ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2003, p. 3368–3370 Vol. 47, No. 10
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.10.3368–3370.2003

Activity of Megazol, a Trypanocidal Nitroimidazole, Is Associated with DNA Damage

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Received 1 May 2003/Returned for modification 19 June 2003/Accepted 18 July 2003

DNA damage associated with the trypanocidal activity of megazol [2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole] was shown in experiments in which DNA repair-deficient RAD51$^{-/-}$ *Trypanosoma brucei* mutants were found to be hypersensitive to the drug. Parasites resistant to megazol were selected and showed modest cross-resistance to other trypanocides, although neither drug efflux nor changes to intracellular thiols correlated with resistance.

New drugs are urgently required for treatment of human African trypanosomiasis (22). One compound with promise is megazol (5, 12, 13, 18), a nitroheterocyclic compound that forms a nitro radical anion upon reduction (31). Megazol appears to enter *Trypanosoma brucei* via passive diffusion (3), but little is known about its mode of action. We determined the effect of megazol in assays that measured oxidative and reductive stress. We also selected parasites resistant to megazol in order to assess cellular changes associated with resistance.

Bloodstream forms (17) and procyclic forms (6) of *T. brucei brucei* (strain 427) were cultivated by using standard techniques. Assays measuring drug sensitivity were carried out to determine the drug concentration producing a 50% decrease in cell proliferation (IC_{50}) and the resistance factor (average ratio of the IC_{50} for a resistant clone to the IC_{50} for a wild-type clone). The Alamar Blue test (28) was used for bloodstream forms, while cell counting by using an improved Neubauer chamber was required for procyclics. For procyclic cells the IC_{50} of megazol was 0.28 ± 0.01 μM, and for bloodstream forms the IC_{50} was 0.15 ± 0.02 μM.

To induce megazol resistance in vitro, trypanosomes were exposed to drug concentrations that doubled every 2 weeks, starting at 0.008 and 0.01 μM for bloodstream forms and procyclics, respectively. After 6 months of cultivation, procyclic and bloodstream forms that tolerated 10 and 1 μM megazol, respectively, were cloned by limiting dilution.

The IC_{50} for the cloned, resistant, procyclic line was 29.24 ± 3.2 μM (105-fold-reduced sensitivity), while the IC_{50} for the bloodstream form line was 3.2 ± 0.48 μM (21-fold-reduced sensitivity).

Cross-resistance to megazol and other trypanocides was also studied (Table 1). Moderate levels of cross-resistance to two nitroheterocyclic trypanocides, the nitrofuran nifurtimox and the nitroimidazole benznidazole, were detected. Significant, albeit low-level, cross-resistance to the melaminophenyl arsenical compounds cymelarsan, melarsen oxide, and melarsoprol, the diamidine compounds pentamidine and berenil, and suramin was also observed.

Verapamil (20), PAK-104P (7), and two phenothiazine derivatives (prochlorperazine and trifluoperazine) (14) all inhibit cellular extrusion pumps but, when used at 5 μM, failed to reverse megazol resistance in *T. brucei*. Efflux pumps are thus unlikely to play a significant role in the megazol resistance characterized here.

It was recently shown that megazol exposure reduces trypanothione levels in *Trypanosoma cruzi* (23). Bloodstream form *T. brucei* obtained from infected Wistar rats (21) and procyclic forms grown in vitro (6) were resuspended at a concentration of 10^7 cells ml^{-1} in culture medium. Megazol (0.15 to 30 μM) or buthionine sulfoximine (BSO; 10 to 20 μM) was added to the parasite suspensions, and the suspensions were incubated for 2 h. Reduced thiol levels in control and drug-treated parasites were then determined by derivatization with monobromobimane (thiolyte) and separation by high-pressure liquid chromatography as previously described (30).

BSO (16) reduces glutathione (2) and trypanothione (15) levels in trypanosomatids. When added to cell cultures 24 h prior to evaluation of IC_{50}s, BSO enhanced the susceptibilities of both wild-type and megazol-resistant trypanosomes to megazol. At 10 and 20 μM, respectively, BSO caused the IC_{50} for wild-type procyclics to drop from 0.28 to 0.14 ± 0.02 μM and that for resistant cells to drop from 29.24 to 7.33 ± 0.56 μM. With bloodstream forms, the IC_{50} for wild-type cells shifted from 0.15 to 0.07 ± 0.01 μM while that for resistant cells shifted from 3.2 to 1.03 ± 0.03 μM. The concentration of BSO could not exceed 20 μM since BSO is highly toxic to *T. brucei* (IC_{50}s were 15.15 ± 0.22 μM and 11 ± 0.39 μM for procyclic and bloodstream forms, respectively), although related parasitic trypanosomatids *T. cruzi* (26) and *Leishmania* (19) can tolerate doses of BSO of 1 mM. The BSO effect was greater in resistant than in wild-type *T. brucei*.

