Intracellular Release of Nitric Oxide by NCX 972, an NO-Releasing Metronidazole, Enhances In Vitro Killing of *Entamoeba histolytica*

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The activity of NCX 972, a new molecule obtained by adding a nitric oxide (NO) moiety to metronidazole, was tested against six isolates of *Entamoeba histolytica* in xenic cultures. NCX 972 released NO into trophozoite cells and enhanced killing of amoebas in a dose- and time-dependent manner compared to metronidazole.

Entamoeba histolytica is a protozoan parasite causing amoebic colitis, dysentery and liver abscess. Transmission is by fecal-oral route, with cysts able to survive in the environment and trophozoites which penetrate the large intestine mucosa and cause tissue damage. Although the global prevalence of *E. histolytica* infections has been overestimated due to asymptomatic infections by the morphologically identical, noninvasive species *Entamoeba dispar* (2), this parasite is responsible for approximately 100,000 deaths per annum worldwide, being second in importance only to malaria among the pathogenic protozoa (23).

The first-line drugs for invasive amoebiasis are nitroimidazoles, with metronidazole (MTZ) being the drug of choice for efficacy and low cost (10). Although resistance has not yet been proven in *E. histolytica* strains isolated from patients, treatment failures following MTZ therapy have been documented (8). In contrast, resistance to this drug is common in bacteria and the protozoan flagellates *Giardia intestinalis* and *Trichomonas vaginalis*, and in vitro, trophozoites of *E. histolytica* readily adapt to therapeutic levels of MTZ (24). Hence, should resistance to MTZ become widespread, new amoebicidal compounds as well as the improvement of existing drugs will be needed.

Nitric oxide (NO) has been found to possess microbicidal effects against a number of pathogens, including DNA and RNA virus families (1), bacterial pathogens (13), and intracellular and extracellular protozoa (3, 4). Since 1992, NO has been demonstrated to be the major cytotoxic molecule released by activated macrophages for the in vitro cytotoxicity against *E. histolytica* (11). Later evidence suggests that NO is involved in host defense in vivo, this molecule being required for the control of amebic liver abscess in a murine model (19); and also, NO levels are increased in patients with acute intestinal amoebiasis (14).
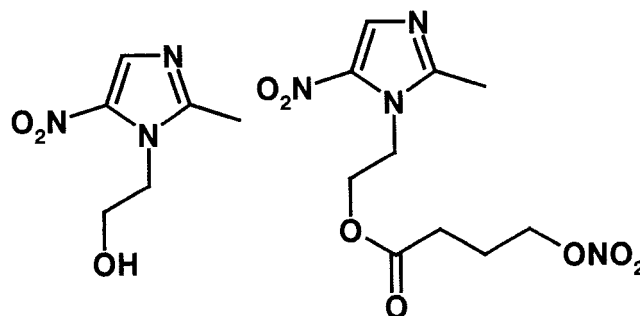
Recently, novel NO-releasing drugs have been proposed for pharmaceutical uses. For example, NO-aspirin is currently tested as an anti-inflammatory and antithrombotic agent (22). In the literature there is no evidence of NO-releasing molecules being effective in treating amoebiasis. To investigate whether a newly synthesized MTZ derivative releasing NO (NCX 972) may be used for the treatment of amoebiasis, here we have compared the in vitro effect of MTZ and NCX 972 against *E. histolytica*.

NCX 972 [4-(nitrooxy)butanoic acid 2-methyl-5-nitroimidazole-1-ethyl ester; molecular weight, 302.26] was from the Nicox Research Institute (Milan, Italy). MTZ (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole; molecular weight, 171.2) was purchased by Sigma (Milan) (Fig. 1).

