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Drug susceptibility testing in microaerophilic parasites: Cysteine strongly affects the effectivities of metronidazole and auranofin, a novel and promising antimicrobial

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ABSTRACT

The microaerophilic parasites *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Giardia lamblia* annually cause hundreds of millions of human infections which are treated with antiparasitic drugs. Metronidazole is the most often prescribed drug but also other drugs are in use, and novel drugs with improved characteristics are constantly being developed. One of these novel drugs is auranofin, originally an antirheumatic which has been relabelled for the treatment of parasitic infections. Drug effectivity is arguably the most important criterion for its applicability and is commonly assessed in susceptibility assays using *in vitro* cultures of a given pathogen. However, drug susceptibility assays can be strongly affected by certain compounds in the growth media. In the case of microaerophilic parasites, cysteine which is added in large amounts as an antioxidant is an obvious candidate because it is highly reactive and known to modulate the toxicity of metronidazole in several microaerophilic parasites.

In this study, it was attempted to reduce cysteine concentrations as far as possible without affecting parasite viability by performing drug susceptibility assays under strictly anaerobic conditions in an anaerobic cabinet. Indeed, *T. vaginalis* and *E. histolytica* could be grown without any cysteine added and the cysteine concentration necessary to maintain *G. lamblia* could be reduced to 20%. Susceptibilities to metronidazole were found to be clearly reduced in the presence of cysteine. With auranofin the protective effect of cysteine was extreme, providing protection to concentrations up to 100-fold higher as observed in the absence of cysteine. With three other drugs tested, albendazole, furazolidone and nitazoxanide, all in use against *G. lamblia*, the effect of cysteine was less pronounced. Oxygen was found to have a less marked impact on metronidazole and auranofin than cysteine but bovine bile which is standardly used in growth media for *G. lamblia*, displayed a marked synergistic effect with metronidazole.

1. Introduction

The microaerophilic protist parasites *Entamoeba histolytica*, *Trichomonas vaginalis* and *Giardia lamblia* (syn. duodenalis, intestinalis) jointly cause hundreds of millions of infections in man every year (Buret, 2008; Leitsch, 2015a; Morgado et al., 2016). As no vaccine is available for the prevention of either infection, treatment is exclusively based on chemotherapy (Upcroft et al., 2001; Leitsch, 2015b). All three parasites are most commonly treated with metronidazole or other 5-nitroimidazole drugs which are active against most microaerophilic or anaerobic pathogens (Upcroft et al., 2001). In most countries, the 5-nitroimidazoles metronidazole and tinidazole are the only approved treatment options for *T. vaginalis* and *E. histolyitca* infections. *Giardia* infections, however, are also routinely treated with albendazole, a benzimidazole that is mainly in use as an antihelminthic. Other treatment options, such as nitazoxanide or furazolidone (Leitsch, 2015a) do exist but are less often chosen, either due to legal constraints or due to a suboptimal performance of the drug as compared to metronidazole or albendazole. In addition to these drugs, auranofin, originally an antirheumatic but reprofiled for the treatment of parasitic infections, has emerged as a promising future alternative (Andrade and Reed, 2015) for the treatment of all three microaerophilic parasites.

Regardless of the drug concerned, tools are necessary to quantify and, thereby, assess its efficacy, not only prior to its introduction

