CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

21-618

21-681

21-682

MICROBIOLOGY REVIEW

MICROBIOLOGY REVIEW
DIVISION OF SPECIAL PATHOGEN AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

NDA #: 21-618, 21-681 and 21-682

REVIEWER : Kalavati Suvarna
Avery Goodwin
CORRESPONDENCE DATE : 07-16-03
CDER RECEIPT DATE : 07-17-03
REVIEW ASSIGN DATE : 07-28-03
REVIEW COMPLETE DATE : 03-01-04

SPONSOR: Presutti Laboratories,
1607 N. Douglas Avenue,
Arlington Heights, IL 60004.

SUBMISSION REVIEWED: N-000

DRUG CATEGORY: Anti-protozoal

INDICATION: Treatment of trichomoniasis [redacted], giardiasis [redacted] and intestinal amoebiasis and amoebic liver abscess.

DOSAGE FORM: Oral Tablets

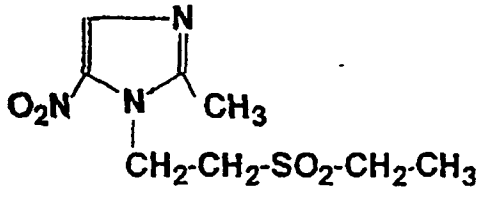
PRODUCT NAMES:

a. **PROPRIETARY:** Tindamax

b. **NONPROPRIETARY:** Tinidazole
CAS: 19387-91-8

c. **CHEMICAL:** 1- [2-(Ethylsulfonyl)ethyl]-2-methyl-5-nitroimidazole.

STRUCTURAL FORMULA:



Molecular weight: 247.28
Empirical formula: $C_8H_{13}N_3O_4S$

SUPPORTING DOCUMENTS: IND # 62,292; DMF #s [redacted]

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EXECUTIVE SUMMARY:

The subject of this NDA is tinidazole for the treatment of trichomoniasis [redacted] giardiasis [redacted] and amoebiasis (intestinal and hepatic).

Mechanism of Action:
Tinidazole is a 5-nitroimidazole compound and belongs to the same class of drugs as metronidazole. Tinidazole is reduced by cell extracts of trichomonads. The reduction of the nitro group results in generation of free radical anion which may be responsible for the anti-protozoal activity of the drug. Chemically reduced drug was shown to cause damage to purified bacterial (*Escherichia coli*) DNA *in vitro*. Supportive evidence that DNA is the target for action comes from base changes observed in bacterial cells and DNA strand breakage observed in mammalian cells. Although the pyruvate: ferredoxin oxidoreductase enzyme plays a role in the reductive activation of metronidazole in protozoa, the enzyme responsible for reduction of tinidazole by protozoa has not been identified. The mechanism by which tinidazole exhibits activity against *Giardia lamblia* and *Entamoeba histolytica* is not known.

Protozoa:
The activity of tinidazole against *Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica* was examined. Please note that the methods for susceptibility testing of anti-protozoal drug have not been standardized. Additionally, the correlation between *in vitro* activity and clinical outcome has not been established.

Trichomonas vaginalis:
The life cycle of *T. vaginalis* consists of only the trophozoite stage. The *in vitro* activity of tinidazole was comparable to metronidazole against the trophozoite stage of *T. vaginalis* laboratory strains and clinical isolates.

In mice infected intravaginally with *T. vaginalis* mixed with *C. albicans*, a 1.41 mg/kg oral dose of tinidazole reduced trophozoite counts by 50%. A higher dose (7.5 to 18.8 mg/kg) was required to have a similar effect in mice infected intraperitoneally or subcutaneously. However, metronidazole was effective at a higher dose (1.4 - 2.6 fold) compared to tinidazole for treatment of mice infected intravaginally or intraperitoneally with *T. vaginalis*.

The suppression of infection in 95% of mice infected intraperitoneally with the *T. vaginalis* [redacted] strain required a dose of 8.8 mg/kg tinidazole. A 3 fold higher dose of tinidazole was required for suppression of infection in mice infected subcutaneously with the same strain. [redacted]

[redacted]

The development of resistance to tinidazole by *T. vaginalis* was not examined *in vitro* or *in vivo*.

[redacted]

In patients treated with a single dose of 2 gm tinidazole, the parasitological outcome was measured using culture and/or wet mount methods in 34 studies. Overall, a successful parasitological outcome was observed in 2131/2271 (95%) patients with vaginitis. Information on symptom resolution or relief was available for 357 of the 2271 patients. A correlation was observed between clinical and parasitological outcome in these 357 patients. A relapse rate of 5% was observed at 1 month post therapy, when both female patients and their male partners were treated with tinidazole. Tinidazole was as effective as metronidazole and other experimental drugs such as ornidazole and carnidazole.

In patients with urethritis, a successful parasitological outcome was observed in 96% (240/250) men treated with tinidazole. Information on resolution of symptoms was available for 105 of the 250 patients. The parasitological outcome correlated with clinical outcome in these 105 patients. The efficacy of tinidazole was similar to metronidazole. The relapse rates were not measured in male patients with urethritis.

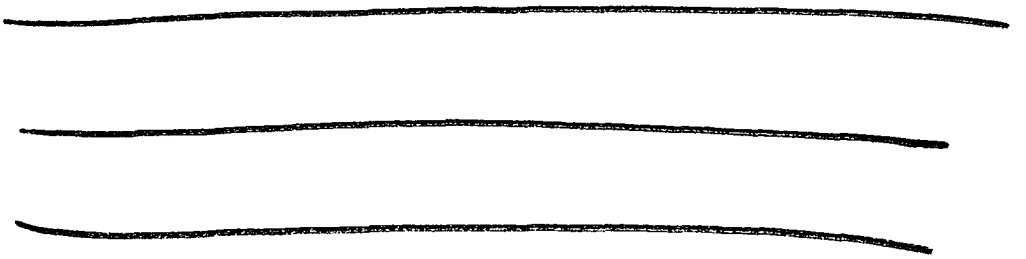
Overall, the parasitological outcome to tinidazole treatment in the trichomoniasis patients evaluated by culture versus those evaluated by wet mount was similar. A direct comparison between wet mount and culture was made in one study. The study showed 40% patients with negative wet mount to be positive by culture, suggesting that the culture method is more sensitive than wet mount. Comparative analysis of the two methods in the literature also suggests that the culture method is more sensitive than wet mount.

Giardia lamblia:
The *in vitro* activity of tinidazole was similar to metronidazole and furazolidone against the trophozoite stage of *G. lamblia* strains and isolates. The activity of tinidazole against the cyst stage of *G. lamblia* was not measured *in vitro*. Tinidazole in combination with doxycycline, mefloquine or furazolidine does not appear to be antagonistic against the P1 and BRIS/82/HEP/41 strains of *G. lamblia* by the growth inhibition and adherence assays.

In suckling mice infected intragastrically with *G. lamblia,* tinidazole (2.8 mg/kg) was more active than metronidazole (40.8 mg/kg) in reducing trophozoite counts in the intestinal tissue at day 2 after discontinuation of treatment. However, the effect of the drug on cyst count was not measured.

The development of resistance to tinidazole by *G. lamblia* and cross-resistance between tinidazole and other drugs was not examined *in vitro* or *in vivo*.

In patients with giardiasis, the parasitological outcome after treatment with tinidazole was measured using unconcentrated and concentrated stool samples in 13 studies. Two or more stool samples were examined in 10 of the 13 studies. Tinidazole was effective in the treatment of giardiasis (710/773, 92%) compared to metronidazole (162/310, 52%) as measured by absence of cysts. Both clinical and parasitological outcomes were measured as therapeutic cure rates in 8 studies, suggesting a correlation between clinical and parasitological outcomes. No information was available on the occurrence of relapse in these patients.



Entamoeba histolytica:
The *in vitro* activity of tinidazole was comparable to metronidazole against the trophozoite stage of *E. histolytica* strains and clinical isolates. The activity of tinidazole against the cyst stage of *E. histolytica* was not measured *in vitro*.

In rats infected intracecally with *E. histolytica*, tinidazole (≥ 50 mg/kg) decreased the severity of infection (based on reduction in trophozoite count in intestinal tissue and improvement in pathology) after 24 hours of treatment. Additionally, tinidazole (100 mg/kg) was effective in preventing the development of amoebic liver abscess in hamsters. The activity of tinidazole was similar to metronidazole in these two *E. histolytica* animal models.

The development of resistance to tinidazole by *E. histolytica* and cross-resistance between tinidazole and other drugs was not examined *in vitro* or *in vivo*.

A successful clinical and parasitological outcome was observed in 92% (342/369) patients with intestinal amoebiasis treated with tinidazole compared to 55% (114/209) treated with metronidazole in 9 studies. The parasitological outcome was measured using unconcentrated and concentrated stool samples. Tinidazole showed similar parasitological outcome in trophozoite passers and cysts passers. The parasitological outcome in trophozoite passers with metronidazole (73%) was comparable to tinidazole (88%). However, the parasitological outcome in cyst passers with metronidazole (47%) was lower than tinidazole (93%). Overall, good correlation was observed between parasitological and clinical outcomes in patients treated with tinidazole on day 30 after initiation of therapy. However, the correlation was poor (parasitological, 51%; clinical 100%) in one study that measured the outcomes 6 day after discontinuation of therapy. The occurrence of relapse in these patients was not measured.

In patients with hepatic amoebiasis, a successful clinical outcome was observed in 94% (260/275) patients treated with tinidazole compared to 74% (89/120) patients treated with metronidazole at 30 days post-treatment. The parasitological outcome was not measured in these

1. INTRODUCTION AND BACKGROUND:

The subject of this NDA is Tinidazole for the treatment of trichomoniasis , giardiasis and amoebiasis (intestinal and hepatic). Tinidazole is marketed in the United Kingdom, France and Australia for the treatment of trichomoniasis and giardiasis in adults by Pfizer under the trade name Fasigyn®. In addition, tinidazole is marketed in other countries (Austria, Belgium, Costa Rica, El Salvador, Finland, Germany, Guatemala, Honduras, Italy, India, Japan, Mexico, Netherlands, New Zealand, Nicaragua, Panama, South Africa, Spain, Sweden, and Switzerland) for the treatment of trichomoniasis, giardiasis, intestinal and hepatic amoebiasis.

For the treatment of trichomoniasis, the sponsor has proposed a single 2 gm dose of oral tinidazole tablets with food for female patients with the disease and their male partners.

Metronidazole is the only drug approved for trichomoniasis in the United States.

For the treatment of giardiasis, a single 2 gm dose with food was proposed for adults and a single 50 mg/kg (up to 2 gm) dose for children over 3 years of age.

In the US, nitazoxanide, quinacrine, and furazolidine are approved for the treatment of giardiasis. However, quinacrine production has been discontinued. Besides these drugs, metronidazole, albendazole, and paromomycin are available for the treatment of giardiasis. Although metronidazole is mostly effective, cases of giardiasis failing treatment with high doses of metronidazole have been reported.

For the treatment of intestinal amoebiasis, a 2 gm dose for 3 days with food was proposed for adults and a 50 mg/kg (up to 2 gm) dose for 3 days in children over 3 years of age. For amoebic liver abscess, the dose was the same as for intestinal amoebiasis but the drug may be administered for 3 to 5 days. Metronidazole, iodoquinol, chloroquine phosphate, paromomycin and erythromycin are approved for the treatment of intestinal amoebiasis. Dehydroemetine is available on a compassionate use basis for the treatment of intestinal and extraintestinal amoebiasis.

Tinidazole is a 5-nitroimidazole derivative. It is insoluble in water but soluble in chloroform, methanol, and dimethyl sulfoxide (DMSO). The peak concentrations of tinidazole (42 - 48 μg/ml) in the plasma are reached within 2 - 6 hours of administration of a 2 gm oral dose. The half-life of the drug is 12.7 ± 0.5 hours and twice that of metronidazole (6 - 7 hours). About 12% of tinidazole is bound by serum proteins compared to 20% of metronidazole. The drug has been shown to cross the blood-brain barrier. In the plasma, tinidazole is the major constituent observed after treatment along with trace amounts of the metabolite, 2-hydroxymethyl tinidazole. The metabolite was shown to be active against the anaerobic bacteria, *Gardnerella vaginalis.*

1.1. *Trichomonas vaginalis*:

T. vaginalis is a protozoan that causes the sexually transmitted disease, trichomoniasis. *T. vaginalis* has only the trophozoite stage in its life cycle. The trophozoites infect the squamous epithelium of the genital tract. The major clinical manifestation is vaginitis in women and urethritis in men.

The identification of *T. vaginalis* is usually based on the microscopic examination of vaginal or urine specimens in females, and urine sediment, urethral discharges and prostatic secretions in male partners. Besides wet mount preparations, culture methods can be used for detection. Culture methods have greater sensitivity (92-95%) than wet mount (45-60%)[1,2]. Diamond's medium, Trichomonas medium no. 2 ——— and the InPouch TV culture system are commonly used to culture *T. vaginalis*[3]. Culture of *T. vaginalis* is considered the gold standard as a negative wet mount does not rule out trichomoniasis.