The effects of BSO and megazol (used at their in vitro IC_{50}s) on the combined thiol levels (total levels of glutathione, trypanothione, and glutathionylspermidine and, measurable only in procyclics [1], of ovothiol) in wild-type and resistant trypanosomes of both forms were determined. Megazol treat-

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TABLE 1. Cross-resistance of bloodstream and procyclic forms of *T. brucei brucei* strain 427 to megazol and other trypanocides[a]

Drug	IC_{50} (μM)			
	Procyclic forms		Bloodstream forms	
	Wild type	Resistant (factor of resistance)	Wild type	Resistant (factor of resistance)
Megazol	0.28 ± 0.01	29.24 ± 3.18 (104)*	0.15 ± 0.02	3.20 ± 0.48 (21)*
Nifurtimox	4.89 ± 0.12	37.12 ± 3.84 (7.6)*	5.58 ± 0.86	34.41 ± 4.91 (6.2)*
Benznidazole	2.89 ± 0.20	12.87 ± 1.29 (4.5)**	15.26 ± 3.85	77.36 ± 4.92 (5.2)*
Suramin	15.59 ± 2.61	66.06 ± 4.15 (4.2)**	0.13 ± 0.01	0.32 ± 0.21 (2.5)***
Berenil	10.99 ± 1.50	41.14 ± 1.31 (3.7)*	0.99 ± 0.12	5.18 ± 1.14 (5.2)*
Pentamidine	2.14 ± 0.03	5.06 ± 0.23 (2.4) (NS)	0.04 ± 0.07	0.15 ± 0.01 (3.8)*
Melarsen oxide	0.02 ± 0.00	0.07 ± 0.08 (3.5)*	0.01 ± 0.00	0.05 ± 0.01 (5.0)*
Melarsoprol	2.38 ± 0.08	2.60 ± 0.02 (1.1) (NS)	0.13 ± 0.01	0.30 ± 0.01 (2.3)***
Cymelarsan	0.06 ± 0.00	0.13 ± 0.00 (2.2)***	0.02 ± 0.02	0.07 ± 0.03 (3.5)***
Ethidium bromide	12.15 ± 1.52	16.89 ± 1.73 (1.4) (NS)	2.07 ± 0.68	4.29 ± 0.71 (2.1) (NS)
Isometamidium	9.80 ± 0.79	11.44 ± 0.79 (1.2) (NS)	3.35 ± 0.56	4.33 ± 0.67 (1.3) (NS)
Brilliant green	0.016 ± 0.00	0.053 ± 00 (3.3)	0.003 ± 00	0.003 ± 0.000 (1.0)

[a] Sensitivities of wild-type and selected megazol-resistant lines to a number of drugs were determined. The factors of resistance indicated in parentheses were calculated as ratios of IC_{50}s for resistant parasites to those for wild-type parasites. Values that were significantly different from that for the control, by Student's *t* test, are designated by an asterisk(s) (*, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$; NS, not significant at $P = 0.05$). Values are means ± standard deviations ($n = 3$ independent experiments).

ment, for 2 h, did not have a significant impact on thiol content in bloodstream or procyclic forms, while BSO, at the concentrations used here, affected only the thiol content of procyclic forms (reducing it about twofold from 3.2 nmol $[10^8$ cells$]^{-1}$).

No significant difference in total thiol levels was observed between wild-type and resistant forms, indicating that alterations to thiol levels are not involved in the development of resistance characterized here.

Nitroheterocyclic compounds are generally believed to exert their cytotoxic effects only after activation by single electron reduction of their corresponding nitro anion radicals (27). In bacteria, the toxic effects of metronidazole are accompanied by damage to DNA (25, 29) and cells defective in DNA repair become hypersensitive to the action of metronidazole (11). A principal enzyme involved in eukaryotic DNA repair is RAD51. *T. brucei* mutants lacking this enzyme (24) were hypersensitive to megazol, indicating that the toxic effects of megazol are accompanied by damage to DNA (Table 2). In contrast, the susceptibility of RAD51 deletion mutants to the trypanocidal nitrofuran nifurtimox was similar to that of wild-type cells. *N*-acetylcysteine (NAC) antagonizes oxidative stress by interacting with a number of the reduced oxygen species (4, 8). When coadministered at 0.5 mM (the highest concentration at which no detrimental effect on trypanosomes was induced by this compound), NAC had no antagonistic effect on the action of megazol while it did have a modest protective effect against the action of nifurtimox (Table 2).

TABLE 2. Activity of megazol against wild-type and RAD51$^{-/-}$ *T. brucei* bloodstream forms[a]

Drug	IC_{50} (μM)	
	Wild type	RAD51$^{-/-}$ mutants
Megazol	0.13 ± 0.0025	0.029 ± 0.002
Megazol with NAC	0.13 ± 0.0020	ND
Nifurtimox	3.37 ± 0.19	3.40 ± 0.15
Nifurtimox with NAC	12.72 ± 1.1	ND

[a] Values are means ± standard deviations ($n = 3$ independent experiments). The effect of 0.5 mM NAC was assessed by adding this concentration to wild-type cells only prior to addition of drug. ND, not determined.

The activity of nifurtimox in *T. brucei* therefore appears to be mediated through reduced oxygen species, produced during futile redox cycling with reduced drug. Megazol's activity, however, derives from direct damage exerted by the reduced megazol nitro anion radical derivatives. In *T. cruzi*, nifurtimox is also known to exert its activity through redox cycling (9, 10). Recently it was shown that nifurtimox stimulated an increase in oxygen uptake into *T. cruzi* cells while megazol did not (23). Thus, experiments using different approaches and different species of trypanosomes indicate that the activities of the nitrofuran nifurtimox and the nitroimidazole megazol arise through different mechanisms.

Bertin Enanga was a Wellcome Trust Traveling Fellow.

The excellent assistance of Flora Logan (funded by a Society for General Microbiology summer studentship) and Janice Brock is gratefully acknowledged. Richard McCulloch (University of Glasgow) kindly donated the RAD51$^{-/-}$ strain of *T. brucei*.

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