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but also later when resistance has spread. Normally, drug susceptibilities of protist parasites to drugs are measured in proliferation assays in the presence of the drug, the most informative readout being the IC_{50}. However, susceptibility testing is not performed in the host but *in vitro* and the composition of the growth medium can affect the outcome considerably. Two factors known to greatly affect metronidazole effectivity are oxygen and cysteine. High levels of oxygen are known to impede metronidazole reduction in all three parasites (Lindmark and Müller, 1976; Gillin and Reiner, 1982), a step which is necessary to render metronidazole toxic (Upcroft et al., 2001). However, oxygen by itself is toxic to microaerophilic parasites and they are not exposed to high oxygen concentrations in their niches in the human body or under normal *in vitro* culture conditions. In the laboratory, microaerophilic parasites are grown in sealed culture flasks filled with growth media containing antioxidants such as cysteine and ascorbic acid. This excludes ambient oxygen and enables a gradual removal of oxygen which is initially present in the medium after preparation. Cysteine is normally added in concentrations of approximately 6 mM (*T. vaginalis*, *E. histolytica*) or 12 mM (*G. lamblia*). Importantly, however, cysteine is not only an antioxidant but also a major constituent of these organisms. They use it as a thiol buffer (Krauth-Siegel and Leroux, 2012), quite analogous to glutathione in most aerobes, and as a frequently incorporated residue in proteins. In *G. lamblia*, e.g., the cell surface is coated with cysteine-rich variant surface proteins (VSP), which can contain more than 100 cysteine residues per molecule (Gargantini et al., 2016). Further, cysteine is an important component of iron-sulphur clusters which are integral parts of many essential proteins including pyruvate:ferredoxin oxidoreductase (PFOR) and ferredoxin (Upcroft et al., 2001). Unfortunately, cysteine is quite reactive and does not only react with oxygen and thiols but also with other compounds, e.g. metronidazole (Willson and Searle, 1975; Mason and Josephy, 1985). Indeed, cysteine was repeatedly shown to have a protective effect against metronidazole (Gillin and Reiner, 1982; Leitsch et al., 2007). Since cysteine does not occur in such high concentrations in the host, metronidazole susceptibility assays in standard medium are prone to error and to deliver underestimates of metronidazole's effectivity *in vivo*. Potentially, also other drugs with or without nitro groups in use against microaerophilic parasites could react with cysteine, such as furazolidone, nitazoxanide, and auranofin.

It was the goal of this study to obtain a better understanding of cysteine's impact on the effectivities of commonly used drugs for the treatment of *E. histolytica*, *T. vaginalis*, and *G. lamblia* and on the effectivity of auranofin. To that end, drug susceptibilitiy assays were conducted under strictly anaerobic conditions in an anaerobic cabinet which enabled the reduction of cysteine concentrations because no protection against oxygen was necessary. The resulting IC_{50} values were compared to values obtained through standard culture in tightly sealed culture flasks which infers the presence of oxygen at the start of the experiment and, therefore, the necessity for cysteine as an antioxidant.

2. Materials and methods

2.1. Antimicrobials

Metronidazole, auranofin, furazolidone and auranofin were purchased from Sigma. Nitazoxanide was a generous gift from Prof. Norbert Müller from the Institute of Parasitology, Bern, Switzerland. Stocks of metronidazole and furazolidone were prepared in water. Stocks of auranofin, nitazoxanide and albendazole were prepared in DMSO.

2.2. Parasites

The strains used in this study were established standard strains, i.e. *E. histolytica* HM-1:IMSS (ATCC 30459), *T. vaginalis* C1 (ATCC 30001), and *G. lamblia* WB C6 (ATCC 50803).

2.3. Growth media

All media were based on LS Diamond's media as described (Diamond, 1957; Diamond et al., 1978; Keister, 1983). All growth media were supplemented with 10% serum (horse serum for *T. vaginalis*, adult bovine serum for *E. histolytica*, and fetal calf serum for *G. lamblia*). The compositions of the media used are summarised in Supplementary Table S1. Cysteine was not added during preparation but during subculture and directly prior to susceptibility assays in the amounts indicated. In the case of *E. histolytica*, not cysteine (5.7 mM) but cystine (2 mM) (both from Sigma) was added during subculture or prior to susceptibility assays. Cystine stocks (400 mM) were prepared in water with small concentrations of NaOH, necessary to solubilise cystine. The small amounts of cystine stock added to the growth medium did not alter pH significantly as determined with pH strips. *G. lamblia* medium was prepared either with or without bovine bile salts (0.5 mg/ml) (Sigma). All growth media were sterile-filtered.

2.4. Culture conditions

Parasites were either grown at 37 °C under standard conditions, i.e. in fully filled and sealed culture flasks (Falcon) or tubes (Nunc) in an incubator, or anaerobically in 24 well cell culture plates (Cellstar, Greiner) (2 ml/well) in an anaerobic cabinet ("Bugbox", Baker Ruskinn). The applied gas mixture contained 80% N_2, 10% CO_2, and 10% H_2. *T. vaginalis* and *G. lamblia* cultures were subcultured every second day, *E. histolytica* cultures twice a week.

2.5. Drug susceptibility assays

Growth media were inoculated with 10,000 parasites/ml and incubated in the presence of appropriate amounts of drug for either 48 h (*T. vaginalis* and *G. lamblia*) or for 72 h (*E. histolytica*). After the indicated time, cultures were placed on ice and cells were counted in a Bürker-Türk counting chamber. Susceptibility assays for each drug concentration tested were performed at least three times in duplicate. At least four concentrations were tested per drug and per parasite. Inhibitory concetrations (IC_{50}) were calculated with Grafit 7 software (Erithacus).