In addition to the above methods, direct immunofluorescence assay, latex agglutination assay enzyme immunoassay, DNA probe assays, and PCR assays are available but mainly used in research settings. The sensitivities of these methods are comparable or better than wet mount but lower than culture method[4-7]. However, the usefulness of these methods in measuring drug efficacy is not known.

Majority of men with *T. vaginalis* infection are asymptomatic. The detection of *T. vaginalis* in urine sediments or urethral scrapings in men by the wet mount and culture method is highly specific but lacks sensitivity (30-45%). A large organism burden is required for a positive test. Hence, asymptomatic male sexual partners of infected women are usually treated with standard dose of metronidazole[8].

1.2. *Giardia lamblia:*

G. lamblia is a flagellated protozoan found in the intestinal tract of human. *G. duodenalis* or *G. intestinalis* are alternate names for the same pathogen. The major clinical manifestations of *G. lamblia* infection are diarrhea and malabsorption. Infection is caused by ingestion of contaminated food or water containing *G. lamblia* cysts. Following ingestion, the cysts pass through the stomach to the small intestine where they excyst to give rise to trophozoites. The trophozoites attach to the epithelial cells in the duodenum and bile duct of the host, divide by longitudinal fission and encyst on reaching the colon. In cases of severe infection, trophozoites are more commonly observed than cysts in diarrheic stool samples. The severity of infection is affected by the number of cysts ingested and the strain causing the infection.

Excystation can be initiated *in vitro* by culture at an alkaline pH or by increasing the concentration of bile[9].

The standard method for diagnosis of giardiasis is based on detection of trophozoites or cysts by microscopic examination of unconcentrated and concentrated stool samples. Examination of 3 stool samples has a sensitivity of >90%[10,11]. Since the trophozoites attach to the duodenal walls and the cysts are shed intermittently in stool, the possibility of missing the parasites exists. In such cases where the level of cysts is low, examination of more than 3 stool samples may be necessary [NCCLS approved guidelines for detection of parasites in stool samples (M28-A), 1997]. Staining of fecal smears is recommended as it improves the sensitivity of the microscopic

examination. Besides microscopic examination, immunofluorescence and enzyme linked immunosorbent assays are available for the detection of *Giardia* cysts and have been shown to be more sensitive than microscopic examinations of iodine stained stool sample. Although these tests need specialized equipment, they are easy to perform and less prone to error. In cases where stool samples are negative, testing of duodenal aspirates or duodenal contents using the Entero-test capsule (the capsule contains a string that can be used to retrieve duodenal contents) may be used to rule out infection.

Methods detecting anti-Giardia IgG antibodies have been used more for epidemiological purposes than diagnosis due to the prolonged persistence of the antibodies in circulation. PCR methods have been developed for detection of *Giardia* cysts, however, the usefulness of PCR for diagnosis and evaluation of drug efficacy is not known.

1.3. *Entamoeba histolytica*:

E. histolytica is a protozoan parasite that causes amoebic dysentery and liver abscess. Its life cycle consists of two stages: cysts and trophozoites. Humans are infected by ingesting cysts, most often via food or water contaminated with human feces. The cyst excysts within the lumen of the small intestine and give rise to 8 trophozoites. The trophozoites can adhere to the mucus and epithelial layers of the colon and invade the colonic epithelium, causing amoebic colitis. Amoebic dysentery with symptoms [such as abdominal pain and tenderness, and painful sudden bowel evacuation (tenesmus), diarrhea, weight loss] usually develops over a period of one to several weeks. Re-encystation of the trophozoites occurs within the lumen of the colon, resulting in the excretion of cysts in the feces and continuation of the life cycle. In some cases the trophozoites will enter the circulatory system and infect other organs such as the liver (hepatic amoebiasis or amoebic liver abscess), or they may penetrate the gastrointestinal tract resulting in acute peritonitis leading to fatality.

The diagnosis of intestinal amoebiasis relies mainly on microscopic examination of stool samples. A minimum of 3 stool samples collected within a 10 day period may be necessary for diagnosis [NCCLS approved guidelines for detection of parasites in stool samples (M28-A), 1997]. Permanent staining of fecal smears with trichrome or iron hematoxylin improves the sensitivity of the microscopic examination. Additionally, concentration of stool samples improves detection of cysts. Histological evaluation of sigmoidoscopy specimens is sometimes used in conjunction with other laboratory finding for detection of trophozoites. However, the microscopic techniques are unable to differentiate between *E. histolytica* and *E. dispar* (non-pathogenic form) in the absence of ingested erythrocytes in the trophozoites. Culture (diphasic Locke's egg slant medium, Diamond's medium or TYI-S-33 medium) with zymodene analysis of the culture lysates is considered the gold standard for differentiating the pathogenic and non-pathogenic forms but is not routinely performed in the laboratory. Besides these methods, enzyme linked immunosorbent assays (ELISAs) that detect the *E. histolytica* specific antigens are available. The antigen detection assays and PCR are more sensitive than routine microscopic examination of stool for the detection of *E. histolytica*. However, the usefulness of these assays in measuring drug efficacy is not known.

The diagnosis of hepatic amoebiasis (liver abscess) is based on demonstration of hepatic lesions by imaging techniques such as computed tomography, ultrasonography, magnetic resonance imaging, technectium-99 liver scan and detection of trophozoites in the liver aspirate

microscopically. As the organism may not be excreted in the stool, serological tests may be useful in the diagnosis of amoebic liver abscess. Serological tests such as indirect hemagglutination assay (IHA), counterimmunoelectrophoresis (CIE), amoebic gel diffusion, complement fixation, indirect fluorescence assay (IFA), latex agglutination assay and ELISAs are available. However, as these are experimental approaches for the diagnosis of extraintestinal amoebiasis, they should be used in conjunction with clinical findings. High background level of seropositivity can be problematic in interpretation of the test results as antibodies can persist for longer than 6 months. Also, antibody titers do not correlate with severity of the disease or response to therapy.

The sensitivities and specificities of the different tests in intestinal and hepatic amoebiasis (liver abscess) are shown in Table 1[12].

Table 1: Sensitivity and specificity of tests used in the diagnosis of amoebiasis.

Test	Colitis		Liver abscess
	Sensitivity	Specificity	Sensitivity
Microscopy (stool)	<60%	10–50%	<10%
Microscopy (abscess fluid)	NA[b]	NA	<25%
Culture with isoenzyme analysis	Lower than antigen or PCR tests	Gold standard	<25%
Stool antigen detection (FI ISA)	>95%	>95%	Usually negative
Serum antigen detection (ELISA)	65% (early)	>90%	~75% (late), ~100% (first 3 days)
Abscess antigen detection (ELISA)	NA	NA	~100% (before treatment)
Salivary antigen detection	Not done	Not done	70%
PCR (stool)	>70%	>90%	Not done
Serum antibody detection (ELISA)	>90%	>85%	70–80% (acute), >90% (convalescent)

[a] Reprinted from reference 220 with permission of the publisher
[b] NA, not available

2. PRECLINICAL MICROBIOLOGY:

2.1. Mechanism of action:

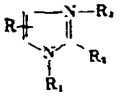
Tinidazole, like metronidazole, belongs to the 5-nitroimidazole class of compounds. The basis for the anti-protozoal and anti-bacterial action of 5-nitroimidazoles is the reduction of the nitro group of the drugs by the susceptible organisms and generation of short-lived nitroimidazole radicals which can oxidize DNA by causing strand breakage. Most of the information on the mechanism of action of 5-nitroimidazole class of compounds is based on studies with metronidazole. Briefly, studies examining the mechanism of action of metronidazole include the binding of ^{14}C-metronidazole to *E. coli* DNA *in vitro* [13,14], inhibition of thymidine uptake by *T. vaginalis* and *Clostridium* in the presence of metronidazole[14], single strand breakage in DNA measured using sucrose gradient sedimentation and spectrophotometric melting profiles of DNA exposed to chemically reduced metronidazole[15], fragmentation of metronidazole to acetamide upon nitro group reduction in *E. histolytica* and *T. vaginalis*[16], generation of metronidazole radical anion by pyruvate:ferredoxin oxidoreductase (PFOR) enzyme in the hydrogenosomal fractions of *Trichomonas foetus*[17], uptake of metronidazole by *Trichomonas* and *Entamoeba*[18], and relationship between metronidazole and bactericidal activity[19]. Although electron-spin magnetic resonance studies have implicated the existence of short-lived nitroimidazole radicals, they have not been isolated or characterized. Also, 5-nitroimidazole derivatives without the nitro group were stated to be inactive[20]. Please note that this review includes details of the studies examining mechanism of action of tinidazole.

Studies using tinidazole have examined the reduction of the drug by extracts of trichomonads, effect of the drug on *E. coli* DNA *in vitro* and effect of the drug on DNA base changes and strand breakage in bacterial and mammalian cells *in vitro*. The mechanism by which tinidazole exhibits activity against *G. lamblia* and *E. histolytica* was not examined.

Reduction of tinidazole by cell extracts of *Trichomonas*:

The ability of trichomonad homogenates to reduce tinidazole *in vitro* was examined[21]. The *T. foetus* or *T. vaginalis* cell homogenates (amount of homogenate used in the test was not specified) were incubated in the presence of pyruvate, methyl viologen (200 nmol), tinidazole or other nitroimidazoles under anaerobic conditions. The reduction of the drug was monitored by the change in absorbance measured spectrophotometrically at 25°C. The results for the reduction of nitroimidazoles by cell extracts were shown as positive or negative in Table 2. Both, tinidazole (compound no. 11) and metronidazole (compound no. 8) were reduced by trichomonad homogenates *in vitro*. However, the criteria for a positive or negative result were not specified and data on the absorbance values in the presence and absence of drugs were not included.

Table 2: Activity of nitroimidazole derivatives on trichomonads and on *S typhimurium* as well as their reducibility in the methyl-viologen-mediated *in vitro* assay.



Compound no.	R	R_1	R_2	R_3	Nonproprietary name	Reference	Relative inhibition of growth[a]		Relative mutagenesis in *S. typhimurium*[c]			Reduction in the in vitro assay
							T. foetus	*T. vaginalis*	TA 100		TA 100 FR$_1$	
									Aerobic	Anaerobic	Anaerobic	
1	4 (5)-NO_2	H	H			37	0	0	0	0	0	+
2	4 (5)-NO_2	H	CH_3			2, 37	0	0	0	0	0	+
3	4-NO_2	CH_2CH_2OH	CH_3			16, 37	0[d]	0[d]	0	0	0	+
4	4-NO_2	CH_3	H	CH_3		38	0	0	0	0	0	−
5	4-NO_2	CH_3	CH_3	CH_3		38	0	0	0	0	0	−
6	5-NO_2	CH_3	H			2, 5, 37	270	220	60	80	50	+
7	5-NO_2	CH_3	CH_3		Dimetridazole	2, 5, 37	300	310	200	160	150	+
8	5-NO_2	CH_2CH_2OH	CH_3		Metronidazole	2, 5, 16, 31, 37	100[d]	100[d]	100	100	100	+
9	5-NO_2	CH_2CH_2OH	CH_2OH			31, 36, 37	30[d]	20[d]	30	20	20	+
10	5-NO_2	CH_2COOH	CH_3			31, 36, 37	0[d]	0[d]	0	0	0	+
11	5-NO_2	$CH_2CH_2SO_2CH_3$	CH_3		Tinidazole	4, 27	50	60	110	140	120	+
12	5-NO_2	CH_2CH_2N (morpholino)	H		Nimorazole	3, 12	100	100	40	30	30	+

[a] Activities are expressed relative to metronidazole (no 8)
[b] Minimum inhibitory concentration values for metronidazole on *T. foetus*, 0 9 μg/ml, on *T. vaginalis*, 0 4 μg/ml.
[c] Mutagenetic effect of metronidazole, expressed as histidine revertants per microgram of drug, is 6.0 for aerobic TA 100, 7 8 for anaerobic TA 100, and 3 6 for anaerobic TA 100 FR,
[d] Confirmed in minimum inhibitory concentration test in liquid medium

Effect of tinidazole on purified *Escherichia coli* DNA:

The ability of tinidazole to cause DNA damage was examined by ^{14}C-deoxythymidine (^{14}C-dT) release assay[22]. For this, a mixture of ^{14}C-labeled *E. coli* DNA (10 μg), unlabeled *E. coli* DNA (10 mg) and tinidazole or other 5-nitroimidazoles (20 μmoles) in trisodium citrate buffer (pH

7.1) were purged with nitrogen gas. The reduction of the drug was carried out using a mercury (Hg) pool cathode and silver chloride (AgCl) anode at 30 microampere current and the reduction monitored by measuring the loss of absorbance of drug spectrophotometrically. The amount of ^{14}C-dT released after dialysis of the mixture against water for 18 hours was measured to determine the damage to DNA. The results in Table 3 show that 12.7% ^{14}C-dT was released from DNA exposed to tinidazole while 9.5% ^{14}C-dT was released from DNA exposed to metronidazole, suggesting that the extent of damage to DNA caused by tinidazole was similar to metronidazole.

Table 3: Redox values of and DNA damage produced by reduced nitroimidazoles.

