J Antimicrob Chemother 2010; **65**: 512–519
doi:10.1093/jac/dkp490 Advance publication 19 January 2010

Journal of Antimicrobial Chemotherapy

Application of an *in vitro* drug screening assay based on the release of phosphoglucose isomerase to determine the structure–activity relationship of thiazolides against *Echinococcus multilocularis* metacestodes

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Received 19 September 2009; returned 20 November 2009; revised 9 December 2009; accepted 10 December 2009

Objectives: The disease alveolar echinococcosis (AE), caused by the larval stage of the cestode *Echinococcus multilocularis*, is fatal if treatment is unsuccessful. Current treatment options are, at best, parasitostatic, and involve taking benzimidazoles (albendazole, mebendazole) for the whole of a patient's life. In conjunction with the recent development of optimized procedures for *E. multilocularis* metacestode cultivation, we aimed to develop a rapid and reliable drug screening test, which enables efficient screening of a large number of compounds in a relatively short time frame.

Methods: Metacestodes were treated *in vitro* with albendazole, the nitro-thiazole nitazoxanide and 29 nitazoxanide derivatives. The resulting leakage of phosphoglucose isomerase (PGI) activity into the medium supernatant was measured and provided an indication of compound efficacy.

Results: We show that upon *in vitro* culture of *E. multilocularis* metacestodes in the presence of active drugs such as albendazole, the nitro-thiazole nitazoxanide and 30 different nitazoxanide derivatives, the activity of PGI in culture supernatants increased. The increase in PGI activity correlated with the progressive degeneration and destruction of metacestode tissue in a time- and concentration-dependent manner, which allowed us to perform a structure–activity relationship analysis on the thiazolide compounds used in this study.

Conclusions: The assay presented here is inexpensive, rapid, can be used in 24- and 96-well formats and will serve as an ideal tool for first-round *in vitro* tests on the efficacy of large numbers of antiparasitic compounds.

Keywords: alveolar echinococcosis, *in vitro* culture, chemotherapy, medium-throughput drug screening

Introduction

Echinococcus multilocularis is a cestode parasite, the metacestode (larval) stage of which causes alveolar echinococcosis (AE), a mainly hepatic disease in humans. AE is fatal if not treated appropriately. The benzimidazoles albendazole and mebendazole are presently used for chemotherapeutic treatment alone, or prior to and post-surgery. However, in AE these benzimidazoles do not appear to be parasiticidal *in vivo*. In addition, failures in drug treatments as well as the occurrence of side effects have been reported, leading to discontinuation of treatment or to progressive disease. Therefore, new drugs are needed to cure AE.[1,2]

One approach to identify novel and/or chemotherapeutically interesting compounds has been to perform whole organism screening employing *in vitro* cultured metacestodes.[1] The more recent advances in the *in vitro* culture of *E. multilocularis* metacestodes[3] have been instrumental in this respect and have allowed the culture and maintenance of metacestodes in the laboratory on a much larger scale than described previously.[4] The effects of *in vitro* drug treatment can be assessed mainly by visual inspection of morphological alterations and by light microscopy. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have also been used to investigate tissue damage in more detail,[5–8] and nuclear magnetic resonance (NMR) spectroscopy has been applied to investigate metabolic changes imposed upon the parasites by drug treatments.[5] Others have attempted to assess parasite viability and growth by the quantification of the expression of molecular marker genes such as 14-3-3 and II/3-10.[9] However, visual inspection relies on subjective observations and requires experienced personnel, and other techniques such as SEM, TEM, NMR

spectroscopy and real-time RT–PCR are intrinsically time consuming and expensive. Nevertheless, these approaches have led to the identification of compounds with reasonable activities in the micromolar range *in vitro*, including potentially promising drugs such as the thiazolide nitazoxanide[8,10] originally developed as an antihelmintic,[11–13] genistein derivatives,[6] 2-methoxyestradiol, artesunate and dihydroartemisinin,[7,14] and p38 mitogen-acivated protein (MAP) kinase inhibitors.[15] While combinations of some of these drugs with albendazole have led to slightly improved treatment efficacy in experimentally infected mice, none of these compounds has exhibited improved activities compared with albendazole (reviewed in Hemphill and Müller[1] and Hemphill *et al.*[2]). Thus, many more drugs and/or compound classes should be investigated and there is an urgent need for a reliable, but also easy-to-handle and rapid *in vitro* drug screening assay for the identification of chemotherapeutically interesting compounds *in vitro*.