2.6. Statistics

In order to validate differential inhibitory concentrations (IC_{50}) as calculated by Grafit 7 software, two-tailed, unpaired Student's t-tests were performed by using the cell numbers determined at the highest joint drug concentration of two given conditions as matrices. For example, the validity of the lower IC_{50} to metronidazole in *T. vaginalis* under anaerobic conditions without cysteine as compared to the IC_{50} in the presence of cysteine was tested using the obtained cell counts under both conditions at a metronidazole concentration of 0.5 μM. This constituted the highest concentration under which *T. vaginalis* was assayed under both conditions. Only in the case of auranofin inhibitory concentrations were so low in the absence of cysteine, or in the presence of low cysteine concentrations respectively, that no overlapping drug concentration range could be established with the same drug in the presence of higher cysteine concentrations.

3. Results

3.1. Establishment of strictly anaerobic culture conditions for E. histolytica, T. vaginalis, and G. lamblia

Initially, it was tested if *E. histolytica*, *T. vaginalis*, and *G. lamblia* could grow in their respective growth media in an anaerobic cabinet with a strictly anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2). Parasites were not incubated in sealed flasks but in 24 well culture plates (2 ml volume/well). The growth medium had been pre-incubated in the anaerobic cabinet for 24 h in order to guarantee full removal of oxygen. Evaporation was minimal due to the high humidity in the cabinet. Growth was readily observed with *T. vaginalis* and *G. lamblia* (Fig. 1B and C) but not with *E. histolytica*. As it had been reported before that *E. histolytica* is sensitive to poorly adjusted cysteine concentrations (Gillin and Diamond, 1981), we tested if *E. histolytica* could grow under strictly anaerobic conditions with a lower concetration of cysteine (3 mM instead of 5.7 mM), no added cysteine, or cysteine substituted with cystine (2 mM), the oxidised form of cysteine. Surprisingly, growth was inhibited in the presence of the lower concentration of cysteine but also in the total absence of cysteine, whereas cystine enabled ample and steady growth. The ability of *E. histolytica* to grow with cystine had been reported earlier (Gillin and Diamond, 1981) but the effect observed was very marked. In fact, sterile-filtered medium with cystine was found to be far superior to standard medium with cysteine as cell counts were more than doubled (Fig. 1A) also under normal conditions, i.e. growth in sealed culture flasks.

Further, it was attempted to decrease the concentrations of added cysteine in the media for *T. vaginalis* and *G. lamblia* as it was hypothesized that the absence of oxygen would reduce the need of cysteine as an antioxidant. In the case of *T. vaginalis*, cysteine could be omitted altogether while still enabling ample growth. Indeed, cell counts were only about 15% lower after two days of culture in the absence of cysteine (Fig. 1B) as compared to culture in the presence of cysteine (5.7 mM). *Giardia lamblia*, however, could not grow in the absence of cysteine or with cysteine replaced by cystine. It was not even possible to reduce the cysteine concentration to 50% of the normal level (i.e. 5.7 mM instead of 11.4 mM) without negatively affecting growth and cell viability, as judged by aberrant cell shapes and smaller numbers of trophozoites attached to the bottom of the wells. It seemed very unlikely that *G. lamblia* would require such high amounts of cysteine (actually, double the concentration that is used for *T. vaginalis* and *E. histolytica* culture) merely for its metabolism, so it was hypothesized that an ingredient in the growth medium other than oxygen necessitated such high levels of cysteine. This component was found to be bovine bile which is routinely added to *G. lamblia* growth media. In the absence