No	Drug	$E_{\frac{1}{2}}$	E_7^1	Damage	Log 1/C*	Reduction potential (mV)
1	Benznidazole	−200	−380	2·7	−0·569	−700
2	Misonidazole	−272	−389	5·0	−0·301	−800
3	Nimorazole	−345	−457	4·05	−0·393	−850
4	Ornidazole	−345	−467	7·4	−0·131	−850
5	Tinidazole	−340	−464	12·7	0·104	−850
6	Azomycin	−374	−418	6·0	−0·222	−900
7	Metronidazole	−382	−486	9·5	−0·022	−900
8	Dimetridazole	−388	−475	10·4	0·017	−900
9	8609 RP	−475	−550*	10·3	0·013	−1000
10	4(5) Nitroimidazole	−540	−527	7·5	−0·125	−1000

$E_{\frac{1}{2}}$ is the polarographic half-wave potential in mV measured against an Ag/AgCl reference electrode at pH 7·0.
E_7^1 is the one-electron redox potential in mV measured against the normal hydrogen electrode
Damage measured as the percentage release of [^{14}C]-dT from DNA.
† C is the calculated drug nucleotide ratio to produce a 10% release of dT from DNA
* The value is computer-calculated on the basis of structural similarities to other drugs (Wardman, personal communication)

The damage to DNA (cytotoxicity) and amount of nitrite released from reduced nitroimidazoles in the presence and absence of DNA was examined in another study[23]. The cytotoxicity was determined by measuring the amount of thymidine released when DNA was exposed to electrolytically reduced drugs including tinidazole. The results in Figure 1 show that the cytotoxicity (expressed as the reduced drug-nucleotide ratio which produces a 10% release of thymidine from DNA) correlates linearly with the one-electron redox potential of the drug. The cytotoxicity of tinidazole (#5) was similar to metronidazole (#7) in this assay. The release of nitrite indicates a direct interaction between a one-electron radical anion ($R\text{-}NO_2^{\cdot -}$) and DNA. Nitrites were released from reduced tinidazole ($\%NO_2^-$ = 4.25%) and reduced metronidazole ($\%NO_2^-$ = 16.1%) in the presence of DNA (Table 4), suggesting involvement of a one-electron radical anion. However, the amount of nitrite released from DNA in the presence of reduced tinidazole was lower than with reduced metronidazole. The correlation of nitrite released from DNA, in the presence of reduced tinidazole or metronidazole, with activity is unclear.

Figure 1: Correlation of electron affinity of nitroimidazole and cytotoxicity.

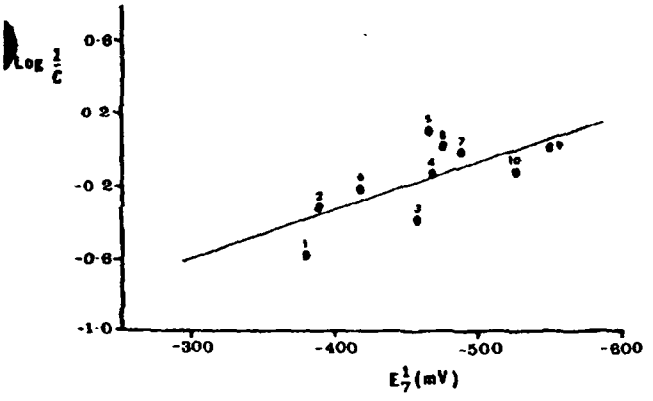


Table 4: Production of nitrite from reduced nitroimidazoles in the absence and presence of *E. coli* DNA at a drug nucleotide ratio of 1:1.

No.	Drug	E_7^1	% NO_2^-	% NO_2^- + DNA
1	Benznidazole	−380	0.05	0
2	Misonidazole	−389	0.5	3.2
3	Nimorazole	−457	0	0
* 4	Ornidazole	−467	5.9	13 3
* 5	Tinidazole	−464	4.25	4.6
6	Azomycin	−418	0.75	1 0
* 7	Metronidazole	−486	16.1	20.7
* 8	Dimetridazole	−475	2.1	19.4
9	8609 RP	−550	0.5	1.3
10	4(5)-nitroimidazole	−527	0	0
*11	2,5-dinitroimidazole		1.0	15.4

*a 5-nitroimidazole.

Ability of tinidazole to cause DNA base changes and strand breakage within bacterial or mammalian cells:

The ability of tinidazole to cause base changes in DNA was evaluated by the Ames test using the *Salmonella typhimurium* strains TA100 and TA100 FR_1[21] These strains require histidine for growth. The bacterial cells were incubated in the presence of tinidazole or other nitroimidazoles, aerobically for 46 hours or anaerobically for 16 hours. The number of cells reverting to histidine prototrophy (lack of histidine requirement) was determined. The results in Table 2 (page 11) show that tinidazole produced more revertants (more base changes) than metronidazole using the *S. typhimurium* TA100 and TA100 FR_1 strains.

In another study[24], the ability of tinidazole to cause base changes in DNA was examined by the same test using the *S. typhimurium* strains TA100 and TA98 (histidine requiring strains). *S. typhimurium* strain TA100 is sensitive to mutagens causing base-pair substitutions and TA98 is sensitive to mutagens causing frame-shift mutations. The bacterial strains were incubated in the presence of tinidazole or other nitroimidazole for 2 to 3 days at 37°C and the number of revertants (cells that do not require histidine for growth) determined. The typical background number of spontaneous revertants for strains TA100 and TA98 were stated to be 150-200 and 20-40 cells per agar plate, respectively. The results in Table 5 show that tinidazole caused DNA base changes in strain TA100 but not in strain TA98. As observed in the previous study, the number of revertants per nmol of tinidazole with strain TA100 was greater than that observed per nmol of metronidazole.

Table 5: Partition co-efficient (PC), one-electron potential and mutagenicity assay results of nitroimidazoles.

Drug	Octonal/water partition	One-electron attachment potential#	Revertants/nmole of drug*/plate	
			TA100	TA 98
Metronidazole	0.93	-0.482	0.82 (0.68-1.1) (4X)	0.016
Tinidazole	0.44	-0.464	2.2 (1.7-3 8) (4X)	0 015
Ro 07-0207	4.0	-0.467	2.0	0.066
Ro 11-3696	1.1	-0.479	1.0	-

* Some drugs are shown as the average value with the range of values observed in brackets and the number of experiments to give an indication of experimental variation.

One-electron attachment potential is a measure of electron affinity and the values shown were from published data.

The 5-nitromidazoles reduced by bacterial nitroreductases may result in the formation of hydroxylamine that can be activated by bacterial or liver cytosolic O-acetyltransferases. The ability of tinidazole to cause base changes in the DNA of 4 *S. typhimurium* strains [TA100 (nitroreductase enzyme containing strain) and TA100NR (nitroreductase enzyme deficient strain), TA100/1, 8-DNP$_6$ (nitroreductase and O-acetyltransferase enzymes containing strain), and YG1029 (strain with high O-acetyltransferase enzyme)] was examined in the presence and absence of mammalian liver S9 mixture[25]. The Ames test under aerobic or anaerobic conditions was used. The ability of the drug to cause DNA base changes was calculated from the average number of induced revertants per plate for each drug concentration. A positive response was defined as a 2 fold-increase in the number of revertants with the drug compared to drug-free control. The results in Table 6 show that tinidazole caused DNA base changes in the *S. typimurium* strain TA100 under aerobic and anaerobic conditions both in the presence and absence of mammalian liver S-9 mixture. The number of TA100NR strain revertants induced by tinidazole in the presence and absence of mammalian liver S9 mixture was 20-fold lower than with the TA100 strain under aerobic conditions, suggesting the involvement of bacterial nitroreductases in the action of tinidazole (Table 6). However, the number of revertants induced by tinidazole using the TA100NR strain was similar to that of the TA100 strain under anaerobic conditions, suggesting that other bacterial enzymes may be responsible for action of tinidazole under anaerobic conditions. Revertants were also observed with strains TA100/1, 8-DNP$_6$ and YG1029 (strains containing bacterial O-acetyltransferase and nitroreductase enzymes) under aerobic conditions in the absence of liver enzymes. The number of revertants in these two strains did not change in the presence of pentachlorophenol (PCP, an inhibitor of O-acetyltransferase), suggesting that bacterial O-acetyltransferase did not play a role in activation of hydroxylamine possibly generated within bacterial cells by the reduction of tinidazole. As observed in previous studies, a greater number of revertants were induced by tinidazole compared to metronidazole.

Table 6: Observed revertant count of tinidazole (TNZ) and metronidazole (MNZ) in TA100,TA100NR, TA100/1,8-DNP$_6$ and YG1029 strains of *S. typhimurium*

Amount in nmol/plate	His$^+$ Revertants per plate[a]									
	TA100				TA100NR				TA100/1,8-DNP$_6$	YG1029
	Aerobic		Anaerobic		Aerobic		Anaerobic		Aerobic	Aerobic
	−S9	+S9[b]	−S9	+S9[b]	−S9	+S9[b]	−S9	+S9[b]	−S9	−S9
00 (Solvent control)	104±9	125±10	133±9	108±8	135±11	122±11	116±10	127±11	138±11	144±13
For TNZ										
50	150±11	156±11	175±11	184±11	140±11	176±11	146±13	196±17	232±18	235±17
200	215±19	320±28	378±29	386±22	139±9	185±12	198±19	268±23	250±18	258±18
800	1032±92	1080±98	2188±190	2328±209	176±13	260±24	1220±110	2448±217	1104±106	1156±109
3200	4264±274	4508±376	7258±584	7376±617	297±21	386±28	4626±423	9632±768	3120±310	4688±376
12800	Inhibition	NT	Inhibition	NT	NT	NT	NT	NT	NT	NT
Rev /nmol	1 32	1.38	2 25	2.29	0 05	0 07	1 43	3 01	0 93	1 44
Rev /nmol with PCP[c]	1 25	–	–	–	–	–	–	–	0.90	1.36
For MNZ										
00 (Solvent control)	115±8	125±10	113±9	110±9	135±11	122±11	116±10	127±11	138±11	144±13
50	156±11	191±12	209±17	214±16	131±12	172±15	165±12	204±19	168±12	215±12
200	190±17	216±19	394±30	669±58	137±13	207±16	279±25	354±33	248±16	251±21
800	714±58	667±60	1708±187	2584±252	155±12	231±21	1425±135	1912±175	561±47	536±44
3200	2392±225	2720±210	7140±526	7224±720	253±21	476±46	4258±417	5984±492	2172±253	2872±280
12800	Inhibition	NT	Inhibition	NT	NT	NT	NT	NT	NT	NT
Rev /nmol	0 72	0.82	2 21	2 21	NC	0 10	1 30	1 84	0 63	0 86
Rev /nmol with PCP[c]	0 69	–	–	–	–	–	–	–	0 59	0 81
NQNO (0 5 μg/plate)	2696±126	–	–	–	2762±132	–	–	–	2796±152	2816±97
2-Aa (5 μg/plate)	–	2860±175	–	–	–	2840±246	–	–	–	–

[a] Mean values and SE for triplicates assayed on 2 occasions ($n = 6$) in aerobic conditions where as $n = 4$ in anaerobic conditions for duplicate plates assayed twice.
[b] 20 μl of S9 was used in 0 5 ml S9 mix per plate
[c] Added in 50 μl DMSO containing 0.12–1 5 μg/plate
NQNO, 4-nitroquinoline-*N*-oxide 2-Aa, 2-anthramine, NT not tested· NC not calculated being inactive

The ability of tinidazole and other nitroimidazoles to cause DNA base changes in *Klebsiella pneumonia*, *E. coli* K12, and *Citrobacter freundii* was examined using the Luria and Delbruck fluctuation test[26]. Briefly, nutrient broth containing 100 bacterial cells/ml and drug was divided into 105 portions of equal volume and incubated at 37°C for 20 hours. The mutation frequency was then determined by plating cells in the presence or absence of streptomycin and incubating at 37°C for 3 days. The following formula was used to calculate mutation frequency:

$$M = \frac{^{10}\log A/B}{0.4343} \cdot \frac{^{e}\log 2}{N \times V}$$

in which M = mutation rate; A = number of portions without mutants; B = total number of portions poured with streptomycin agar; N = number of bacteria per ml, V = volume of a portion.

100 portion plated with streptomycin and 5 portion plated in the absence of streptomycin

Tinidazole and the other nitroimidazole at concentration of ≥ 0.2 mM (≥ 49 μg/ml) increased the mutation frequency in *K. pneumonia*, *C. fruendii* and *E. coli* K12 strain (Table 7). These results point to DNA being the target for the action of tinidazole in bacteria.