Pathogenic *E. histolytica* strains used (255, AX1, AX2, AX3, 128, and 735), as isolated from different areas of the world (Table 1), cloned and typed by isoenzyme electrophoresis and PCR (A. R. Sannella, M. Gramiccia, M. C. Angelici, G. Dettori, and E. Viani, Letter, J. Eukaryot. Microbiol. **45:**137, 1998), were from the cryostabilate collection of Istituto Superiore di Sanità, Rome, Italy. For routine maintenance the strains were grown in tubes as xenic cultures in 2.5 ml of Robinson's medium supplemented with *Escherichia coli* bacteria (16). This medium, rather than an axenic culture medium (e.g., TYI-S-33 medium [18]), was chosen also for drug testing assays, in order to verify any possible interference between NCX 972 and common intestinal bacterial flora. Overgrowth of bacterial flora was controlled by 1 μg/ml penicillin-streptomycin mixture, a dosage proven nontoxic to amoeba cells.
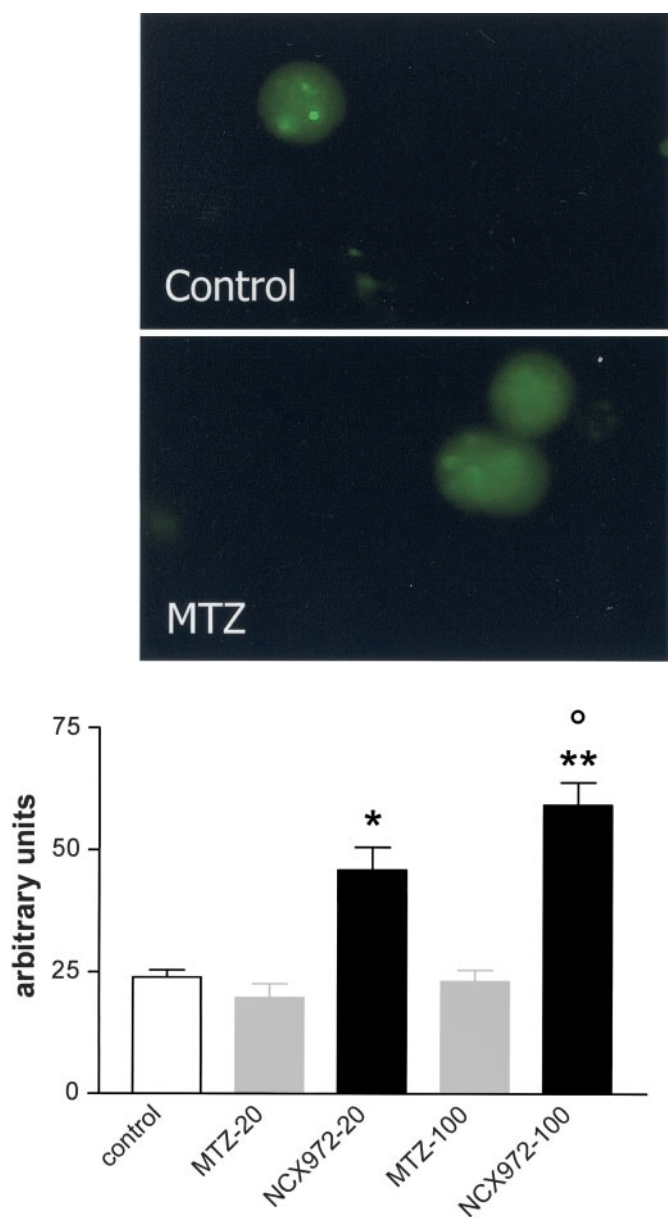
Firstly, we have observed that NCX 972 was able to release NO into amoebic trophozoites, as measured by using the DAF-