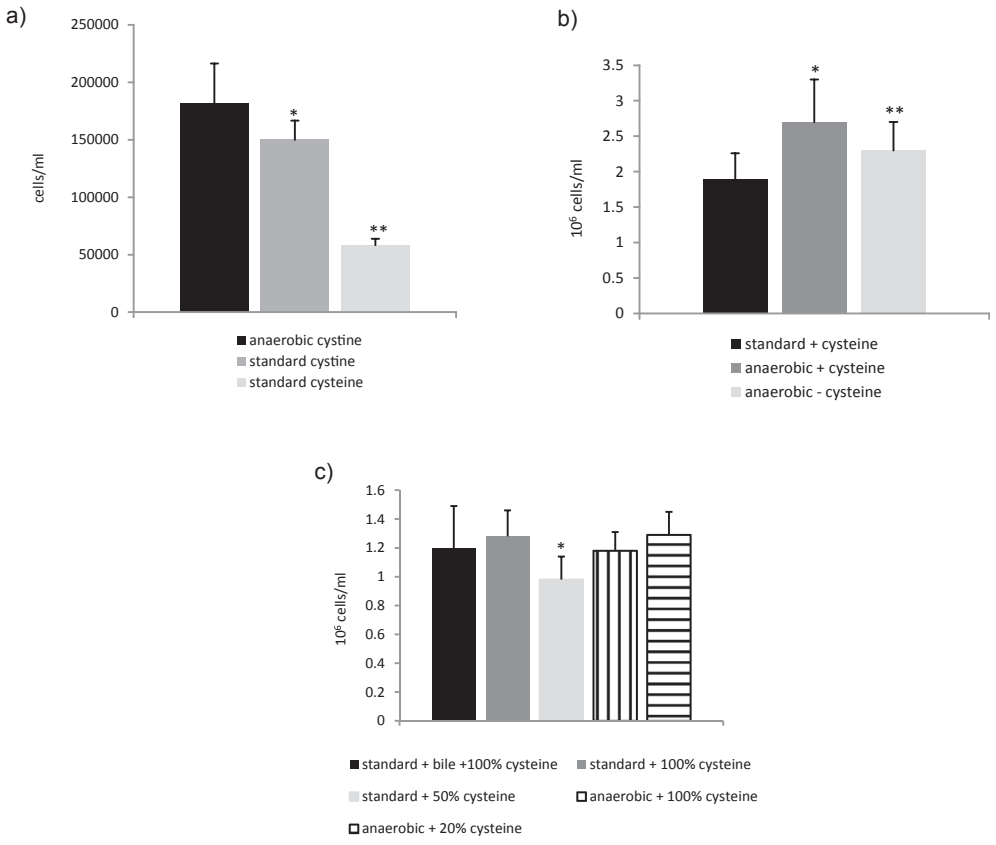


Fig. 1. **A**, growth of *E. histolytica* in all growth media tested as indicated. Cultures were inoculated with 10,000 trophozoites/ml and incubated at 37 °C for 72 h. Afterwards, cell were counted in a Bürker-Türk counting chamber. All measurements were done in duplicate in at least three independent experiments. *, indicates slower growth than under anaerobic conditions with cystine, $p < 0.05$. **, indicates slower growth than under normal conditions with cysteine, $p < 0.003$. **B**, growth of *T. vaginalis* in all growth media tested as indicated. Cultures were inoculated with 10,000 trophozoites/ml and incubated at 37 °C for 48 h. Afterwards, cell were counted in a Bürker-Türk counting chamber. All measurements were done in duplicate in at least three independent experiments. *, indicates faster growth than under normal conditions with cysteine, $p < 0.003$. **, indicates faster growth than under normal conditions with cysteine, $p < 0.05$. **C**, growth of *G. lamblia* in all growth media tested as indicated. Cultures were inoculated with 10,000 trophozoites/ml and incubated at 37 °C for 48 h. Afterwards, cell were counted in a Bürker-Türk counting chamber. All measurements were done in duplicate in at least three independent experiments. *, indicates slower growth than under normal conditions without bile and 100% cysteine, $p < 0.003$.
All *p*-values were determined with unpaired, two-sided Student's t-test.

of bile, cysteine concentrations could be lowered to 20% of the normal level without negatively affecting growth (Fig. 1C) when trophozoites were grown anaerobically. When trophozoites were grown under standard conditions without bile, the cysteine concentration could be reduced to 50% of the normal level (5.7 mM instead of 11.4 mM) with only a mild slow-down of growth observed as compared to standard *G. lamblia* medium containing bile and 11.4 mM cysteine and modified medium without bile and with 11.4 mM cysteine (Fig. 1C).