Table 7:

INCREASE OF THE MUTATION FREQUENCY OF DIFFERENT TEST ORGANISMS TREATED WITH NITROIMIDAZOLES

Test organism	Tinidazole concentration mM/l					Ipronidazole concentration mM/l						Panidazole concentration mM/l					Ornidazole conc		
	1	0.5	0.2	0.1	0.05	1	0.5	0.2	0.1	0.05	0.02	1	0.5	0.2	0.1	0.05	0.5	0.2	0.
Klebsiella pneumoniae	6.9[b]	4.3[a]	3.9[a]	3.4[b]	2.2[a]	39.1[b]	14.7[b]	5.7[b]	3.0[b]	2.0[b]	1.6[b]	5.1[b]	5.6[a]	6.1[a]	3.8[b]	2.2[a]	16.8	5.5[b]	3..
Escherichia coli	8.3[a]	5.2[a]	2.7[b]	1.7[b]		22.4	13.5	4.6	4.1	3.8	1.8	3.7	4.3	3.1	1.9				
Citrobacter freundii	4.4[a]	3.3[a]	2.6[a]	1.6[a]		6.1	4.2	1.9	1.3			3.5	2.9	3.6	2.4	1.6			

[a] Average of 2 experiments.
[b] Average of 3 experiments.

The ability of tinidazole to induce DNA single strand breaks was examined in peripheral blood lymphocytes[27]. For this, whole blood (80 µl) was incubated with different concentrations of tinidazole, metronidazole or ornidazole at 37°C for 30 minutes. The blood samples were then transferred to the dark and incubated under 1 atmosphere pressure (the time period was not specified). Lympho-prep medium was added to the blood samples and lymphocytes separated by centrifugation at 900 x g. The lymphocytes were then subjected to alkaline single cell electrophoresis using the method of Singh[28]. A fluorescent microscope was used to analyze 200 cells and the damage classified as 0 to 4 based on the amount of DNA in the 'comet' tail. Bleomycin was used as a positive control. The results in Table 8 show that tinidazole, metronidazole and ornidazole caused DNA damage in lymphocytes to the same extent as the bleomycin.

Table 8:

Distribution of damage levels in PBL exposed to different treatments

Drug	Concentration (µg ml)	Level of DNA damage[a]					Damaged cells[a]	WDI[b]
		0	1	2	3	4		
Control	*0*	*26*	*46*	*15*	*7*	*6*	*74 0*	*121*
Bleomycine	100	1 1	12.8	27 4	25.9	32 7	98 9	276
Metronidazole								
	10	4.5	19.0	27.0	28 0	21 5	95.5*	243
	100	4 5	21 0	27.5	24.5	22.5	95 5*	240
	500	0	20 1	30.8	17.1	32 0	100 0*	261
Ornidazole								
	10	5 5	27.4	27 3	24.5	15 3	94 5*	217
	100	1.1	7.0	25 8	37 9	27.7	98 9*	283
	500	0	16 7	6.3	28 2	48 9	100 0*	309
Tinidazole								
	10	0	7 3	32 7	28.0	32 0	100.0*	285
	100	0	3 3	23 0	27 4	46 2	100 0*	317
	500	0	1 3	12.0	42 0	44 7	100 0*	330

Overall, the nitro group of tinidazole is reduced by extracts of trichomonads. Chemically reduced tinidazole releases nitrite and thymidine from *E. coli* DNA *in vitro*. Tinidazole caused DNA base changes in bacterial cells and DNA strand breakage in lymphocytes, suggesting that tinidazole acts mainly on DNA. Although nitroreductases have been implicated in the reduction of tinidazole in bacteria, the enzyme responsible for the reduction of tinidazole in protozoa has not been identified. The mechanism by which tinidazole exhibits activity against *Giardia* and

Entamoeba was not examined. Thus, the mechanism by which tinidazole exhibits anti-protozoal activity is not completely understood.

2.2. Activity *in vitro*:

2.2.1. Activity against protozoa:

The *in vitro* activity of tinidazole was measured against 3 protozoa, namely, *T. vaginalis*, *G. lamblia,* and *E. histolytica*.

2.2.1.1. *T. vaginalis*:

The *in vitro* activity of tinidazole against *T. vaginalis* was examined in 14 studies conducted in different laboratories. Different media [Diamond's medium, Cysteine-peptone-liver infusion-maltose (CPLM) medium, or Thioglycollate medium] were used to test the activity under aerobic or anaerobic conditions. The exponential phase of growth for *T. vaginalis* (300 trophozoites/ml) in Diamond's medium lasts for 120 hours with a generation time of 6 hours[29]. The endpoint for determination of minimum inhibitory concentration (MIC) in these studies was absence of motility unless specified otherwise. Viability of the protozoa was not examined. In some studies the minimum lethal or cidal concentration (MLC, also referred to as MCC), defined as the concentration at which no motile organisms were observed upon subculture, was determined unless specified otherwise. Please note that there are no standardized methods for *in vitro* susceptibility testing of anti-trichomonad drugs.

Laboratory strains:

The activity of tinidazole against 5 *T. vaginalis* strains (including a laboratory generated strain A with metronidazole MIC of 100 μg/ml under aerobic conditions) was examined[30]. Other anti-protozoal agents were used as comparators. The cultures (inoculum size not specified) in Diamond's medium were incubated in the presence or absence of drug aerobically or anaerobically at 37°C for 72 hours. The cultures were examined microscopically for motility and the MIC determined. An aliquot was subcultured to fresh medium and the MLC values determined after 5 days. The MIC and MLC values for tinidazole and other antiprotozoal agents are shown in Table 9. Under aerobic conditions, the tinidazole MIC values ranged from 0.4 to 25 μg/ml and MLC values ranged from 0.8 to 50 μg/ml. Under anaerobic conditions, the tinidazole MIC and MLC values were lower (≤ 6.25 μg/ml). The tinidazole MIC and MLC values against a strain with high metronidazole MIC (strain A) were higher (60 fold higher under aerobic conditions and 7 fold higher under anaerobic conditions) than against the strains with low metronidazole MIC, suggesting cross-resistance between metronidazole and tinidazole. The activity of tinidazole against the strains with low metronidazole MICs was similar to furazolidone, mebendazole and anisomycin.

Table 9: *In vitro* susceptibilities of 5 strains of *T. vaginalis* to 5 antimicrobial agents.

Strain and growth conditions	Metronidazole		Tinidazole		Furazolidone		Mebendazole		Anisomycin	
	MIC[a]	MLC[b]	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
A[c]										
Anaerobic[d]	6.2	12.5	6.2	6.2	3.1	6.2	6.2	0.2	0.2	1.6
Aerobic	100	50	25	50	3.1	3.1	3.1	0.8	<0.1	0.8
B										
Anaerobic	0.4	1.6	0.4	1.6	3.1	6.2	1.6	0.4	0.2	1.6
Aerobic	3.1	1.6	0.4	0.8	3.1	3.1	0.8	0.2	<0.1	<0.2
C										
Anaerobic	0.2	3.1	0.8	3.1	1.6	3.1	1.6	0.2	0.2	1.6
Aerobic	0.8	1.6	0.4	0.8	3.1	3.1	1.6	0.2	<0.1	0.2
D										
Anaerobic	0.4	0.8	0.8	1.6	1.6	6.2	3.1	0.2	0.2	1.6
Aerobic	0.8	0.8	0.4	0.8	3.1	6.2	3.1	<0.1	0.2	0.4
E										
Anaerobic	0.4	3.1	0.4	3.1	3.1	6.2	6.2	0.2	0.2	0.8
Aerobic	0.8	1.6	0.4	0.8	1.6	6.2	6.2	0.4	0.4	0.8

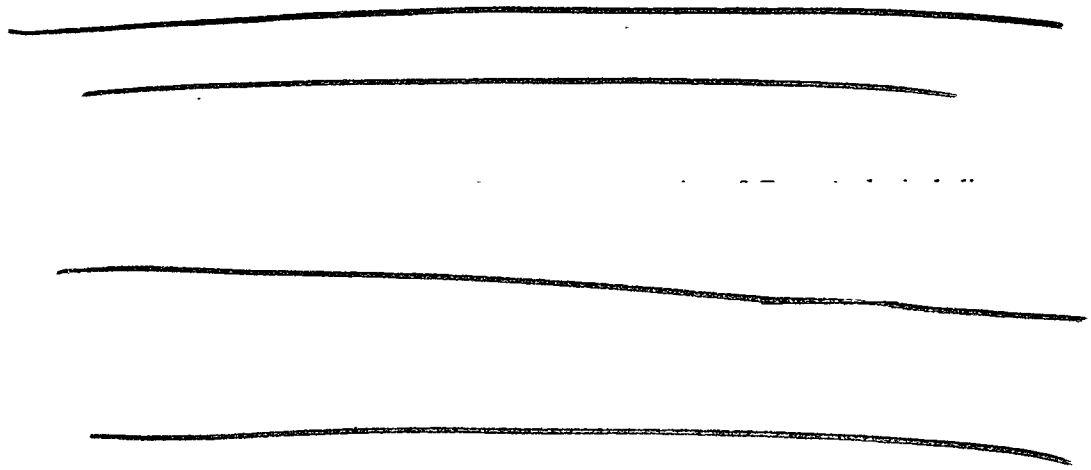
[a] The MIC was defined as the concentration of drug (in micrograms per milliliter) at which no trichomonal motility was observed.
[b] The MLC was defined as the concentration of drug (in micrograms per milliliter) at which no viable organisms were detected by subculture.
[c] Strain A was a laboratory isolate known to be
[d] An anaerobic environment was maintained by adding ascorbic acid to the medium in tightly capped screw-top jars

In another study[31], the activity of tinidazole against a strain of *T. vaginalis* was examined using the same medium as described above. Trophozoites (4 to 10 x 10^4) in Diamond's medium were incubated in the presence of tinidazole or metronidazole at 37°C for different time points. The authors did not specify if the cultures were incubated under aerobic or anaerobic conditions. The number of motile trophozoites was counted and the ratio of motile organisms in drug-treated cultures to drug-free controls was calculated. The MIC was defined as the lowest concentration of the drug that produced a ratio of ≤ 0.5. For determining the MLC, a 10 fold higher inoculum was used. The MLC was defined as the lowest concentration of the drug which gave a count of $<1.25 \times 10^3$ trophozoites/ml. The results in Table 10 show that both the tinidazole MIC and MLC values against *T. vaginalis* at 48 hours were 1.25 µg/ml. The metronidazole MIC and MLC values against *T. vaginalis* were 2 to 8 fold higher than the tinidazole MIC and MLC values.

Table 10: Minimum inhibitory concentration (MIC) and minimum cidal concentration (MCC = MLC) against *T. vaginalis* in Diamond's medium.

Incubation time in hours	Tinidazole			Metronidazole			Metronidazole ratio/tinidazole ratio
	MCC	MIC	Ratio MCC/MIC	MCC	MIC	Ratio MCC/MIC	
6	40.0	10.0	4	>80.0	20.0	>8 (>4)*	>2
12	5.0	2.5	2	80.0	5.0	16	8
24	2.5	1.25	2	20.0	2.5	8	4
48	1.25	1.25	2 (1)*	10.0	2.5	4	2

*discrepancies in the tinidazole MCC/MIC ratio at 48 hours and metronidazole MCC/MIC ratio at 6 hours. The correct ratios based on the MCC and MIC values are shown within parenthesis.



In another study[33], the *in vitro* activity of tinidazole and other nitroimidazoles was tested against *T. vaginalis* using a different medium. Trophozoites (2.5 x 10^3/ml) in thioglycollate medium were incubated anaerobically in the presence of drug at 37°C for 24 hours and the MICs determined. The endpoint used to determine MIC was not specified. The results in Table 12 show that the tinidazole MIC against 5 strains of *T. vaginalis* ranged from 1.25 to 10 µg/ml. The activity of tinidazole was similar to metronidazole but 2-fold lower than the experimental drug, HOE239, against most strains.

Table 12: *In vitro* activity of tinidazole, metronidazole, and HOE239 against *T. vaginalis*.

***T. vaginalis* strain**	**MIC (µg/ml) in thioglycollate medium**		
	Tinidazole	**Metronidazole**	**HOE 239**
Carneri	1.25	5.0	0.625
Rd	2.5	2.5	0.31
VW	2.5	5.0	1.25
BC 7	10.0	10.0	5.0
TR 14	5.0	10.0	5.0

Clinical isolates:

The *in vitro* activity of tinidazole against 8 clinical isolates of *T. vaginalis* was examined[34]. Metronidazole was used as a comparator. Cultures (4 x 10^4 trophozoites/ml) in thiogylcollate medium were incubated in the presence of two fold dilutions of the drug (dissolved in saline) at 37°C for 3 days and the MIC determined. It is unclear if the cultures were incubated under aerobic or anaerobic conditions. The MLC was also determined 5 days after subculture into Diamond's medium. The results in Tables 13 and 14 show that the tinidazole MIC and MLC values against the 8 isolates were in the range of 0.12 - 1.25 µg/ml and 1.25 – 2.5 µg/ml, respectively. The metronidazole MIC values were similar (0.31 to 1.25 µg/ml) to tinidazole while the metronidazole MLC values were 1.3 to 8 fold higher (1.88 -10 µg/ml).

