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Drug library screening against metronidazole-sensitive and metronidazole-resistant *Trichomonas vaginalis* isolates

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Abstract

Objectives—Metronidazole and tinidazole are effective treatments for most patients with trichomoniasis but not for individuals who are infected with very resistant strains of *Trichomonas vaginalis* or persons with hypersensitivity to the 5-nitroimidazole drugs. Thus, there is a need for additional oral therapies to treat trichomoniasis.

Methods—We screened the US Drug Collection Library against metronidazole-susceptible and resistant strains of *T vaginalis*. Activity was measured by incubating parasites and drugs for 48 h in the presence of tritiated thymidine. Growth inhibition was determined by the reduction of incorporated radioactivity by compounds at 20 μM in comparison to media control. Drugs that showed good initial activity were further tested to calculate IC_{50} values. Drugs with the most promise were tested together with metronidazole to see if there was any combinatorial effect.

Results—Of the 1040 drugs in the library, 83 (8%) reduced growth of a metronidazole-susceptible *T vaginalis* strain by at least 20%. Of these, IC_{50} values were calculated for 27 compounds and 8 drugs were evaluated in combination with metronidazole. Disulfiram and nithiamide were non-5-nitroimidazole drugs that showed the best activity against parasites when used alone. Albendazole and coenzyme B12 were the most promising compounds to boost the efficacy of metronidazole.

Conclusions—No one drug was as effective as any of the 5-nitroimidazole compounds. However, disulfiram and nithiamide may be useful to treat individuals with hypersensitivity to 5-nitroimidazole drugs and albendazole and coenzyme B12 may be helpful in combination with metronidazole or tinidazole for treatment of persons with highly resistant *T vaginalis* infections.

INTRODUCTION

Metronidazole was introduced as a treatment for *Trichomonas vaginalis* infections in 1959. The first description of trichomoniasis treatment failure for this drug was in 1962.[1] A recent

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study in six cities distributed throughout the USA estimated that 4.3% of *T vaginalis* isolates obtained from persons attending sexually transmitted disease (STD) clinics show some degree of resistance to metronidazole.[2] In 2004, another 5-nitroimidazole, tinidazole, was approved in the USA for treatment of *T vaginalis* infections. Tinidazole has better in vitro activity against trichomonas isolates and seems to have better clinical efficacy with fewer side effects.[3] However, because it is in the same drug class as metronidazole, infections that are highly resistant to metronidazole can also fail to resolve following tinidazole treatment. The 5-nitroimidazoles are the only Food and Drug Administration (FDA) approved oral medications with demonstrated efficacy against trichomoniasis.

In addition to treatment failure, allergy to the 5-nitroimidazoles can complicate use of metronidazole or tinidazole for persons with trichomoniasis. For most people, hypersensitivity is relatively mild and treatment can be effected using a short course desensitisation protocol.[4] However, severe allergic reactions such as Stevens-Johnson syndrome or anaphylaxis can occur in response to 5-nitroimidazoles, precluding their use in certain individuals.[5 6]

Intravaginal treatments for trichomoniasis using non-5-nitroimidazoles have some efficacy and are the only alternative in cases of very high drug resistance or severe hypersensitivity to metronidazole and tinidazole.[4] However, topical therapy is not as successful as systemic treatment, especially for patients with parasites in their periurethral glands. Thus, there is a population of patients with *T vaginalis* for whom no effective oral therapy exists, indicating a need for new drugs to treat trichomoniasis.

A number of experimental and natural compounds have been evaluated against trichomonads, with several demonstrating good potency.[7–10] Unfortunately, none of them have progressed to clinical trials, usually as a result of inadequate funding to perform the appropriate testing. Like parasitic diseases in developing countries, trichomoniasis is most common in persons with less purchasing power, limiting the potential profitability of any new therapy and, thus disincentivising pharmaceutical companies to invest in their development.

One alternative to developing a new drug is to repurpose existing compounds for use in a new application. For example, the antitrypanosomal drug eflornithine was originally patented for reduction of unwanted facial hair. We wished to test if any compounds that are already in use or are in development had adequate activity against *T vaginalis* that they may have utility as an alternative to, or in conjunction with, 5-nitroimidazoles for treating trichomoniasis. Assessment of compounds already approved for human use in vitro could lead to more rapid evaluation in infected individuals than testing of a completely new drug.