The rationale of our study is that such an assay could be based on the detection of vesicle fluid components, which are released by the metacestodes once they are structurally impaired by drug treatment. *E. multilocularis* metacestodes are basically fluid-filled vesicles that are surrounded by an outer, acellular laminated layer, and the inner surface of this laminated layer is delineated by the actual parasite tissue, the germinal layer. The germinal layer secretes the components of the laminated layer towards the metacestode periphery into the laminated layer and also secretes and/or releases metabolites into the vesicle fluid. One enzyme that represents an intrinsic component of vesicle fluid, and which is also found on the laminated layer, is alkaline phosphatase (AP).[16,17] The detection of AP activity in medium supernatants of drug-treated metacestode cultures has been proposed as a method to screen for active drugs.[18] AP activity has indeed been found to be increased in culture supernatants treated with nitazoxanide,[8] 2-methoxyestradiol and artemisinin derivatives,[7,14] but the sensitivity of this assay is not always satisfactory.[15] Thus, detection of AP activity does not represent a reliable measure for *in vitro* drug screening.

Here, we present an assay, which is based on the detection of phosphoglucose isomerase (PGI) activity, thus of a key enzyme of glycolysis. Interestingly, PGI activity was detected not only in cells, but also in the vesicle fluid of intact metacestodes, and not in medium supernatants of intact vesicles. Upon mechanical damage or *in vitro* drug treatment, metacestodes release PGI into the medium supernatant and the corresponding activity can be easily measured. In addition, we present the screening results obtained with a series of 30 thiazolides in order to show the potential of this screen for high-throughput assays.

Materials and methods

Media and biochemicals

If not stated otherwise, all culture media were purchased from Gibco-BRL (Zurich, Switzerland) and biochemical reagents were from Sigma (St Louis, MO, USA).

In vitro cultivation of E. multilocularis metacestodes

Culture of *E. multilocularis* (isolate H95) was carried out as described previously.[3,19] In short, metacestodes dissected from experimentally infected BALB/c mice were crushed by pressing through a metal tea strainer. The metacestodes were incubated in antibiotic solution [20 mg/L levofloxacin (Aventis, Meyrin, Switzerland) and 20 mg/L ciprofloxacin (Bayer, Zürich, Switzerland) in PBS] overnight. The next day, 5×10^6 rat hepatocytes/mL (kindly provided by Klaus Brehm, Institute for Hygiene and Microbiology, University of Würzburg) were added to 1 mL of metacestodes, and medium [Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), 100 U/mL penicillin G, 100 mg/L streptomycin sulphate, 10 mg/L tetracycline-HCl and 20 mg/L levofloxacin] was added to 50 mL. These co-cultures were incubated in culture flasks at 37°C and 5% CO_2, with medium changes once a week. Splitting of cultures was carried out when a total metacestode volume of 15 mL was exceeded. Metacestodes were used for experimental procedures when they reached diameters of 2–4 mm.

In vitro drug treatment of E. multilocularis metacestodes

E. multilocularis metacestodes were collected at 1–2 months of culture and were washed three times in PBS (Fluka Chemie, Buchs, Switzerland) in order to remove medium, debris and broken vesicles. Medium without phenol red (RPMI, 100 U/mL penicillin G and 100 mg/L streptomycin sulphate) was added to the same volume of vesicles and distributed to 24-well plates (Greiner Bio-One, Frickenhausen, Germany; 2 mL per well, ~25–35 vesicles) or 96-well plates (Greiner Bio-One; 200 μL per well, ~4–6 vesicles), respectively. Nitazoxanide and other thiazolides were prepared as stocks of 10 g/L in DMSO. Pre-dilutions to 500 mg/L were prepared in medium and added to the metacestodes as indicated. As a negative control, the corresponding dilution of DMSO was used. As a positive control, in some experiments, albendazole was applied. After the indicated periods of drug treatments *in vitro*, damage was observed by eye and classed into the following groups: 0, <10%; 1, 10%–50%; 2, 50%–90%; and 3, >90% of damaged metacestodes. Samples of medium supernatants (200 μL out of wells from 24-well plates and 100 μL out of wells from 96-well plates, respectively) were collected and stored at −20°C until further measurement of PGI activity.

PGI assay

Damage of vesicles was measured indirectly by detecting the release of PGI, a prominent component of vesicle fluid. PGI activity was measured by employing an indirect assay described by Gracy and Tilley.[20] The assay was performed in 96-well microtitre plates (Greiner Bio-One). Per well, 95 μL of assay buffer [100 mM Tris-HCl (pH 7.6), 0.5 mM NAD (Fluka), 2 mM EDTA (Merck) and 1 U of glucose-6-phosphate dehydrogenase] was mixed with 20 μL of each sample (medium supernatants, see above). Measurements were performed in triplicate. As an assay inhibition control, 5 mg/L of each compound was added to a reaction mixture including metacestode fluid. The reaction was started by addition of fructose-6-phosphate (Fluka) to a concentration of 1 mM. NADH formation was measured by reading the absorbance at 340 nm (A_{340}) at various timepoints (0–20 min) on a 96-well plate reader (MRXII, Dynex, Chantilly, VA, USA). Enzyme blanks (without addition of substrate) and substrate blanks (without enzyme) were also included. Absorbance values of these blanks were subtracted from the enzyme reaction values afterwards. Enzyme activities were calculated from the corresponding linear regression parameters ($\Delta A_{340}/\Delta t$) and expressed as μmol substrate transformed per s (μkat). For the determination of specific PGI activity when mechanically destroyed vesicles were compared with drug-damaged vesicles, the protein content of the supernatant was determined.