Table 13: Minimum inhibitory concentration (MIC) of metronidazole and tinidazole for fresh isolates of *T. vaginalis* at the end of 3 days

Strain of T. vaginalis	MIC (μg./ml.) Metronidazole	Tinidazole	Percentage activity of metronidazole relative to tinidazole
1	1·25	1·25	100
2	0·31	0·12	37·5
3	1·25	0·31	25
4	0·47	0·16	33
5	1·25	0·47	37·5
6	1·25	0·94	75
7	0·94	0·63	67
8	0·47	0·31	67
Mean value	0·90	0·52	57·8

Table 14: Minimum trichomonicidal concentration (MCC) of tinidazole and metronidazole for fresh isolates of *T. vaginalis* upon subculture

Strain of T. vaginalis	MCC (μg./ml.) Metronidazole	Tinidazole	Percentage activity of metronidazole relative to tinidazole
1	10·0	1·25	12·5
2	2·5	1·25	50
3	2·5	1·88	75
4	2·5	1·25	50
5	5·0	1·88	37·5
6	3·75	2·5	67
7	1·88	1·25	67
8	2·5	1·25	50
Mean value	3·83	1·56	40·7

MCC= MLC = minimum lethal concentration.

In another study[35], the activity of tinidazole against 91 *T. vaginalis* clinical isolates was examined using the same medium and inoculum size as described in the previous study. However, the cultures were incubated under aerobic conditions for 48 hours and the MIC determined. Metronidazole and ornidazole were used as comparators. The MIC values of tinidazole against these isolates ranged from 0.05 to 12.5 μg/ml and were similar to that observed in the previous study (Tables 13 and 15). It was stated that the tinidazole MICs were 1.56 μg/ml against the 2 isolates with high metronidazole MIC values (50 μg/ml).

Table 15: Minimum inhibitory concentrations of metronidazole, ornidazole and tinidazole against *T. vaginalis*.

5-Nitroimidazole	MIC Range
Metronidazole	0.2–50 (5.5 ± 8)
Ornidazole	0.05–12.5 (1.2 ± 1.9)
Tinidazole	0.05–12.5 (0.6 ± 1.3)

Data are μg/ml (mean ± SD).
*N = 91.

In another study[36], the *in vitro* activity of tinidazole against 63 isolates of *T. vaginalis* collected over a 20 month period was examined using a different medium. It is unclear whether the number of isolates represents the number of patients. The activity of the drug was measured using 2 inoculum sizes (10,000 trophozoites/ml or 100,000 trophozoites/ml). The cultures were incubated in the presence of tinidazole, metronidazole or nifuratel in Diamond's medium for 48 hours. The condition (aerobic or anaerobic) under which the cultures were incubated was not specified. The number of motile trophozoites was counted and MICs determined. The cidal concentration was determined by subculture in the same medium without drug for 1 to 3 days. The tinidazole MIC and MLC values were similar to metronidazole or nifuratel and ranged from 0.12 to 0.25 μg/ml, and 0.50 to 1.50 μg/ml, respectively (Table 16). The geometric mean MIC and MLC values for tinidazole using the lower concentration of inoculum were 6-fold lower than with a higher concentration of inoculum. The tinidazole MLC values were 3 to 7 fold higher than the MIC values against these isolates.

Table: 16: Sensitivity of *T. vaginalis in vitro* to three drugs (read after 48 hours incubation).

Inoculum (*T. vaginalis*/ml.)	Drug	No. of strains	Static concentration (μg./ml.) Range	Static concentration (μg./ml.) Geometric mean	Cidal concentration (μg./ml.) Range	Cidal concentration (μg./ml.) Geometric mean
Small about 10,000	Metronidazole	19	0·08–1·00	0·14	0·25–1·50	0·59
	Tinidazole	18	0·12–0·25	0·13	0·50–1·50	0·71
	Nifuratel	19	0·06–0·25	0·09	0·12–1·50	0·52
Large about 100,000	Metronidazole	44	0·50–1·50	0·88	0·50–7·50	2·79
	Tinidazole	44	0·50–2·25	1·08	1·50–7·50	3·96
	Nifuratel	32	0·50–1·50	0·99	1·50–5·00	3·05

In another study[37], the activity of tinidazole against 55 clinical isolates was examined using a different medium. The isolates were obtained at baseline from patients successfully treated with a single dose (1.8 gm) of tinidazole. Susceptibility testing was performed by incubating cultures (0.5 to 2 x 10^4 trophozoites/ml) in CPLM medium in the presence or absence of drug at 37°C. The MIC was determined at the end of 24 hours of incubation. The results in Table 17 show that the tinidazole MIC values against the 55 clinical isolates ranged from 2 to 6 μg/ml. No comparator was used in this study.

Table 17: *In vitro* activity of tinidazole against isolates from 55 patients at the end of 24 hour incubation.

Patient number	Tinidazole MIC (μg/ml)	Patient number	Tinidazole MIC (μg/ml)	Patient number	Tinidazole MIC (μg/ml)
1	2	20	4	39	4
2	3	21	4	40	6
3	2	22	2	41	3
4	3	23	3	42	6
5	2	24	3	43	6
6	2	25	4	44	2
7	3	26	5	45	2
8	5	27	4	46	5
9	4	28	3	47	2
10	3	29	4	48	5
11	4	30	5	49	4
12	6	31	2	50	5
13	3	32	4	51	6
14	4	33	5	52	3
15	5	34	5	53	5
16	4	35	5	54	3
17	5	36	3	55	3
18	3	37	2		
19	5	38	4		

Mean MIC = 3.76 ± 1.25 μg/ml; Range MIC = 2 - 6 μg/ml.

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A-5

2.2.1.2. *G. lamblia*:

The *in vitro* activity of tinidazole against the trophozoite stage of *G. lamblia* strains and clinical isolates was measured in 10 studies using different methods such as viability, adherence, colorimetric, ^{3}H-thymidine uptake or motility assays. The inoculum sizes, media, incubation period and the endpoint for activity determination varied with the assay performed in different laboratories. However, all studies measured either the minimum inhibitory concentration (MIC, defined as concentration that altered trophozoite morphology, viability or adherence) or the concentration required for 50% inhibition (IC_{50}) based on the assay used. The doubling time for the trophozoite stage of different strains of *G. lamblia* varies from 12 to 44 hours. Please note that there are no standardized methods available for susceptibility testing of anti-giardial drugs.

Laboratory strains:

The *in vitro* activity of tinidazole, metronidazole and albendazole was examined against the 2 strains (P1 and P1C10) of *G. lamblia*[46]. Trophozoites (30,000 to 50,000 cells/ml) in B1-S-33 medium containing bovine serum without antibiotics or vitamin mixture NCTC 135 were incubated in the presence of drug for 4 or 24 hours. At the end of the incubation period, the cultures were centrifuged. The cell pellet was resuspended in drug-free growth medium and incubation continued for up to 7 days. The experiments were performed in duplicate. The MIC defined as the lowest concentration of drug that altered trophozoite morphology, multiplication or adherence was determined. Additionally, the minimum lethal concentration (MLC) defined as the lowest concentration of the drug in which no morphologically normal or dividing trophozoites were observed by day 7 was determined.

The tinidazole MIC and MLC values against the P1 strain exposed to the drug, for 24 hours, were 0.5 and >10 μg/ml, respectively (Table 21). The activity of tinidazole was similar to metronidazole but 20 fold lower than albendazole.

Table 21: Effects of albendazole, tinidazole and metronidazole *in vitro* on *G. lamblia* P1 strain after 24 hours exposure.

	24 h exposure		Relative activity of albendazole, unidazole and metronidazole[a]	
	MIC[b] (μg/ml)	MLC[b] (ug/ml)	MIC[b] (%)	MLC[b] (%)
Albendazole	0·1	0·5	100	100
Tinidazole	0·5	>10·0	20	<5
Metronidazole	1·0	>10·0	10	<5

[a]Activity of albendazole is taken as 100% for comparative purposes.
[b]MIC=minimum inhibitory concentration; MLC=minimum lethal concentration.

The activity of tinidazole against the P1C10 strain was similar to that of the P1 strain exposed to the drug for 24 hours (Table 22). The MIC and MLC values against the trophozoites of P1C10 strain exposed to tinidazole for 4 hours were 4 times higher than that of trophozoites exposed to the drug for 24 hours. The activity of tinidazole against the P1C10 strain was similar to metronidazole but lower (12.5 fold) than albendazole.

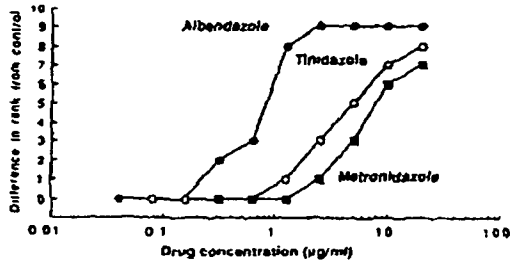
Table 22: Effects of albendazole, tinidazole and metronidazole *in vitro* on *G. lamblia* P1C10 strain after 4 and 24 hours exposure.

	4 h exposure		24 h exposure	
	MIC[a]	MLC[a]	MIC[a]	MLC[a]
Albendazole	0·32 (100)	1·28 (100)	0·04 (100)	0·64 (100)
Tinidazole	1·28 (25)	>20·48 (<6·25)	0·32 (12·5)	20·48 (3·12)
Metronidazole	2·56 (12·5)	>20·48 (<6·25)	0·64 (6·25)	>20·48 (<3·12)

[a]MIC=minimum inhibitory concentration; MLC=minimum lethal concentration. Values shown are activities in μg/ml after 4 and 24 h exposure; numbers in parentheses are percentages of the activity of albendazole, which is taken as 100% for comparative purposes.

For adherence measurement, the cultures were scanned microscopically at 200x magnification and the average number of trophozoites adhering to the glass tube in 5-10 fields was counted and given a rank (see chart next to Figure 5). Using the rank, difference in adherence between cultures in the presence and absence of drug was plotted against the drug concentration (Figure 5). The activity of tinidazole was lower than albendazole but higher than metronidazole. Overall, the results of the viability assay (Tables 21 and 22) and adherence assay (Figure 5) were comparable.

Figure 5: The drug related effect of albendazole, tinidazole and metronidazole on ranked trophozoite adherence of *G. lamblia* plotted as difference from control at 24 hours after 4 hours drug exposure.



Rank	Number adhering
0	None
1	<1
2	1– 10
3	10– 25
4	25– 50
5	50– 100
6	100– 250
7	250– 500
8	500–1000
9	1000–2000
10	Monolayer

In another study[47], the *in vitro* activity of tinidazole was measured against the same *G. lamblia* strain (strain P1) using a different method (colorimetric). For this, 3 x 10^4 trophozoites were incubated with different dilutions of drugs in TYI-S-33 medium containing 30 mM glucose supplemented with 10% heat inactivated calf serum and 5 mM arginine under anaerobic conditions at 37°C for 3 days. At the end of the incubation period, cells were lysed using 20% Triton X-100. The lysate was allowed to stand at 4°C for 1 hour, and the amount of nucleoside hydrolase (an essential enzyme for biosynthesis of nucleic acids) released by lysis was measured by monitoring the change in absorbance at 405 nm after addition of the substrate 4-nitrophenyl-β-D-ribofuranoside (NPR). The raw data were not shown but the tinidazole IC_{50} value was stated to be 0.4 μM (0.10 μg/ml). The results in Figure 6 show that the metronidazole and furazolidine IC_{50} values were 1.0 μM (0.17 μg/ml) and 0.5 μM (0.11 μg/ml), respectively.

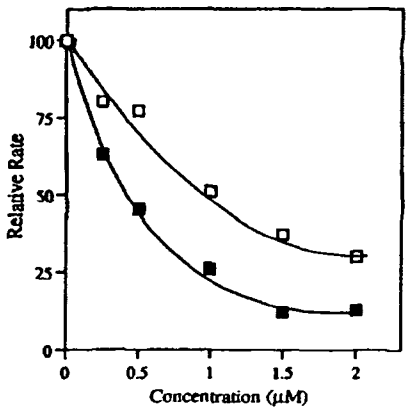


Figure 6: Cells were grown in the presence of varying concentrations of metronidazole (❑) and furazolidone (■) for 3 days. The cells were then lysed and the nucleoside hydrolase activity determined. The IC_{50} represents the concentration of compound at which the activity in the presence of the drug is 50% of the control rate (without compound).

In another study[48], the activity of tinidazole against the same strain was measured by ^{3}H-thymidine uptake. For this, 5 x 10^4 trophozoites in modified TY1-S-33 medium were incubated in the presence of drug at 37°C for 20 hours. The incorporation of ^{3}H-thymidine over an additional 4 hour incubation period was measured. The IC_{50} value based on 50% inhibition of ^{3}H-thymidine uptake was calculated. The tinidazole IC_{50} value (0.53 μM i.e., 0.13 μg/ml) against the P1 strain (Portland 1) using the ^{3}H-thymidine uptake assay was similar to that using the colorimetric assay (Table 23 and previous study). Also, the activity of tinidazole against the P1 strain was similar to furazolidone and metronidazole.