MATERIALS AND METHODS

Compound library selection

The US Drug Collection Library (Microsource Discovery Systems, Gaylordsville, Connecticut, USA) was screened for activity against susceptible and resistant strains of trichomonas. The library consists of 1040 drugs that have been approved for human use in

the USA or that are being tested in clinical trials. It contains multiple drug classes and is supplied as 10 mM stocks in dimethyl sulfoxide (DMSO).

Trichomonas strain selection and culture

Standard metronidazole-sensitive (CDC 520) and standard metronidazole-resistant (CDC 085) isolates were maintained in Diamond's trypticase-yeast-maltose media at 37°C. Anaerobic conditions were generated by using a GasPak jar and CO_2-generating GasPak Plus anaerobic system envelopes (Becton Dickinson, Sparks, MD) and monitored with GasPak disposable anaerobic indicator strips (Becton Dickinson).

Determination of drug activity by thymidine incorporation inhibition

Drug activity was evaluated using a tritiated thymidine incorporation inhibition assay.[7] For the initial screen of the US Drug Collection library all drugs were tested in triplicate against the reference susceptible *T vaginalis* strain under aerobic conditions. Drugs were diluted to a final concentration of 20 μM in Diamond's media in a 96-well round bottom plate. Each screening plate contained internal negative 0.2% (w/v) DMSO (matching the concentration of the vehicle for test drugs) and positive metronidazole controls in serial twofold dilutions from 400 μg/mL to 0.1 μg/mL (2.34 mM to 1.14 μM). A total of 10 000 trichomonads were added to each well. Tritiated thymidine (Perkin Elmer, Waltham, Massachusetts, USA) was added at 0.5 μCi per well at culture initiation and plates were incubated at 37°C in a 5% CO_2 environment. After 48 h, wells were visually assessed on an inverted microscope and drugs that appeared to have killed or severely inhibited parasite motility were noted. The trichomonads were then harvested onto glass fibre filters and washed with DiH_2O (Filtermate Harvester, Perkin Elmer). Filter mats were dried overnight before processing for scintillation counting on the MicroBeta2 scintillation counter (Perkin Elmer). Data were collected in counts per minute.

Drugs that inhibited growth of parasites in media alone by 20% or more were included for further testing against sensitive and resistant strains under aerobic and anaerobic conditions. Selection of drugs for secondary screening also emphasised those that could be administered orally with good bioavailability and low host toxicity, although some with high toxicity, were included for comparison. Drugs were screened at 12 doses between 0.02 μM and 40 μM in triplicate to determine IC_{50} values with both strains under both conditions. As before, DMSO and metronidazole controls were included. Tritiated thymidine incorporation and readout were carried out as described in the first round of screening. Dose response curves were generated and IC_{50} values determined using GraphPad Prism V.5.0 (GraphPad Software, San Diego, California, USA).

Drug combination screening

Checkerboard microdilution assays were performed with drugs that yielded good activity in secondary screening to assess efficacy of drug combinations. Selected drugs were screened in combination with metronidazole with doses near the IC_{50}s for both drugs for both strains under aerobic and anaerobic conditions. Inclusion in the third round of screening was determined by IC_{50} values, toxicity, absorbability and method of delivery. Measurement of

drug combination activity was performed in the same manner as the primary and secondary screens.

RESULTS

Primary screening

The 1040 drugs in the compound library were screened against the reference susceptible reference strain of *T vaginalis* at 20 μM (figure 1). The library metronidazole inhibited thymidine incorporation by 80.9%. Of the other library drugs, 83 inhibited thymidine uptake by at least 20%, with 11 drugs having activity equal to or greater than that of metronidazole. The drug classes with the best efficacy against *T vaginalis* growth included antibiotics, antineoplastics and antiparasitic agents (figure 1). The growth inhibitory activity of all test compounds is reported in online supplementary table S1.