3.2. Drug susceptibility testing under anaerobic and standard conditions with modified media

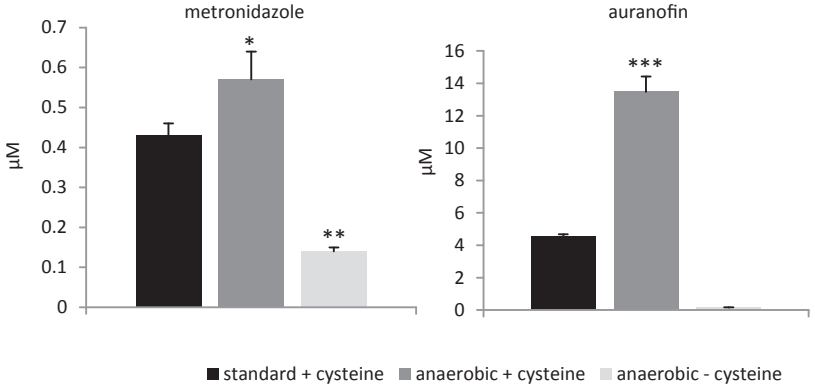
After definition of the growth conditions and the compositions of the growth media used for each parasite had been determined (Fig. 1), drug susceptibility assays were performed. However, before testing the drugs, tolerance to DMSO, used to solubilise nitazoxainde, auranofin, and albendazole, was assessed in cysteine-free media. Indeed, both parasites that could grow anaerobically without cysteine, i.e. *T. vaginalis* and *E. histolytica*, proved to be sensitive to concentrations of DMSO above 0.5% (not shown), whereas in the presence of cysteine even DMSO concentrations above 1% did not negatively affect growth (not shown). In order to rule out any synergistic effect of DMSO with the drugs tested, stocks were prepared in appropriate concentrations so that DMSO concentrations in assays without cysteine never exceeded 0.1%.

All three parasites were assayed for sensitivity to metronidazole and auranofin. Metronidazole is the most widely used drug against anaerobic/microaerophilic pathogens worldwide and auranofin is a novel and promising drug shown to be active against each parasite (Debnath et al., 2012; Tejman-Yarden et al., 2013; Hopper et al., 2016). In addition, *G. lamblia* was assayed for albendazole, nitazoxanide and furazolidone. Albendazole is an established alternative to metronidazole in the treatment of giardiaisis, and the two other drugs are also in use against *G. lamblia*, albeit far less frequently. Importantly, nitazoxanide and furazolidone are also nitro drugs, a nitrothiazolide and a nitrofuran, respectively, and are believed to share several characteristics with metronidazole, e.g. the dependence on reduction of the nitro group for toxicity (Leitsch, 2015b).

Due to the clearly superior growth performance of *E. histolytica* in the presence of cystine, the susceptibilities for metronidazole and auranofin were only determined under anaerobic conditions in growth medium containing cystine. The IC_{50} of metronidazole was determined to be 2.35 ± 0.11 μM, and that of auranofin 0.19 ± 0.05 μM, thus very low as compared to metronidazole. Drug susceptibilities of *T. vaginalis* (Fig. 2) and *G. lamblia* (Fig. 3) were determined in presence of different cysteine concentrations and under anaerobic conditions as well as standard conditions. In addition, *G. lamblia* was assayed for susceptibility for all five drugs under standard conditions with and without bile.