Table 23: Nitrohetercycle sensitivities in isolates of *G.lamblia.*

Isolate	Nitroheterocycle ID_{50} (μm) Met	Tin	Fur
BRIS/85/HEPU/436	0.45	1.18	0.61
BRIS/84/HEPU/343	0.60	0.49	2.24
BRIS/83/HEPU/141*	0.61	0.38	1.10
BRIS/85/HEPU/452	0.66	0.53	1.20
BRIS/83/HEPU/210*	0.68	0.50	1.50
BRIS/83/HEPU/153*	0.71	0.55	1.50
BRIS/83/HEPU/120*	0.78	0.43	4.23
BRIS/85/HEPU/449	0.83	1.00	1.70
BRIS/83/HEPU/106*	0.86	0.57	0.87
BRIS/83/HEPU/161*	0.91	0.37	0.85
BRIS/84/HEPU/353*	0.92	0.70	1.96
BRIS/85/HEPU/497	1.04	0.54	1.37
BRIS/83/HEPU/99*	1.07	1.16	0.56
BRIS/86/HEPU/592	1.15	1.37	2.56
BRIS/82/HEPU/41*	1.77	0.45	0.60
PORTLAND 1*	1.79	0.53	0.47
BRIS/83/HEPU/136*	2.21	1.18	0.43
BRIS/86/HEPU/567	4.48	1.32	1.34

Abbreviations: Met, metronidazole; Tin, tinidazole; Fur, furazolidone.

*The drug sensitivities for these isolates have also been published elsewhere (Boreham *et al.*, 1984; Boreham & Shepherd, 1985). Values for Spearman's Ranked Correlation Coefficient: Met vs Fur, $r_s = -0.31$, not significant; Tin vs Fur, $r_s = -0.05$, not significant; Met vs Tin, $r_s = 0.42$, significant correlation $P < 0.05$.

$ID_{50} = IC_{50}$

In another study[49], the *in vitro* activity of tinidazole and other anti-parasitic drugs was examined against *G lamblia* P1 strain using a different medium. For this, 5 x 10^4 trophozoites in

Diamond's medium were incubated in the presence of 2-fold dilutions of drugs at 37°C for 48 hours in microcentrifuge tubes. At the end of the 48 hour incubation period, the trophozoites were detached by chilling and subcultured into fresh medium without drug for 48 hours. The number of trophozoites upon subculture was counted with a haemocytometer and the number of viable trophozoites deduced using the haemcytometer count and a linear regression plot of cell density versus inoculum. The MLC and the concentrations required for inhibition of growth by 50% (IC_{50}) and 90% (IC_{90}) were determined. The results in Table 24 show that the tinidazole MLC against the P1 strain of *G. lamblia* ranged between 0.5 and 1 µg/ml and was similar to that observed in previous studies. The tinidazole IC_{50} and IC_{90} values against the P1 strain of *G. lamblia* were 0.14 and 0.44 µg/ml, respectively. The tinidazole IC_{90} value was >3-fold lower than metronidazole but ≥ 3-fold higher than albendezole or mebendazole.

Table 24: Susceptibility of *G. lamblia* to 13 antimicrobial agents.

Antimicrobial agent	IC50 (mg/L)	SD	IC90 (mg/L)	SD	MLC (mg/L)
Albendazole	0·01	0·001	0·02	0·002	0·03 0·04
Mebendazole	0·06	0·020	0·13	0·030	0·20–0·50
Ornidazole	0·12	0·030	0·39	0·040	0·50-1·00
Tinidazole	0·14	0·027	0·44	0·090	0·50-1·00
Metronidazole	0·21	0·030	1·28	0·200	1·00-5·00
Mepacrine	0·39	0·050	1·72	0·350	1·50-5·00
Furazolidone	0·62	0·015	2·10	0·150	5·00-10·00
Secnidazole	0·62	0·140	2·10	0·300	5·00-10·00
Hemezole	1·55	0·440	3·80	0·500	10·00 15·00
Nifuroxazide	3·84	1·000	12·25	2·500	10·00 20·00
Etofamide	5·98	1·400	16·00	2·200	40·00 50·00
Nalidixic acid	12·13	1·860	30·20	5·500	45·00–50·00
Quinfamide	>200	.	.	.	.

IC50, 50% inhibitory concentration, IC90, 90% inhibitory concentration MLC, minimum lethal concentration; SD, standard deviation.

In another study[50], the activity of tinidazole and other anti-parasitic drugs against 6 strains of *G. lamblia* (including the P1 strain) was examined by the ^{3}H-thymidine uptake assay. Trophozoites (5 x 10^4/150 µl) in modified TYI-S-33 medium were incubated for 37°C in microtiter plates for 24 hours prior to addition of different concentration of drug. Radiolabeled thymidine was added after cultures were incubated in the presence of drug for 20 hours. The uptake of ^{3}H-thymidine was measured by incubating the cultures for additional 4 hours and the IC_{50} values calculated. The results in Table 25 show that the tinidazole IC_{50} values against the 6 strains ranged from 0.12 to 1.16 mM (0.03 – 0.29 µg/ml). Tinidazole was more active than metronidazole or quinacrine but similar to furazolidine against most strains.

The results in Table 25 show that the generation time (required to undergo an asexual multiplication) for the 6 strains varied from 12.5 ± 3.3 to 44.2 ± 12.2 hours. The authors have stated that a decrease in generation time was noted in strains from the time of first culture to maintenance of an established culture, suggesting that isolates were heterogeneous when first obtained. However, there appears to be no significant difference in the activity of the drugs using established cultures with different generation times.

Table 25: The 50% inhibitory concentrations (IC_{50}) of 4 anti-giardial drugs at 24 hours.

Strain	Generation time (hours)	IC_{50} (µmole/L)			
		Tinidazole	Metronidazole	Furazolidone	Quinacrine
Portland 1	12.8 ± 2.5	0.53	1.79	0.47	1.51
BRIS/82/HEPU/41	12.5 ± 3.3	0.45	2.14	0.60	1.09
BRIS/83/HEPU/99	44.2 ± 12.2	1.16	1.61	0.56	2.85
BRIS/83/HEPU/106	13.2 ± 6.6	0.53	0.91	0.74	1.18
BRIS/83/HEPU/120	20.0	0.64	1.08	2.50	1.59
BRIS/83/HEPU/136	25.7	0.12	2.21	0.43	4.02

In another experiment, the time-kill curves for the different drugs were determined using strain BRIS/83/HEPU/106. For this, 10^4 trophozoites were incubated with 5 x 10^{-6} M drug for 4, 8, 16, 24, or 48 hours. The viability of the trophozoites was determined by the ^{3}H-thymidine uptake assay, motility and trypan-blue dye-exclusion methods. The results in Table 26 show that the time required by the drug to have an inhibitory effect on 50% of the trophozoites was shorter using the ^{3}H-thymidine uptake assay compared to the motility and dye-exclusion assays, suggesting that it is a more sensitive method for determining activity. However, the clinical relevance of such measurements is not known.

Table 26: Time (hours) required by drugs *in vitro* to elicit 50% inhibitory effect on trophozoites of strain BRIS/83/HEPU/106 by 3 methods.

Drug	Thymidine incorporation	Motility	Dye exclusion
Metronidazole	8	12	48
Tinidazole	4	4	20
Furazolidone	9	41	>48
Quinacrine	13	>48	>48

All drugs were present at 5×10^{-6} molar

In another study[51], the activity of tinidazole and other antiprotozoal agents against 8 strains of *G. lamblia* (originally isolated from humans) and 1 strain of *G. lamblia* (originally isolated from cat) was examined using a semi-solid medium. For this, the trophozoites (50,000/ml) were incubated in TPS-1 medium containing different dilutions of the drugs. The medium was then semi-solidified with 0.16% agar and cultures incubated at 37°C for 2 - 7 days for determination of MIC. The MIC of the drug was defined as the lowest concentration of the drug at which no visible growth was detected. The tinidazole MIC against all 9 strains (8 human and 1 cat) was <0.5 µg/ml while that for metronidazole and furazolidone varied from <0.5 to 2.5 or 5.0 µg/ml (Table 27). Tinidazole was more active than the other antiprotozoal drugs i.e., paramomycin, chloroquine and pyrimethamine (Table 27).

Table 27: *In vitro* activity of six antiprotozoal agents against nine *G. lamblia* strains.

Strain	MIC (μg/ml)					
	Metronidazole	Tinidazole	Paromomycin	Chloroquine	Furazolidone	Pyrimethamine
HP34	<0.5	<0.5	50.0	10.0	<0.5	100.0
HP63	0.5	<0.5	100.0	100.0	5.0	100.0
HP89	<0.5	<0.5	50.0	100.0	<0.5	100.0
HP100	1.0	<0.5	50.0	100.0	1.0	100.0
HP102	<0.5	<0.5	50.0	100.0	1.0	100.0
HP103	<0.5	<0.5	<1.0	100.0	0.5	100.0
HP104	1.0	<0.5	>100.0	10.0	<0.5	100.0
HP105	<0.5	<0.5	50.0	100.0	<0.5	100.0
CAT1	2.5	<0.5	>100.0	>100.0	1.0	100.0

The activity of tinidazole against the *G. lamblia* strain GC41 was measured using the adherence and growth inhibition assays[52]. For the adherence assay, the trophozoite suspension (6-10 x 10^6 cells/ml) in Diamond's medium supplemented with 10% heat inactivated calf serum was incubated in the presence of drug at 37°C for 2 hours and 130 μl of the suspension passed through nylon fiber micro column. The columns were incubated at 37°C for an additional 30 minutes and the effluent collected by applying suction pressure. The number of trophozoites in the effluent was counted and % adherence and % adherence inhibition calculated as follows:

$$\%\text{Adherence} = 100 - \left\{ \frac{\text{Giardia conc. in effluent}}{\text{Giardia conc in original suspension}} \times 100 \right\}$$

$$\%\ \text{Adherence Inhibition (AI)} = 100 - \left\{ \frac{\text{Adherence in drug-treated suspension}}{\text{Adherence in untreated suspension}} \times 100 \right\}$$

For the growth inhibition assay, 1 x 10^6 trophozoites in the same medium were incubated in the presence of drugs and the trophozoite concentration in treated vs untreated cultures was determined using a coulter counter. The % growth inhibition was calculated as follows:

$$\%\ \text{Growth Inhibition (GI)} = 100 - \left\{ \frac{\text{Giardia conc in drug-treated culture}}{\text{Giardia conc. in untreated culture}} \times 100 \right\}$$

The results in Table 28 show that exposure of *G. lamblia* to tinidazole (100 μg/ml) resulted in inhibition of adherence by 55% and inhibition of growth by 87%. The activity of tinidazole was similar to metronidazole by the growth inhibition assay but 1.6 fold lower by the adherence assay.

Table: 28: Activity of tinidazole against *G. lamblia* by adherence and growth inhibition assays.

Chemotherapeutic agent (100 μg/ml)	Adherence inhibition (% ± S E)	Growth inhibition % ± S E)
Imidazoles		
Tinidazole	54.5 ± 4.7*	86.9 0.2*
Metronidazole	87.7 ± 10.1*	94.3 ± 0.9*

*p<0.001

In another study[53], the *in vitro* activity of tinidazole against 2 strains (BRIS/83/HEPU/106 and BRIS/83/HEPU/120) of *G. lamblia* was examined by sub-culturing from stocks on different days. The ^{3}H-thymidine uptake assay described earlier on page 29 was used to measure activity. The results in Table 29 show that the tinidazole IC_{50} values varied 3 fold against strain BRIS/83/HEPU/106 (range 0.20 to 0.64 x 10^{-6} M or 0.05 to 0.16 µg/ml) and 4 fold against the strain BRIS/83/HEPU/120 (range 0.53 to 2.23 x 10^{-6} M or 0.13 to 0.55 µg/ml) when measured on different days using the ^{3}H-thymidine assay.

Table 29: Activity of tinidazole (TIN), metronidazole (MET), furazolidone (FUR) and quinacrine (QUIN) against *G lamblia* BRIS/83/HEPU/106 and BRIS/83/HEPU/120 *in vitro*.

Strain BRIS/83/HEPU/106

Clone	Doubling time (h)	MET	TIN	FUR	QUIN
	15·4	0·91	0 62	0·75	1·17
106/1/1	21·0	0 94	0·59	0 93	2·67
106/1/2	16·6	0 69	0 20	0 89	0·76
106/1/3	17·1	0·74	0 56	0 69	0·76
106/1/4	16·7	0·83	0 41	0·58	1·49
106/1/5	13·2	0 68	0·60	0·39	1·48
106/1/6	17·6	0·94	0·55	0 34	1·00
106/1/7	15·5	1·00	0 55	0·58	1·36
106/1/8	14·9	0·83	0·64	0·71	0·65
106/1/9	13·3	0·73	0·60	0·70	0·79
106/1/10	14 3	0·80	0·55	0·69	0·78
106/1/11	15·0	0 90	0·53	0·58	0·92
Range	13·2-21·0	0 68-1·0	0·20-0·64	0·34-0·93	0·65-2 67

Strain BRIS/83/HEPU/120

Clone	Doubling time (h)	MET	TIN	FUR	QUIN
	16·9	0·98	0·56	4·23	1 36
120/1/1	15·9	0·71	0·53	1·03	1·24
120/1/2	14·5	0·56	0·61	0·85	2·93
120/1/3	15·6	0·62	0·58	1·24	1·03
120/1/4	15·2	0·62	0·56	1 06	1·08
120/1/5	14·3	0·64	0·63	0·68	1·79
120/1/6	16·2	0·71	1·02	1·13	1·13
120/1/7	14·0	0·69	0·68	0·91	0 86
120/1/8	14·0	0·70	0·76	1·33	0·81
120/1/9	25·3	0·57	0·54	1·64	0·73
120/1/0	17·4	0 68	0·88	0·89	2·14
120/1/11	12·4	1·14	2·23	0·52	1·20
120/1/12	13·9	0·83	0·66	1·16	0·93
120/1/13	14·8	1·18	0·99	1·75	0·20
120/1/14	17·5	0·63	0 64	0·88	1·14
120/1/15	12·4	0·93	1·45	1·16	0·42
Range	12·4-25·3	0·56-1·18	0·53-2·23	0·52-1·75	0·20-2·93

In another study[54], the activity of tinidazole and metronidazole against a different strain of *G. lamblia* (strain WB, ATCC 30957) was measured using the same method as the previous study. The IC_{50} values for tinidazole, metronidazole and furazolidone against this strain were 0.78 ± 0.48 µM (0.19 ± 0.11 µg/ml), 2.1 ± 0.80 µM (0.36 ± 0.14 µg/ml), and 1.0 ± 0.03 µM (0.23 ± 0.01 µg/ml), respectively.