Secondary screening

IC_{50} values were determined for 27 drugs, for susceptible and metronidazole-resistant strains of *T vaginalis* under aerobic and anaerobic conditions (table 1). Drugs not included at this level were either highly toxic, are for topical use only, or are poorly absorbed from the intestinal tract. Not surprisingly, compounds that demonstrated the best inhibitory activity in the initial screen had lower IC_{50} values than those drugs with lower activity. However, some drugs that had good activity in the initial screen against the susceptible *T vaginalis* strain were less effective under anaerobic conditions (eg, clofazaimine, niclosamide) or against the resistant *T vaginalis* isolate (eg, ornidazole, emetine). Only disulfiram, ronidazole, fenbendazole, nithiamide and albendazole showed consistent activity for both strains under both growth conditions. Thimerosal was also active under all four strain/condition combinations but is very toxic and primarily used as a preservative for other biologicals. It was included in this round of testing primarily as a non-nitroimidazole with antitrichomonad activity.

Drug combination screening

Because few of the compounds demonstrated clear-cut efficacy against both *T vaginalis* strains under aerobic and anaerobic conditions, we evaluated the most promising drugs in combination with metronidazole to determine if better activity could be achieved when the drugs were used together. To perform these tests, we set up a checkerboard dilution series that included the IC_{50} drug concentration of both compounds. For these tests, only the metronidazole-resistant reference strain of *T vaginalis* was used. Under aerobic conditions (figure 2), a drug combination effect with metronidazole was evident only for coenzyme B12 and albendazole. For the other compounds tested, ether metronidazole or the combination test drug above a certain concentration (eg, disulfiram or ethacrynic acid) demonstrated the dominant effect on trichomonad growth. Under anaerobic conditions (figure 3), a combinatorial effect was seen for metronidazole with niclosamide, minocycline hydrochloride (HCL), nithiamide, coenzyme B12, cloxyquin and albendazole. Disulfiram again demonstrated a dominant effect at concentrations of 1 μM or greater but ethacrynic acid appeared to have no inhibitory effect under anaerobic conditions.

DISCUSSION

It can cost $18.5 million to bring a new drug to the level of clinical trial testing.[11] The cost to perform clinical trials and bring the drug to market can be an additional $250 million and take 7–14 years.[11 12] As a result, before pharmaceutical companies are willing to make large investments in time and money to develop a new drug, they must have a strong financial incentive to do so. For infection agents like *T vaginalis* that disproportionately affect persons at the lower end of the socioeconomic scale,[13] and for which effective treatments are available for over 90% of the infected population, the impetus to make this sort of investment is very small. Nevertheless, without additional treatment options for trichomoniasis, hundreds of thousands of women will remain infected with *T vaginalis*.

Our goal was to identify drugs that had been approved for other purposes for their potential activity against *T vaginalis*, either alone or in combination with a 5-nitroimidazole such as metronidazole. Of the 1040 compounds in the drug collection library, few outside of the 5-nitroimidazole class had good activity against *T vaginalis* while meeting other criteria that would be important for an alternative drug such as low toxicity, oral dosing and systemic bioavailability. Only disulfiram and nithiamide met these criteria while maintaining good activity against metronidazole resistant and susceptible parasite strains under aerobic and anaerobic conditions (table 1). Disulfiram, also known as Antabuse, is used in the treatment of alcoholism and has been shown to have activity against trichomonads in vitro.[14] Nithiamide, also known as aminitrozole, was tested as a treatment for trichomoniasis in humans prior to the availability of metronidazole with moderate to poor results.[15 16]

Lacking a clear orally available, non-toxic and highly effective alternative to the 5-nitroimidazole drugs, we also evaluated compounds with partial activity in combination with metronidazole. Promising effects were shown by niclosamide, minocycline HCL, nithiamide, coenzyme B12, cloxyquin and albendazole. Niclosamide and albendazole are antihelmintic drugs, minocycline is used for the treatment of acne, coenzyme B12 is one of the B complex vitamins, and cloxyquin has activity against fungal and bacterial infections, including *Mycobacterium*. Overall, albendazole and coenzyme B12 showed the most promise, in terms of combinatorial efficacy under aerobic and anaerobic conditions as well as low toxicity, suggesting that their use in conjunction with 5-nitroimidazole drugs would be safe. In contrast, there was no clear benefit of combining disulfiram and metronidazole for increased in vitro activity. Furthermore, use of these two drugs together is contraindicated as it causes acute psychosis in approximately 20% of individuals receiving both drugs.[17] Even for the combinations with metronidazole that appear like they would be well tolerated, additional investigation of their pharmacological properties should be pursued before concurrent use of two drugs is attempted.

