. De Mbo, J. Maldonado, M. P. Crozet, M. Laget, H. Guiraud, and G. Dumenil. 1997. Genotoxicomodulation in 5-nitroimi-
dazole series, abstr. C16. Presented at the 33rd International Meeting on Medicinal Chemistry, Reims, France, 16 to 18 September 1997.
30. Vanelle, P., M. P. Crozet, J. Maldonado, and M. Barreau. 1991. Synthèse par réactions Sn1 de nouveaux 5-nitroimidazoles antiparasitaires et antibacte-
riens. Eur. J. Med. Chem. 26:37–178.
31. Vanelle, P., J. Maldonado, M. Gasquet, F. Delmas, P. Timon-David, O. Jentzer, and M. P. Crozet. 1991. Studies on antiparasitic agents: effect of the lactam nucleus substitution in the 2-position on the in-vitro activity of new 5-nitroimidazoles. J. Pharm. Pharmacol. 43:735–751.
32. Voolmann, T., and P. F. L. Boreham. 1993. Metronidazole resistant *Tricho-
onas vaginalis* in Brisbane. Med. J. Aust. 159:190.