The activity of tinidazole in combination with other antiparasitic drugs was measured using the P1 and BRIS/82/HEP/41 strains of *G. lamblia* by the growth inhibition and adherence assays[55]. For the growth inhibition assay, 1 x 10^5 trophozoites/ml in TYI-S-33 medium supplemented with 10% fetal calf serum were incubated with the different drugs (alone or in fixed combination) at 37°C for 48 hours. The trophozoite concentration was then determined with the aid of a coulter

counter. The experiment was performed 3 times on 3 separate days. The percentage growth inhibition in drug treated culture compared to untreated controls was calculated.

Tinidazole in combination with doxycycline, mefloquine or furazolidine does not appear to be antagonistic against the P1 and BRIS/82/HEP/41 strains of *G. lamblia* by the growth inhibition assay (Table 30).

Table 30: Effect of tinidazole in combination with other antiparasitic drug on growth and adherence of *G. lamblia*.

Drug (µg/ml)	Growth inhibition (%)[a]		Adherence inhibition (%)[a]	
	Portland (P1 strain)	BRIS/82/HEP/41 strain	Portland (P1 strain)	BRIS/82/HEP/41 strain
Doxycycline (20)	31.0 ± 3.3	29.8 ± 1.5	10.8 ± 0.5	12.8 ± 0.3
Mefloquine (5)	24.4 ± 3.3	22.9 ± 2.0	16.7 ± 0.9	22.4 ± 1.1
Tinidazole (0.2)	28.4 ± 2.2	21.2 ± 2.2	20.3 ± 0.8	23.8 ± 1.3
Furazolidone (0.2)	37.0 ± 1.2	23.0 ± 0.7	10.3 ± 0.9	8.1 ± 0.4
Tinidazole (0.2) + Doxycycline (20)	47.0 ± 3.8	38.9 ± 6.2	36.5 ± 0.1*	39.7 ± 2.0*
Tinidazole (0.2) + Mefloquine (5)	51.7 ± 0.2	38.5 ± 4.6	45.7 ± 5.6*	45.7 ± 6.3*
Tinidazole (0.2) + Furazolidone (0.2)	46.4 ± 4.3	31.2 ± 3.3	22.9 ± 1.7	16.9 ± 3.2

* synergism

[a] The results represent mean ± standard error of the mean of 3 or more experiments, each in triplicate.

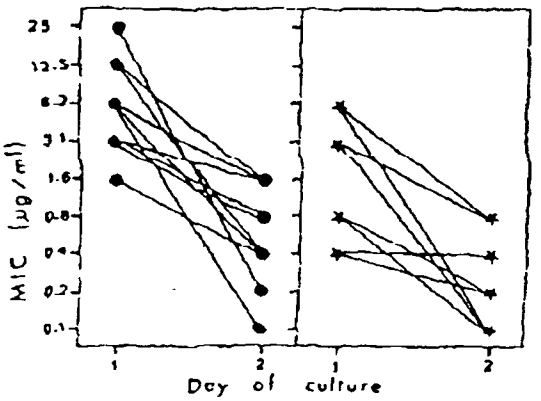
For the adherence assay, the trophozoite suspension (1 x 10^5 cells/ml) in TYI-S-33 medium supplemented with 10% heat inactivated calf serum was incubated in the presence of drug at 37°C for 2 hours and 130 µl of the suspension passed through nylon fiber micro column. The columns were incubated at 37°C for an additional 30 minutes and the effluent collected by applying suction pressure. The experiment was performed 3 to 12 times in triplicate. The % adherence was calculated as described on page 32. The inhibition of adherence of the 2 strains (P1 and BRIS/82/HEP/41) of *G. lamblia* by a combination of tinidazole and doxycycline or mefloquine was 1.7 to 3.4 fold higher than each of the drugs alone (Table 30) Thus, a combination of tinidazole and doxycycline or mefloquine does not appear to be antagonistic against the 2 strains (P1 and BRIS/82/HEP/41) of *G. lamblia* using the adherence assay. The sponsor has stated that the combinations of tinidazole with doxycycline or mefloquine were synergistic. However, the criteria used for characterizing synergistic activity are unclear. Please note that such observations were not made using the growth inhibition assay.

Clinical isolates:

The *in vitro* activity of tinidazole against 4 clinical isolates of *G. lamblia* was examined by immobilization assay[56]. Metronidazole was used as the comparator. For this, *G. lamblia* cultures (1 x 10^3 to 5 x 10^4 trophozoites per ml) in Diamond's medium were incubated in the presence of drug in the wells of leucocyte migration plates. The wells were sealed using a coverslip and the plates incubated anaerobically in an inverted position for up to 3 days. At different time intervals, the coverslips were examined using an inverted microscope at 600x magnification to determine the percentage of motile trophozoites. One hundred trophozoites at each concentration were observed for 15 seconds and the minimal immobilizing concentrations (MIC) defined as the lowest concentration of the drug showing no motility was determined.

The results in Figure 7 show that the tinidazole MICs against the 4 isolates were higher (range 0.4 to 6.2 μg/ml) at 24 hours than at 48 hours (range 0.1 to 0.8 μg/ml). The metronidazole MICs (1.6 to 12.5 μg/ml) were higher than tinidazole values at 24 hours but similar at 48 hours.

Figure 7: Time-dependence of the minimal immobilizing concentration (MIC) of metronidazole (●) or tinidazole (★). The MIC is the lowest concentration of drug at which no motile *G. lamblia* trophozoites among the first 100 (each observed for 15 seconds in situ in the microculture system) were seen. The lines connect cultures of the same isolate of *G. lamblia*.



In another study[57], the *in vitro* activity of tinidazole against 13 isolates of *G. lamblia* was examined by the ^{3}H-thymidine uptake assay described previously (page 29). Other antiprotozoal drugs were used for comparison. The inoculum size was not specified in the publication. The IC_{50} values for metronidazole, tinidazole, furazolidone, and quinacrine against these 13 isolates are shown in Figure 8. The mean IC_{50} values for tinidazole, metronidazole, furazolidone and quinacrine were 0.70 ± 0.09 μM (0.42 ± 0.02 μg/ml), 0.99 ± 0.15 μM (0.17 ± 0.02 μg/ml), 1.30 ± 0.26 μM (0.29 ± 0.06 μg/ml), and 1.50 ± 0.41 μM (0.76 ± 0.21 μg/ml), respectively.

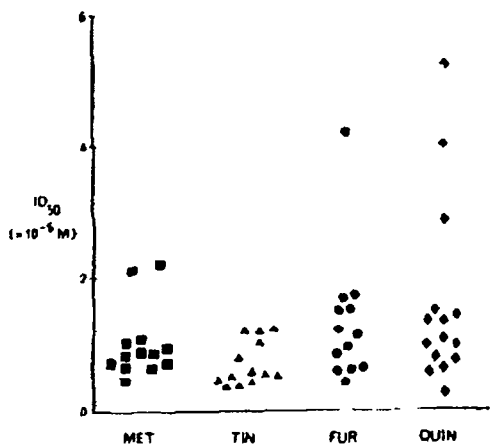


Figure 8: Concentrations of metronidazole (MET), tinidazole (TIN), furazolidone (FUR), and quinacrine (QUIN) required to inhibit uptake of ^{3}H-thymidine by 50% (IC_{50} = ID_{50}) for *Giardia lamblia* isolates from 13 children.

In another study[48], the *in vitro* activity of tinidazole was examined against 17 clinical isolates of *G. lamblia* using the ^{3}H-thymidine incorporation assay as previously described on page 29. The P1 strain was used as a control. The results in Table 23 (page 29) show that the tinidazole IC_{50} values against the 17 isolates ranged between 0.37 μM (0.09 μg/ml) to 1.37 μM (0.34 μg/ml). The activity of tinidazole was similar to metronidazole and furazolidone.

In another study[58], the *in vitro* activity of tinidazole against 25 *G. lamblia* isolates was examined using a different method. For this, cultures (3 x 10^6 trophozoites per ml) in TPS-1 medium were incubated in the presence of different concentrations of drug at 37°C and examined microscopically daily for up to 7 days. The growth of *G. lamblia* trophozoites was confirmed using an inverted microscope at 63x magnification. The MIC was defined as the lowest concentration of the drug at which no visible growth was detected. The results in Table 31 show that the tinidazole MICs against the 25 clinical isolates was <0.5 μg/ml and was similar to metronidazole.

Table 31: Frequency distribution of MIC values (n = 25).

MIC range (μg/ml)	Frequency distribution with antibiotic:		
	Tinidazole	Metronidazole	Furazolidone
0.1<-<0.5	25	24	22
0.5<-≤1.0	0	1	1
1.0<-≤5.0	0	0	0
5.0<-≤10	0	0	2

In summary, the activity of tinidazole was measured against the trophozoite stage of 22 strains and 59 isolates of *G. lamblia* under different experimental conditions such as using different media, incubation periods, inoculum sizes and methods (Table 32). Irrespective of the assay conditions, the tinidazole MIC values against the *G. lamblia* strains ranged from 0.32 to 1.0 μg/ml. The tinidazole IC_{50} values against the strains ranged from 0.03 to 0.29 μg/ml. Against the clinical isolates, the tinidazole MIC values (0.1 to 6.2 μg/ml) and IC_{50} values (0.09 - 25.0 μg/ml) were variable. The different assays gave comparable activity when using the same strain. A 3 - 4 fold variation was observed in the activity of tinidazole measured on different days against the same strain. Overall, the activity of tinidazole was similar to metronidazole and furazolidone.

Table 32: Summary of *in vitro* activity of tinidazole against G. *lamblia* strains and isolates.

Study	No. of strains (name) or isolates	Inoculum size (trophozoites/ ml)	Medium	Method (incubation period at 37°C)	Tinidazole	
					MIC (µg/ml)	IC_{50} (µg/ml)
Strains						
Meloni	2 (P1 and P1C10)	3-5 x 10^4	B1-S-33 media + bovine serum	Growth inhibition (24 hours)	0.32-0.50	
Kang	1 (P1)	3 x 10^4	TY1-S-33 media+ glucose + serum + arginine	Colorimetric (72 hours)		0.1
Smith	1 (P1)	5 x 10^4	Modified TY1-S-33 media	^{3}H-thymidine uptake (24 hours)		0.13
Gordts	9	5 x 10^4	TPS-1 media	Growth inhibition (2-7 days)	<0.5	
Boreham	6 (P1)	5 x 10^4	Modified TY1-S-33 media	^{3}H-thymidine uptake (24 hours)		0.03-0.29
Cedillo-Rivera	1 (P1)	5 x 10^4	Diamond's media	Growth inhibition (48 hours)	0.5-1.0	0.14
Boreham	2 (BRIS/83/HEP /106 and /120)	6-10 x 10^6	Diamond's media	^{3}H-thymidine uptake (2 hours)		0.19 ± 0.11
Summary	**22**	**10^4 - 10^6**	**5 different medium**	**2 hours - 7 days**	**0.32 - 1.0**	**0.03 - 0.29**
Clinical isolates						
Jokipii	4	10^3-5 x 10^4	Diamond's media	Motility (24 or 48 hours)	0.4 - 6.2 0.1 - 0.8	1.6-25.0 0.1 – 1.6
McIntyre	13	3 x 10^4	Modified TY1-S-33 media	^{3}H-thymidine uptake (24 hours)		0.42 ± 0.02
Smith	17	3 x 10^4	Modified TY1-S-33 media	^{3}H-thymidine uptake (72 hours)		0.09-0.34
Gordts	25	3 x 10^6	TPS-1 media	Growth inhibition (7 days)	<0.5	
Summary	**59**	**10^3 - 10^6**	**3 different medium**	**24 hours – 7 days**	**0.1 - 6.2**	**0.09 - 25.0**

Please note that the activity of tinidazole against the cyst stage of *G. lamblia* was not measured *in vitro*.