While this study failed to reveal a currently approved non-5-nitroimidazole drug that is likely to be an effective oral therapy against trichomoniasis infections when used alone, it does suggest some possible new therapies for treatment of persons with highly resistant *T vaginalis* infections or those individuals with severe hypersensitivity reactions to metronidazole and tinidazole. Two other drugs that were not part of the drug library that we tested but may merit investigation for activity against trichomoniasis are miltefosine

and nifuratel. Miltefosine is used for treatment of human visceral leishmaniasis in India, Colombia and Germany. It has good in vitro activity against metronidazole-resistant and metronidazole-susceptible strains of *T vaginalis* but has not been tested in infected people.[18] Even if it is effective against trichomoniasis, its use would need to be closely monitored as it is contraindicated in pregnancy and women who are sexually active but not using contraceptives. Thus, women with *T vaginalis* infections are a group in which miltefosine use could be risky. Nifuratel is used in Europe for a number of vulvovaginal infections as well as *Helicobactor pylori*. It is often used orally and topically and shows similar efficacy as metronidazole;[19] however, its topical use has been associated with contact dermatitis in some individuals.[20]

Continued efforts to identify alternative oral therapies for trichomoniasis are warranted. The number of persons with highly 5-nitroimidazole resistant infections or who are severely hypersensitive is in the hundreds of thousands in the USA alone. This number, along with the ability to make a new compound commercially profitable is likely not sufficient to induce substantial investment by pharmaceutical companies. Nevertheless, the individual and public health impact of trichomoniasis, along with comorbidities with which it is associated (eg, increased risk of HIV transmission) merits attention to these difficult-to-treat infections as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key messages

- The 5-nitroimidazole drugs metronidazole and tinidazole are effective oral therapies for most persons with *Trichomonas vaginalis* infections.
- However, drug resistance and hypersensitivity to 5-nitroimidazoles make them inappropriate treatments for some individuals with trichomoniasis.
- Identification of a new oral therapy for trichomoniasis would be most useful but pharmaceutical companies have little incentive to develop such drugs.
- Evaluation and repurposing of existing drugs may be the most likely approach to identify new oral therapies for *T vaginalis* infections.