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Metronidazole   NCX 972

FIG. 1. Chemical structure of MTZ (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole) and NCX 972.
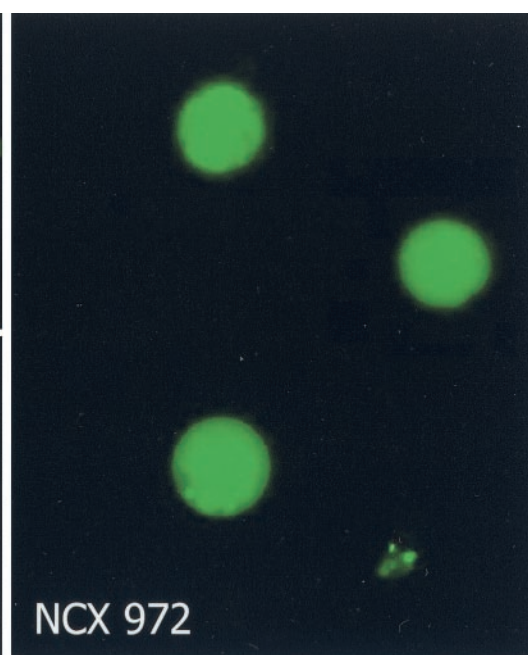
FIG. 2. Intracellular release of NO by NCX 972 in trophozoites of *E. histolytica*. Cells were preincubated with DAF-2DA and then treated with a 20- to 100-μM concentration of either MTZ or NCX 972 for 30 min. Images report DAF fluorescence of trophozoites untreated (control) or treated with 100 μM MTZ and 100 μM NCX 972. Data are expressed as arbitrary units. The gray density was calculated at the single-cell level (about 100 cells/treatment condition/experiment). Bars represent the mean ± SEM (error bars) from two separate experiments. One-way ANOVA followed by Bonferroni's test was used to determine the significant differences. Symbols: *, $P < 0.001$ between 20 μM NCX 972 and 20 μM MTZ; **, $P < 0.001$ between 100 μM NCX972 and 100 μM MTZ; °, $P < 0.05$ between 20 μM and 100 μM NCX 972 treatments. For further details see the text.

cell level. Then, data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (GraphPad Prism Software, version 3.03).

A basal low level of DAF fluorescence was found in untreated trophozoites (23.9 ± 1.5 arbitrary units; digital scale ranging from 0 to 255, minimum to maximum fluorescence intensity, respectively) as well as in cells treated for 30 min with 20 and 100 μM MTZ (19.7 ± 2.8 and 23.1 ± 2.3 arbitrary units, respectively) (Fig. 2) (data are presented as means ± standard

2DA detection system (12). DAF-2DA is a dye that, upon binding an oxidized species of NO, results in irreversible DAF fluorescence, thus permitting identification of the source of local NO formation in living cells. This method has been recently used to demonstrate both the intracellular release of NO an NO-releasing derivative of aspirin (9), and the endogenous production of NO into cells (21).

Briefly, trophozoites of the 255 strain were loaded with 10 μM DAF-2DA for 15 min and then incubated with a 20- to 100-μM concentration of either NCX 972 or MTZ for 30 min. After washings, cells were plated onto glass micro slides, covered with coverslips and viewed with an Axioplan II microscope (Carl Zeiss, Jena, Germany) equipped with a cooled charge-coupled device camera SPOT (Diagnostic Instruments, Inc., Sterling Heights, Mich.). Images were digitally acquired and processed for densitometric determination at the single-

TABLE 1. Strains of *E. histolytica* used and activities of NCX 972 and MTZ after 48-h exposure

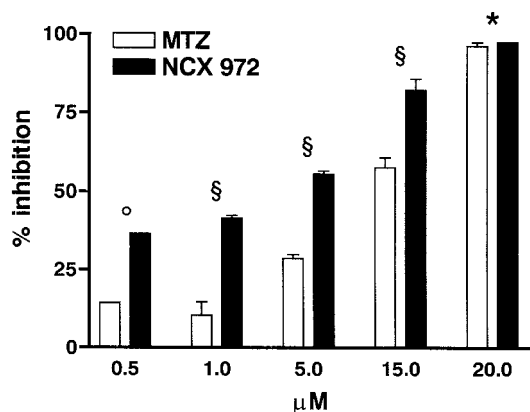| *E. histolytica* strain	Origin	Mean % inhibition ± SEM caused by addition of:			
		NCX 972		MTZ	
		5 μM	20 μM	5 μM	20 μM
255	Zimbabwe	46.1 ± 0.5	97.7 ± 0.1	28.6 ± 1.2	96.4 ± 1.2
AX1	Brazil	47.6 ± 1.0	97.1 ± 0.2	25.1 ± 1.1	97.7 ± 0.8
AX2	Ecuador	44.2 ± 0.6	97.6 ± 1.0	20.3 ± 1.0	97.8 ± 0.4
AX3	Italy	36.0 ± 1.4	96.2 ± 0.8	19.7 ± 2.1	96.4 ± 0.2
128	Mexico	46.7 ± 2.1	100	27.3 ± 1.9	100
735	India	37.3 ± 0.9	97.8 ± 0.5	22.6 ± 1.5	97.8 ± 0.2