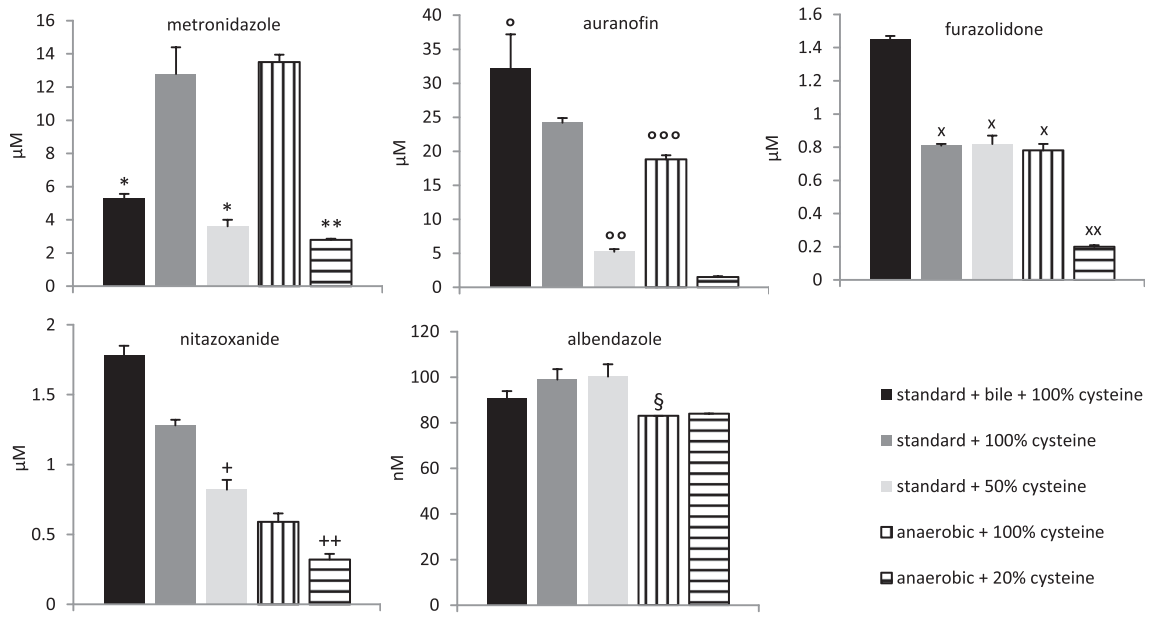
The susceptibility of *T. vaginalis* to metronidazole was practically unaltered under anaerobic conditions as compared to standard conditions (Fig. 2), with a slight but statistically significant increase of tolerance to metronidazole in the total absence of oxygen. If cysteine was omitted, however, *T. vaginalis* became fourfold more sensitive to metronidazole, indicating a strong protective effect of cysteine against metronidazole. This protective effect was even far more pronounced with auranofin (Fig. 2). In the absence of cysteine, the IC_{50} for auranofin was approximately 100-fold lower than observed in the presence of 5.7 mM cysteine. Oxygen, in turn, rendered *T. vaginalis* more sensitive to auranofin, as indicated by a 2.5-fold lower IC50 under standard conditions as compared to anaerobic conditions (both in the presence of cysteine).

Overall, similar observations were made with *G. lamblia* (Fig. 3) but with some surprising differences. The tolerance to metronidazole was greatly enhanced, approximately 2.5-fold, in the absence of bile, indicating a synergistic effect of metronidazole and bile against *G. lamblia*. Total absence of oxygen, however, did not significantly alter the susceptibility to metronidazole. As expected, cysteine had a strongly protective effect, both under anaerobic as well as standard conditions. When *G. lamblia* was assayed for auranofin susceptibility, results partly contrasted with those obtained with *T. vaginalis*. Cysteine had again a very strong protective effect, as indicated by a 12-fold lower IC_{50} in the presence of only 20% cysteine, but now oxygen also had a protective effect instead of an



	standard + cysteine	anaerobic + cysteine	anaerobic - cysteine
metronidazole (μM)	0.43 ± 0.03	0.57 ± 0.07	0.14 ± 0.01
auranofin (μM)	4.51 ± 0.17	13.47 ± 0.95	0.15 ± 0.02

Fig. 2. Susceptibility testing in *T. vaginalis* with metronidazole and auranofin under the conditions indicated. Cultures were inoculated with 10,000 trophozoites/ml and incubated at 37 °C for 48 h. Afterwards, cell were counted in a Bürker-Türk counting chamber and IC_{50} values calculated using Grafit 7 software. All measurements were done in duplicate in at least three independent experiments. The IC_{50} values, including SEM, obtained for both drugs under all conditions tested are given in full in the attached table. *, indicates lowered susceptibility to metronidazole than under normal conditions with cysteine, $p < 0.05$. **, indicates lower susceptibility than under anaerobic conditions with cysteine, $p < 0.003$. ***, indicates lower susceptibility to auranofin than under normal conditions with cysteine, $p < 0.003$. All p-values were determined with unpaired, two-sided Student's t-test.



	standard + bile 100% cysteine	standard 100% cysteine	standard 50% cysteine	anaerobic 100% cysteine	anaerobic 20% cysteine
metronidazole (µM)	5.31 ± 0.25	12.8 ± 1.6	3.62 ± 0.38	13.5 ± 0.45	2.79 ± 0.07
auranofin (µM)	32.2 ± 1.63	24.21 ± 0.68	5.23 ± 0.38	18.79 ± 0.61	1.52 ± 0.11
furazolidone (µM)	1.45 ± 0.02	0.81 ± 0.01	0.82 ± 0.05	0.78 ± 0.04	0.2 ± 0.01
nitazoxanide (µM)	1.78 ± 0.07	1.28 ± 0.04	0.82 ± 0.07	0.59 ± 0.06	0.32 ± 0.04
albendazole (nM)	90.94 ± 2.97	99 ± 4.53	100.3 ± 5.33	83.03 ± 0.06	84.01 ± 0.08