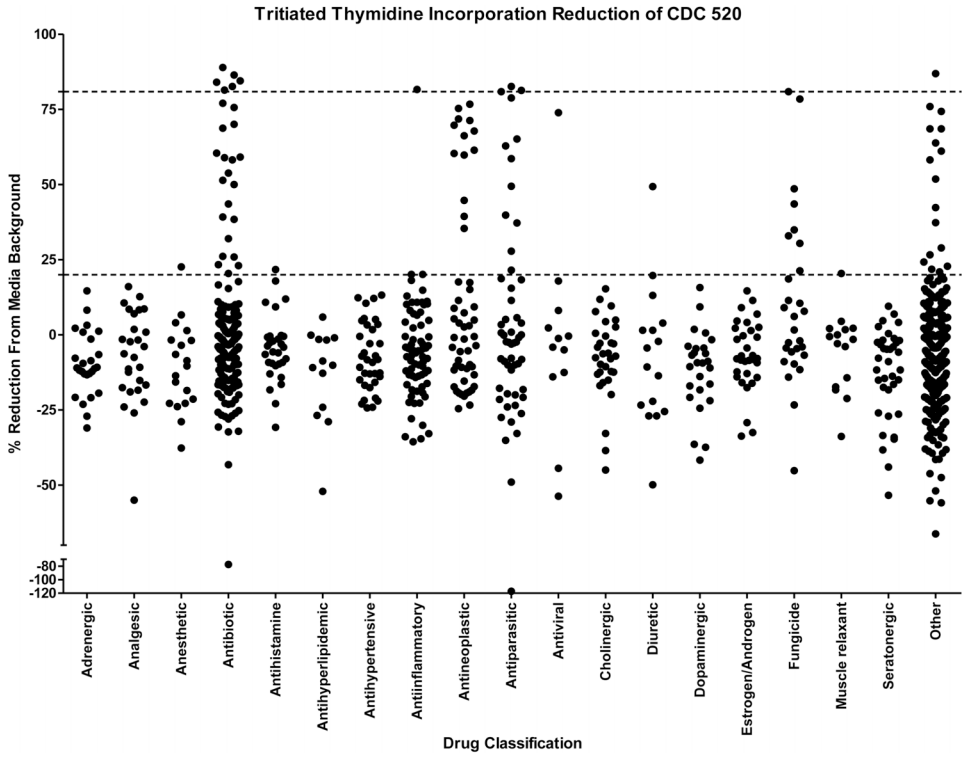

Figure 1.

First round of screening of library drugs against a susceptible *Trichomonas vaginalis* strain. Data represent the per cent reduction in tritiated thymidine incorporation compared with control of incubation for 48 h. Line at 20% reduction indicates cut-off for further testing of compound. Line at 80% reduction indicates level of activity of library metronidazole.