2.2.1.3. *E. histolytica*:

The sponsor has included 4 studies conducted in different laboratories in support of the *in vitro* activity of tinidazole against the trophozoite forms of *E. histolytica*. Two different medium [Liver marmite serum media (LMS) or Locke's media] were used to determine the minimum inhibitory concentration (MIC) i.e., the concentration of the drug required to completely inhibit the growth of the parasite compared to drug-free control at the end of 24 or 48 hours of incubation by microscopic examination. The doubling time for the trophozoite stage of *E. histolytica* is about 8 hours. Please note that there are no standardized methods for determining *in vitro* susceptibility of anti-protozoal drugs against *E. histolytica*.

Laboratory strains:

The *in vitro* activity of tinidazole and metronidazole against the trophozoite stage of 6 strains of *E. histolytica* was examined using the LMS medium but without any supplements[59]. Cultures (200 trophozoites/ml) in LMS medium were incubated in the presence of drug at 37°C for 48 hours and the MIC determined. The MIC value was confirmed by subculturing in LMS medium.

The results in Table 33 show that the tinidazole MIC values (range 0.625 to 1.25 µg/ml) were slightly higher than the metronidazole MIC values (range 0.312 to 0.625 µg/ml).

Table 33: *In vitro* activity of Tinidazole and Matronidazole on six strains of *Entamoeba histolytica.*

Sr. No.	Strain No.	Drug used	In Micrograms/ml					
			10	5	2.5	1.25	0 625	0.312
1.	4/73	Tinidazole	N	N	N	N	++	++
		Metronidazole	N	N	N	N	N	N
2	6/73	Tinidazole	N	N	N	N	+	++
		Metronidazole	N	N	N	N	N	–
3.	9/73	Tinidazole	N	N	N	N	+	++
		Metronidazole	N	N	N	N	N	N
4.	12/73	Tinidazole	N	N	N	N	N	+
		Metronidazole	N	N	N	N	N	N
5	14/73	Tinidazole	N	N	N	N	+	++
		Metronidazole	N	N	N	N	N	N
6	42/73	Tinidazole	N	N	N	N	N	+
		Metronidazole	N	N	N	N	N	N

N= No growth.
+ = growth.

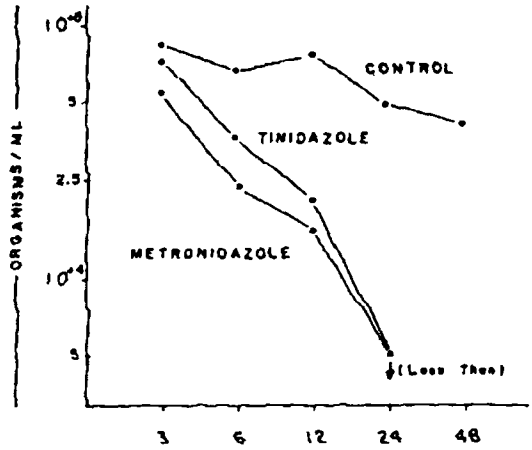
In another study[60], the activity of tinidazole and other antiamoebic drugs against 30 strains of *E. histolytica* was examined using the same medium. For this, 1500 trophozoites were incubated in the presence of drug at 37°C for 48 hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug that showed no growth of amoeba on subculture to Boeck and Drbohalv's medium. If non-motile trophozoites were observed, vital staining with 1% neutral red for 15-20 minutes was performed to detect viable trophozoites. The results in Table 34 show that the tinidazole MICs against the 30 *E. histolytica* strains ranged from 0.0625 to 0.25 µg/ml. The activity of tinidazole was similar to metronidazole and ornidazole.

Table 34: The minimal inhibitory concentration µg/ml of *Entamoeba histolytica* to anti-amoebic durgs

Isolated strains no.	Dehydroemetine	Ornidazole	Metronidazole	Tinidazole
1	0.5	0.0625	0.0625	0.0625
2	0.5	0.0625	0.0625	0.0625
3	0.25	0.125	0.125	0 0625
4	0.25	0.0625	0.125	0.125
5	0.5	0.0625	0.0625	0.0625
6	0.5	0 0625	0.0625	0.0625
7	0.25	0 125	0.0625	0.125
8	0.25	0.125	0.125	0.0625
9	0.125	0.0625	0.0625	0 0625
10	0.25	0.0625	0.125	0.0625
11	0.125	0.0625	0.125	0.0625
12	0.25	0.0625	0.0625	0.0625
13	0.25	0.125	0.125	0 0625
14	0.125	0.0625	0.125	0.0625
15	0.125	0.0625	0.0625	0.0625
16	0.25	0.0625	0.125	0.125
17	0.25	0.0625	0.125	0.0625
18	0.125	0.0625	0.0625	0.0625
19	0.5	0.0625	0.0625	0.125
20	0.25	0.0625	0.0625	0 0625
21	0.25	0.125	0.125	0.125
22	0.5	0.0625	0.125	0.125
23	0.25	0.125	0.125	0.125
24	1	0.125	0.125	0.125
25	1	0.125	0 125	0.125
26	0.5	0 25	0.125	0.25
27	0 25	0.125	0.125	0.125
28	0.125	0 125	0.125	0 125
29	0 5	0 0625	0.0625	0 125
30	1	0 0625	0.0625	0.0625

In another study[31], the activity of tinidazole and metronidazole against a strain of *E. histolytica* was examined using a different medium. The culture (9 x 10^4 trophozoites/ml) was incubated with 80 µg/ml of drug in Locke's medium at 37°C. The number of organisms per ml in drug-treated medium compared to drug-free control was determined after 24 hours of incubation. A 10-fold decrease in parasite count was observed in the presence of tinidazole and metronidazole at 24 hours (Figure 9).

Figure 9: Activity against *E histolytica* trophozoites in Locke's solution.
Drug concentration: 80 µg/ml.



Clinical isolates:
The *in vitro* activity of tinidazole against 14 isolates of *E. histolytica* was examined using the LMS medium with supplements[61]. Other anti-protozoal agents were used as comparators. About 200 amoebae were incubated with different concentrations of the drug in LMS medium supplemented with 10% horse serum and 10 mg of sterile rice starch. The cultures were incubated at 37°C for 48 hours and the MIC determined. The MIC of tinidazole (CP12-574) required to inhibit growth of the 14 isolates ranged from 0.3 to 2.5 µg/ml and was similar to metronidazole 0.16 to 1.25 µg/ml (Tables 35).

In summary, the *in vitro* activity of tinidazole against the trophozoite stage of *E. histolytica* strains was examined in 4 different laboratories. Two studies measured the tinidazole MIC in liver marmite medium at 48 hours. The tinidazole MIC values against 36 strains ranged from 0.063 to 1.25 µg/ml. The tinidazole MIC values against the strains were comparable to metronidazole MIC values. The activity of tinidazole against the 14 clinical isolates (MIC range = 0.3 to 2.5 µg/ml) of *E. histolytica* was similar to that observed against the laboratory strains (MIC range = 0.063 to 1.25 µg/ml) at 48 hours. The activity of tinidazole against the clinical isolates was 2-fold lower than metronidazole. One study showed a 10-fold reduction in trophozoite count in the presence of 80 µg/ml tinidazole at 24 hours using Locke's medium. The activity of tinidazole against the cyst stage of *E. histolytica* was not examined *in vitro*.

Table 35: *In vitro* activity of the antiamoebic drugs against the 14 clinical isolates of *Entamoeba histolytica*.

Strain	Clinical diagnosis	Drug tested *in vitro*	Control (No. of amoebae per 0.05 ml)	Number of parasite (% reduction in parasite count)								
				500 µg/ml	50 µg/ml	5 µg/ml	2.5 µg/ml	1.25 µg/ml	0.625 µg/ml	0.312 µg/ml	0.156 µg/ml	0.078 µg/ml
69-490	Amoebiasis	Oxytetracycline	159	N	N	1(99%)	10 (94%)	ND	ND	ND	ND	ND
		Chloroquine phosphate		ND	N	N	N	35 (88%)	70 (66%)	ND	ND	ND
		Di-iodohydroxyquinoline		ND	N	N	N	N	N	4 (97.5%)	25 (83%)	ND
		Tinidazole		**ND**	**N**	**N**	**N**	**3 (98%)**	**10 (94%)**	**ND**	**ND**	**ND**
66-857	Amoebic hepatitis	Di-iodohydroxyquinoline	285	ND	N	N	N	N	N	2 (99%)	6 (98%)	ND
		Tinidazole		**ND**	**N**	**N**	**N**	**N**	**46 (84%)**	**76 (73%)**	**ND**	**ND**
69-990	Intestinal amoebiasis	Oxytetracycline	350	N	4 (99%)	31 (92%)	25 (93%)	ND	ND	ND	ND	ND
		Chloroquine phosphate		N	1 (>99%)	35 (90%)	70 (80%)	ND	ND	ND	ND	ND
		Di-iodohydroxyquinoline		ND	N	N	N	N .	11 (97%)	31 (92%)	40 (89%)	ND
		Tinidazole		**ND**	**N**	**N**	**N**	**30 (92%)**	**20 (94%)**	**28 (92%)**	**ND**	**ND**
68-3421	Amoebiasis	Oxytetracycline	80	N	N	15 (81%)	13 (84%)	21(74%)	22 (73%)	ND	ND	ND
		Chloroquine phosphate		N	1 (99%)	4 (95%)	5 (94%)	33 (59%)	33 (59%)	ND	ND	ND
		Di-iodohydroxyquinoline		ND	N	N	N	11(86%)	48 (40%)	50 (38%)	ND	ND
		Tinidazole		**ND**	**N**	**N**	**N**	**1 (99%)**	**58 (27%)**	**78 (2.5%)**	**72 (10%)**	**ND**
67-1543	Malabsorption	Oxytetracycline	240	N	N	N	8 (97%)	10 (87.5%)	25 (90%)	53 (44%)	65 (73%)	82 (66%)
		Chloroquine phosphate		N	N	N	5 (98%)	11 (95%)	28 (89%)	38 (84%)	42 (82%)	60 (75%)
		Di-iodohydroxyquinoline		N	N	N	N	N	16 (93%)	18 (93%)	26 (89%)	38 (84%)
		Tinidazole		**N**	**N**	**N**	**N**	**N**	**N**	**30 (88%)**	**28 (89%)**	**58 (76%)**
		Metronidazole		N	N	N	N	N	N	3 (99%)	8 (97%)	12 (95%)
69-1555	Amoebic hepatitis	Oxytetracycline	238	N	N	N	N	N	1 (>99%)	4 (99%)	21 (91%)	131 (45%)
		Chloroquine phosphate		N	N	N	N	3 (99%)	6 (98%)	8 (96%)	14 (94%)	15 (94%)
		Di-iodohydroxyquinoline		N	N	N	N	N	1 (>99%)	2 (>99%)	4 (99%)	12 (95%)
		Tinidazole		**N**	**N**	**N**	**N**	**N**	**N**	**N**	**10 (96%)**	**23 (90%)**
		Metronidazole		N	N	N	N	N	N	5 (98%)	8 (96%)	15 (94%)
69-1757	Pain abdomen	Oxytetracycline	250	N	N	7 (98%)	24 (91%)	27 (90%)	20 (92%)	23 (91%)	27 (90%)	73 (70%)
		Chloroquine phosphate		N	N	N	8 (97%)	10 (96%)	18 (93%)	38 (85%)	60 (76%)	69 (73%)
		Di-iodohydroxyquinoline		N	N	N	N	N	4 (98%)	6 (98%)	7 (98%)	14 (94%)
		Tinidazole		**N**	**N**	**N**	**N**	**5 (98%)**	**23 (91%)**	**51 (80%)**	**53 (79%)**	**102 (59%)**
		Metronidazole		N	N	N	N	N	N	N	1 (>99%)	5 (98%)
69-1926	Intestinal amoebiasis	Oxytetracycline	460	N	N	N	6 (99%)	5 (99%)	10 (98%)	16 (97%)	21 (96%)	35 (93%)
		Chloroquine phosphate		N	N	5 (99%)	22 (95%)	28 (94%)	21(96%)	31(93%)	35 (93%)	43 (91%)
		Di-iodohydroxyquinoline		N	N	N	N	1(>99%)	2 (>99%)	5 (99%)	10 (98%)	17 (97%)
		Tinidazole		**N**	**N**	**N**	**N**	**N**	**1 (>99%)**	**6 (99%)**	**18 (97%)**	**32 (93%)**
		Metronidazole		N	N	N	N	N	6 (99%)	12 (98%)	36 (92%)	48 (90%)
68-2511	Intestinal amoebiasis	Oxytetracycline	280	N	N	6 (98%)	50 (82%)	53 (81%)	58 (79%)	65 (77%)	72 (74%)	80 (72%)
		Chloroquine phosphate		N	N	84 (70%)	112 (60%)	105 (63%)	123 (56%)	130 (54%)	138 (51%)	145 (48%)
		Di-iodohydroxyquinoline		N	N	N	N	N	N	4 (98%)	12 (96%)	36 (87%)
		Tinidazole		**N**	**N**	**N**	**N**	**N**	**2 (99%)**	**8 (97%)**	**16 (94%)**	**30 (89%)**
		Metronidazole		N	N	N	N	N	22 (92%)	25 (91%)	36 (87%)	45 (84%)

The test was read after 48 hours of incubation at 37°C; N = No growth; ND = Not done