Fig. 3. Susceptibility testing in *G. lamblia* with metronidazole, auranofin, furazolidone, nitazoxanide, and albendazole under the conditions indicated. Cultures were inoculated with 10,000 trophozoites/ml and incubated at 37 °C for 48 h. Afterwards, cell were counted in a Bürker-Türk counting chamber and IC_{50} values were calculated using Grafit 7 software. All measurements were done in duplicate in at least three independent experiments. The IC_{50} values, including SEM, obtained for the five drugs under all conditions tested are given in full in the attached table. *, indicates higher susceptibility to metronidazole than under normal conditions without bile and with 100% cysteine, $p < 0.003$. **, indicates higher susceptibility to metronidazole than under anaerobic condition with 100% cysteine $p < 0.003$. °, indicates lower susceptibility to auranofin than under normal conditions without bile and with 100% cysteine, $p < 0.05$. °°, indicates higher susceptibility to auranofin than under normal conditions without bile and with 100% cysteine, $p < 0.003$. °°°, indicates lower susceptibility than under normal conditions without bile and with 100% cysteine, $p < 0.05$. x, indicates higher susceptibility to furazolidone than under normal conditions with bile and 100% cysteine, $p < 0.05$. xx, indicates lower susceptibility to furazolidone than under anaerobic conditions without bile and with 100% cysteine, $p < 0.003$. +, indicates higher susceptibility to nitazoxanide than under normal conditions without bile and with 100% cysteine, $p < 0.05$. ++, indicates higher susceptibility to nitazoxanide than under anaerobic conditions without bile and with 100% cysteine, $p < 0.003$. §, indicates higher susceptibility to albendazole than under normal conditions without bile and with cysteine, $p < 0.003$. All p-values were determined with unpaired, two-sided Student's t-test.

exacerbating one, as observed with *T. vaginalis*. Interestingly, bile also had a modest but significant protective effect, quite in contrast to the situation seen with metronidazole. Unexpectedly indeed, bile also had a protective effect against the nitro drugs nitazoxanide and furazolidone. This was surprising given the opposed synergistic effect of bile and metronidazole. Oxygen had no effect on the IC_{50} of furazolidone whereas susceptibility to nitazoxainde was about twofold enhanced in the absence of oxygen. Cysteine had a fairly mild protective effect against nitazoxanide under anaerobic and standard conditions, whereas it had a protective effect against furazolidone, i.e. about fourfold, in the absence of oxygen only. The susceptibility to albendazole was very similar under all conditions applied, which had been anticipated due the drug's activity as a β-tubulin inhibitor which is unexpected to be unaffected by oxygen or cysteine levels.

4. Discussion

It was the major aim of this study to develop assay conditions for drug susceptibility testing in microaerophilic parasites which reduce the extent of artificially introduced interferences. Although growth media used for laboratory culture of parasites are unavoidably artificial because they cannot substitute for interactions with the host or the host's bacterial flora, some constituents can exacerbate this discrepancy even further. One such constituent is cysteine which functions as an antioxidant and an important cell constituent of microaerophilic parasites in growth media but is freely available in the human host only in very low quantities. Further, it avidly reacts with compounds other than oxygen or thiols and can therefore distort results of drug assays. A profound effect on metronidazole had already been suggested before (Gillin and Reiner, 1982; Leitsch et al., 2007) but the extent was never gauged. In addition, potential interactions of cysteine with other drugs in use against microaerophilic parasites, including nitro drugs like furazolidone and nitazoxanide, but also the novel antiparasitic drug auranofin, had not been assessed previously.

In order to minimise the requirement for cysteine, or any other thiol for that matter, assays were performed under strict anaerobiosis. Indeed, *E. histolytica* and *T. vaginalis* could grow without any added cysteine, at least in its reduced form. However, only *T.*

vaginalis was fully independent of added cysteine/cystine, i.e. in its reduced or oxidised form, when grown without oxygen. In fact, cysteine is already present in the medium as a component of serum proteins and peptides (Gillin and Diamond, 1981), but the concentration might be too low to enable growth of *G. lamblia* and *E. histolytica*. It must be taken into account that cysteine is a fairly rare amino acid in proteins of aerobes but very frequently incorporated into proteins of microaerophiles and anaerobes. Therefore, a large amount of serum proteins and peptides originating from yeast extract must be processed by microaerophilic parasites to meet their requirements. In addition, sulphur from methionine could be utilised but only *T. vaginalis* and *E. histolytica* can synthesize cysteine (Westrop et al., 2006; Jeelani and Nozaki, 2016), whereas *G. lamblia* cannot.