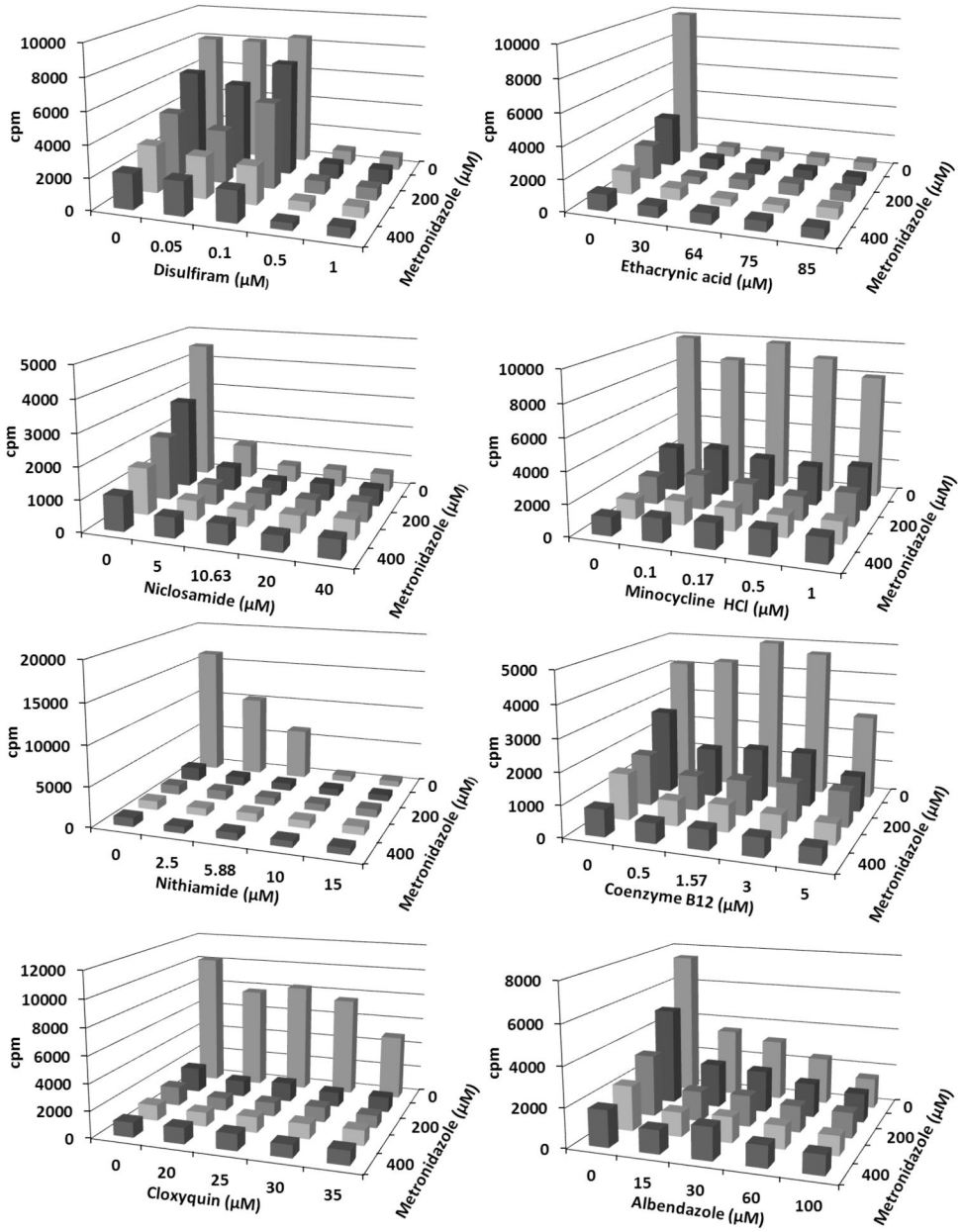

Figure 2.

Drug combination screening under aerobic conditions. Bars represent average counts per minute (cpm) from tritiated thymidine incorporation in triplicate cultures after 48 h incubation in an aerobic environment.