FIG. 3. Activity of NCX 972 and MTZ against *E. histolytica* trophozoites. Drugs were added to the indicated concentrations, and the incubation was continued for 48 h. Data are expressed as percent inhibition and each bar represents the mean + SEM (error bars). Two-way ANOVA followed by Bonferroni's test was used to determine the significant differences. Symbols: °, $P < 0.01$ between 0.5 μM NCX 972 and 0.5 μM MTZ; §, $P < 0.001$ between 1 μM NCX972 and 1 μM MTZ, or 5 μM NCX972 and 5 μM MTZ, or 15 μM NCX972 and 15 μM MTZ; *, not significant. For further details see the text.

errors of the means [SEM]). When the cells were treated with 20 and 100 μM NCX 972 for 30 min, DAF fluorescence was strongly enhanced above the background level (45.9 ± 4.7 and 59.2 ± 4.6 arbitrary units, respectively; $P < 0.001$) (Fig. 2).

To compare the cytotoxic activities of NCX 972 and MTZ, two separate experiments were performed for each of the amoeba strains, and at each time point and concentration, viable trophozoites were counted from triplicate cultures with a phase-contrast microscope in 100 microscopic fields (magnification, ×200). Granular and rounded cells were regarded as dead. The mean viable cell count from treated cultures was expressed as percentage of the mean viable cell count from untreated cultures in each experiment (slightly modified from the method of Seifert et al. [18]).

A 48-h dose-response assay using both drugs at concentrations from 0.5 to 20 μM was performed on the 255 strain (Fig. 3). These data were analyzed by two-way ANOVA with the F statistical test for independent effect of drug concentration and treatment (GraphPad Prism Software, version 3.03). As expected, MTZ produced a log-normal inhibition effect, with a 50% effective concentration of about 11 μM. Compared to MTZ, treatment with NCX 972 showed a significantly higher inhibitory activity, with a 50% effective concentration of about 3 μM (F, 161.0 [$P < 0.0001$]). Also differences in cell number among drug concentrations were significant by two-way ANOVA (F, 294.8 [$P < 0.0001$]). Notably, the efficacy of NCX 972 in killing *E. histolytica* trophozoites was significantly higher at low and mid drug concentrations, i.e., when MTZ possesses a suboptimal activity (e.g., 36.5% ± 0.1% versus 14.4% ± 0.2% at 0.5 μM; 41.5% ± 0.8% versus 10.4% ± 4.2% at 1 μM). On the contrary, at the highest concentration tested (20 μM), the difference between the activity of NCX 972 and MTZ was not significant (97.7% ± 0.1% versus 96.4% ± 1.2%). A similar inhibitory effect was obtained using 5 and 20 μM concentrations of NCX 972 or MTZ on further five *E. histolytica* strains (Table 1).

A time-dependent (6 to 72 h) effect of a sublethal concentration (5 μM) of NCX 972 or MTZ were also assayed on trophozoites of the 255 strain. Data were analyzed by two-way analysis of variance (ANOVA) with the F statistical test for independent effect of time and drug treatment followed by the Bonferroni's test. When compared with MTZ, treatment with NCX 972 showed an extremely significant quicker inhibitory activity (F, 51.7 [$P < 0.0001$]) (Fig. 4). However, the highest difference between NCX 972 and MTZ was observed after 72-h drug exposure (56.5% ± 0.1% versus 24.8% ± 0.7%; $P < 0.001$).