Indeed, cysteine proved to have a profound protective effect against metronidazole and an even more impressive effect on susceptibility for auranofin (Figs. 2 and 3). These results confirm previous assumptions that cysteine effectively counteracts metronidazole toxicity and, therefore, distorts metronidazole susceptibility assays. They further imply that auranofin avidly reacts with cysteine, and that cysteine in the medium acts as a buffer to protect cysteine residues in the parasites' proteins. Further work, however, is needed to add credibility to this claim. Surprisingly, cysteine was only protective against furazolidone in the absence of oxygen, which was unexpected, given the commonly held notion that nitrofurans generate oxidative stress through redox cycling (Leitsch, 2015b). However, the nitrofurans are a comparably poorly studied drug class and more data are needed to frame reliable hypotheses regarding their mode of action. The protective effect of cysteine against nitazoxanide was significant but milder than observed with metronidazole. Susceptibility to the β-tubulin inhibitor albendazole was totally unaffected by cysteine levels.

Oxygen is another factor possibly affecting drug susceptibility in microaerophilic parasites. Under standard conditions oxygen levels are initially high, especially if the growth medium is sterile-filtered and not autoclaved, but decrease gradually by reacting with cysteine and ascorbic acid in the medium or by being scavenged by the parasites. Under strictly anaerobic conditions, however, oxygen is excluded right from the start, potentially altering the effect of the drugs. Oxygen can, for example, prevent the reduction of metronidazole, but obviously only if constantly present in high concentrations. In the setup used in this study, metronidazole was similarly effective under anaerobic and under standard conditions, in *T. vaginalis* as well as in *G. lamblia*, indicating that oxygen levels are not critical in metronidazole susceptibility assays, unless they are very high. Auranofin, however, was far less effective (2.5-fold) in *T. vaginalis* in the absence of oxygen than under standard conditions. In contrast, *G. lamblia* was more susceptible to auranofin in the absence of oxygen. This discrepancy is unexpected and cannot be explained based on the current status of knowledge on auranofin's mode of action. Further, oxygen had no effect on the susceptibility of *G. lamblia* to furazolidone but a fairly pronounced protective one (twofold) against nitazoxanide, suggesting that oxygen might counteract reduction of nitazoxanide. Finally, *G. lamblia* was slightly more susceptible to albendazole in the absence of oxygen. Albendazole is generally believed to target *G. lamblia* β-tubulin (Morgan et al., 1993) but was also suggested to cause oxidative stress in *G. lamblia* (Martínez-Espinosa et al., 2015). The latter notion, however, is not supported by the results of this study because the antioxidant cysteine had no effect on albendazole's effectivity and oxygen even had a slightly protective effect.

Bovine bile proved to be a third factor influencing drug susceptibilities in *G. lamblia*. Bile was found in this study to greatly increase the need of *G. lamblia* for cysteine in the medium and showed a surprisingly pronounced synergistic effect with metronidazole by rendering *G. lamblia* 2.5-fold more susceptible to metronidazole. Even more surprising, bile had the opposite, i.e. modestly protective effect against auranofin, furazolidone, and nitazoxanide. If taking into consideration that cysteine, oxygen, and bile had strongly differing and often even opposed effects on the susceptibilities to metronidazole, furazolidone, and nitazoxanide, it seems likely that these drugs have, at least in *G. lamblia*, different modes of action despite all being nitro drugs.

To conclude, this study demonstrates that growth media components need to be critically assessed when the effectivities of drugs against microaerophilic parasites are determined. The huge differences between the inhibitory concentrations of auranofin in dependence of cysteine are a perfect illustration for this. However, further studies are warranted as also other media components such ascorbic acid could potentially affect the effectivities of drugs that are in use against microaerophilic parasites.

Conflict of interest

I declare that there is no conflict of interests of this manuscript with my position as a research associate at the Medical University of Vienna. There are no commercial interests attached to this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijpddr.2017.09.001.

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