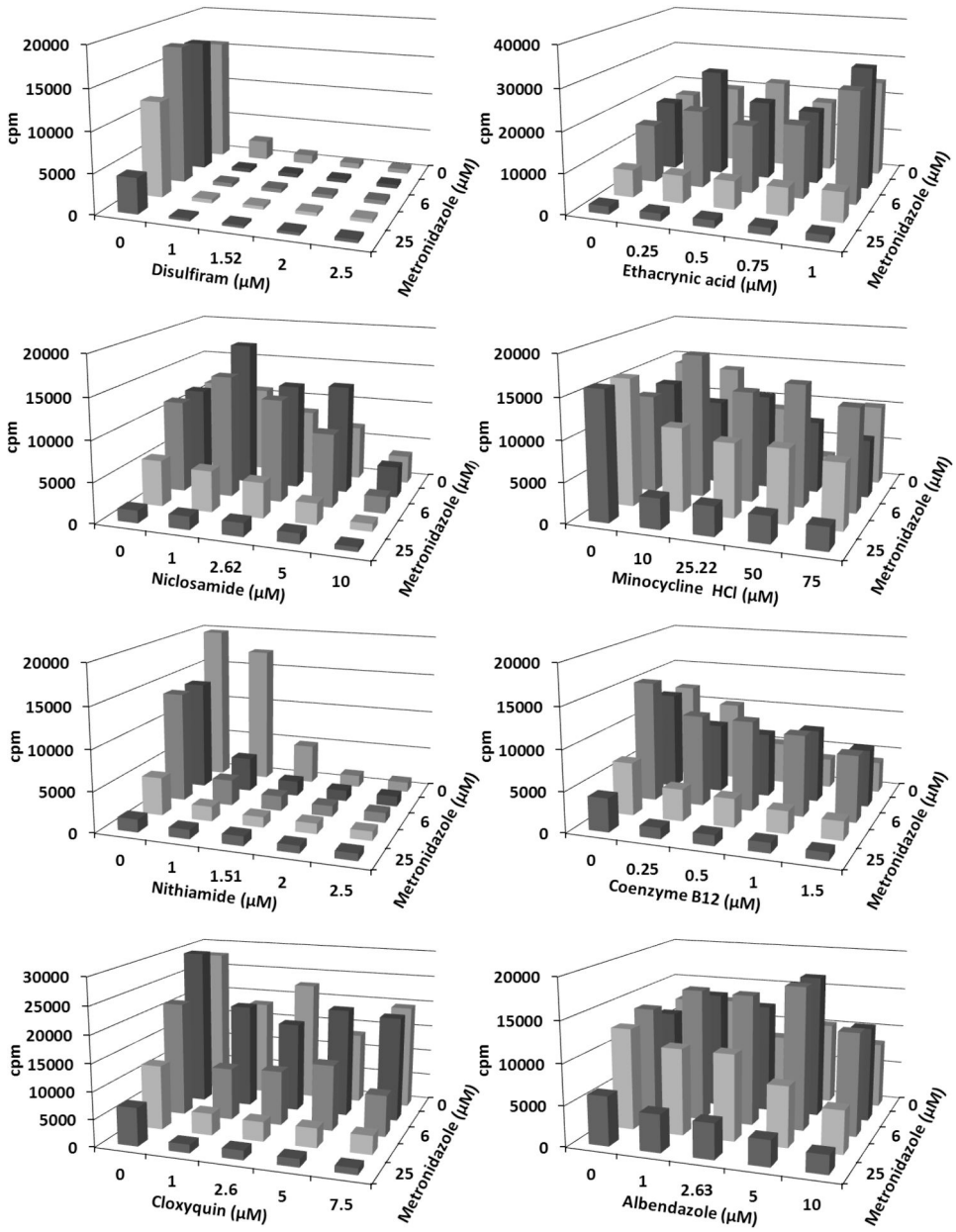

Figure 3.

Drug combination screening under anaerobic conditions. Bars represent average counts per minute (cpm) from tritiated thymidine incorporation in triplicate cultures after 48 h incubation in an anaerobic environment.

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Table 1

IC_{50} values generated in second round of screening

Drug	% Reduction from media (20 μM avg)	IC_{50} 520 aerobic (μM)	IC_{50} 520 anaerobic (μM)	IC_{50} 085 aerobic (μM)	IC_{50} 085 anaerobic (μM)	Third round testing?
Metronidazole	80.9	6.85	0.54	23.90	3.13	Yes
Disulfiram	86.9	0.06	0.09	0.10	1.52	Yes
Niclosamide	78.8	3.85	>40	10.63	2.62	Yes
Nithiamide	58.9	1.33	0.78	5.88	1.51	Yes
Cloxyquin	53.8	>40	2.71	>40	2.62	Yes
Ethacrynic acid	49.3	>40	3.22	>40	0.89	Yes
Minocyline HCL	43.5	>40	>40	0.17	25.22	Yes
Coenzyme B12	42.3	15.63	>40	1.57	0.50	Yes
Albendazole	39.8	18.31	30.83	30.53	2.63	Yes
Thimerosal	86.4	1.06	2.36	0.82	3.64	No[1]
Clofazimine	82.6	0.75	>40	>40	>40	No[2]
Oxyphenbutazone	81.6	1.64	0.89	8.39	>40	No[1,2]
Ornidazole	81.4	3.00	0.78	>40	4.05	No[3]
Ronidazole	81.3	1.07	0.22	3.48	0.36	No[3]
Penfluridol	74.3	39.29	7.38	>40	>40	No[1]
Nitrofurantoin	70.0	22.71	6.71	18.77	>40	No[2,4]
Menadione	68.5	23.95	3.39	>40	0.48	No[1]
Emetine	68.5	23.15	>40	>40	>40	No[1,2]
Oxfendazole	65.1	>40	>40	>40	22.71	No[2,4]
Fenbendazole	62.8	2.95	4.84	12.85	3.06	No[4]
Bismuth subsalicylate	61.1	>40	>40	12.31	<0.01	No[5]
Mebendazole	58.6	9.88	22.78	>40	>40	No[4]
Doxycycline HCL	58.2	23.18	>40	>40	>40	No[2]
Demeclocycline HCL	39.2	<0.01	>40	NC	>40	No[2]

Drug	% Reduction from media (20 μM avg)	IC_{50} 520 aerobic (μM)	IC_{50} 520 anaerobic (μM)	IC_{50} 085 aerobic (μM)	IC_{50} 085 anaerobic (μM)	Third round testing?
Griseofulvin	34.9	>40	>40	>40	4.83	No[2]
Fusidic acid	32.0	37.53	>40	>40	1.34	No[2]
Phenolphthalein	28.9	NC	5.12	3.85	<0.01	No[1]
Pefloxacine mesylate	26.1	NC	0.60	11.89	0.14	No

Drugs were excluded from further screening for reasons of:

(1) toxicity

(2) high IC_{50}S

(3) being a 5-nitroimidazole

(4) poor absorption from the gastrointestinal tract; or

(5) requirement of high dosing for detection in the plasma. If there was no dose-response relationship, the curve did not converge (NC) and no IC_{50} could be calculated. HCL, hydrochloride.