Since NO possesses antimicrobial activity (6), the possibility that the effect of NCX 972 was attributable to a killing of bacterial flora used as natural feed for trophozoites in the culture medium, has been also considered. To address this question, bacteria were counted by agar plating after 24-h exposure to NCX 972 or MTZ at different concentrations (1 to 20 μM). No significant differences were seen between number of *E. coli* colonies developed from untreated cultures and cultures treated with both drugs (data not shown).

Taken together, our data indicate that the NO-releasing MTZ derivative (NCX 972), especially at low doses, exerts an enhanced activity against *E. histolytica*.

Although the effect of NO on parasites has been well established (for reviews, see references 3 and 4), macromolecular targets have not been identified so far. Cysteine proteases from a number of intracellular parasitic protozoa (*Leishmania infantum*, *Plasmodium falciparum*, and *Trypanosoma cruzi*) have been reported to be inactivated by NO-mediated S-nitrosylation (5, 17; M. Colasanti, L. Salvati, G. Venturini, P. Ascenzi, and L. Gradoni, Letter, Trends Parasitol. **17:**575, 2001). *E. histolytica* trophozoites release large amounts of cysteine proteases in extracellular environment, which significantly contribute to amebic virulence (7; for a review, see reference 15).
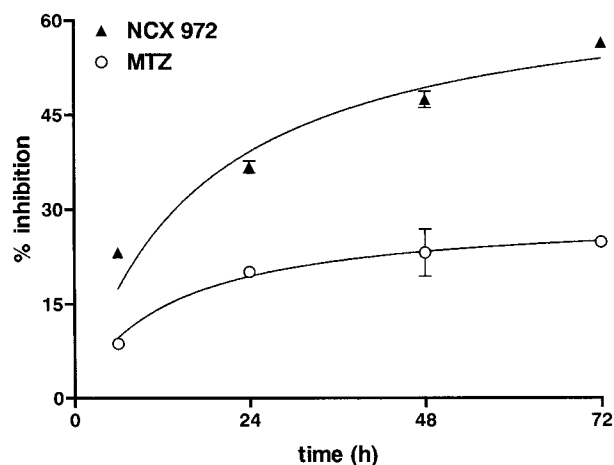

FIG. 4. Time-dependent effect of NCX 972 and MTZ against *E. histolytica* trophozoites. Both drugs (5 μM) were incubated for 6 to 72 h. Data are expressed as percent inhibition and each point represents the mean ± SEM (error bars). Two-way ANOVA followed by Bonferroni's test was used to determine the significant differences: not significant between NCX 972 and MTZ at 6-h treatment; $P < 0.01$ between NCX 972 and MTZ at 24-h treatment; $P < 0.001$ between NCX972 and MTZ at 48-h or 72-h treatments. For further details see the text.

Very recently, *E. histolytica* cysteine proteases have been found to be inhibited by *S*-nitroso-glutathione, a physiological NO donor (20). In addition, alcohol dehydrogenase-2, an enzyme required for the survival of this parasite, has been inactivated by the same NO donor (20). However, the possibility that under our conditions NO may affect other parasite molecular targets and/or enhance sensitivity of *E. histolytica* to MTZ should be taken into account.

In conclusion, NCX 972 displayed higher and faster activity than MTZ in killing *E. histolytica* and this effect appears to be correlated with NO release into parasite cells. These data support the concept that the addition of NO-releasing moiety to an established drug, such as MTZ, significantly improves its activity. Note that other drugs using the same NO-releasing approach have been found to be effective and safe, and are currently employed in clinical trials; also, they exert cytoprotective effects in the gastrointestinal mucosa (22).

Thus, although further studies are needed (including standard drug development processes), NO-releasing MTZ NCX 972 appears to be a promising lead compound to enhance efficacy of the present treatment and to reduce intestinal damage associated with invasive amoebiasis.

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