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

David Leitsch

Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria

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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

E-mail address: david.leitsch@meduniwien.ac.at.

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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.