SEM and TEM

For some drug treatments, metacestodes were processed for SEM and TEM as described by Hemphill and Croft.[21] Briefly, metacestodes were

fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2) for 2 h at room temperature, followed by post-fixation in 2% OsO_4 in 100 mM sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. Then, samples were washed in distilled water and treated with 1% uranyl acetate for 30 min. Subsequently, the specimens were extensively washed in distilled water and dehydrated by sequential incubations in increasing concentrations of ethanol. For SEM analysis, dehydrated specimens were finally immersed in hexamethyl-disilazane and air dried under a fume hood. They were then sputter-coated with gold and inspected on a JEOL 840 scanning electron microscope operating at 25 kV. For TEM, the specimens were fixed and dehydrated as described above and subsequently embedded in Epon 812 resin (Fluka). Polymerization of the resin was carried out at 65°C overnight. Sections were cut on a Reichert and Jung ultramicrotome and were loaded onto 300-mesh copper grids (Plano GmbH, Marburg, Germany). Staining with uranyl acetate and lead citrate was performed as previously described.[21]

Statistics

Linear regression, correlation analysis (Pearson, Spearman), analysis of variance (ANOVA) and *t*-tests were performed using the software package R version 2.8.0.[22]

Results

In preliminary experiments, PGI activity was identified in vesicle fluid of *E. multilocularis* metacestodes. In order to see whether the amount of PGI activity depended on metacestode size—and thus age—metacestodes were kept in culture for different time periods, divided into four size classes (size 1, <2 mm; size 2, 2–4 mm; size 3, 4–6 mm; and size 4, >6 mm) and identical volumes of vesicle fluid were assessed for PGI activity. PGI activity was even detectable in fluids of the smallest metacestodes and significantly correlated to size ($P<0.05$, Figure 1). For further experiments, metacestodes of 2–4 mm in diameter were used.

In order to investigate whether drug-induced structural damage of metacestodes leads to release of vesicle fluid components into the medium supernatant, we treated metacestodes with nitazoxanide (5 mg/L for 4 days). This resulted in distinct alterations within the metacestode tissue as assessed by TEM, including loss of microtriches, accumulation of lipid droplets and progressive disintegration of tissue organization (Figure 2a and b). The release of PGI activity into the medium supernatant was measured and compared with the activity obtained from mechanically lysed metacestodes and with DMSO as a solvent control (Figure 2c). Whereas PGI release from DMSO-treated vesicles was barely detectable, specific PGI activity from nitazoxanide-treated and mechanically destroyed vesicles was nearly 50 times higher. Both values were significantly different from the DMSO value, but not from each other (Figure 2c). Thus, release of PGI activity correlated with drug-induced metacestode tissue damage.

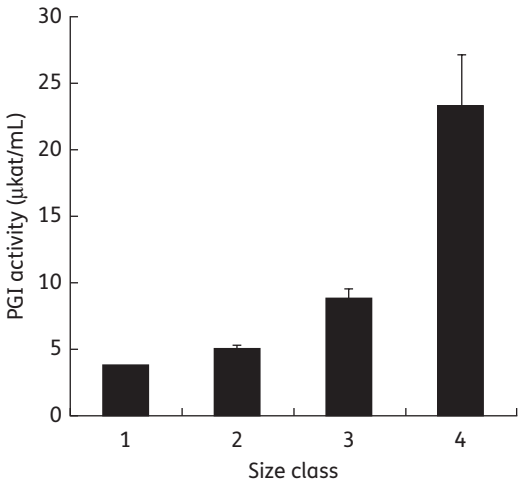

Figure 1. PGI activity in vesicle fluid of *E. multilocularis* metacestodes of various size classes, namely: <2 mm (1); 2–4 mm (2); 4–6 mm (3); and >6 mm (4). Experiments were performed in quadruplicate. Means ± SE are given.

Time course experiments were performed. Metacestodes were incubated with albendazole (5 mg/L), nitazoxanide (5 mg/L) or DMSO (as a solvent control) for 10 days. At various timepoints, PGI activity in the medium supernatants was measured. DMSO did not lead to an increase of PGI activity in medium supernatants. In the case of nitazoxanide, PGI activity increased from 3 days onwards, reaching a plateau between day 5 and day 10 of drug treatment. In the case of albendazole, 5 days of treatment did not lead to a substantial PGI release, while after 10 days PGI activity levels in albendazole-treated cultures were similar to those obtained with nitazoxanide treatment (Figure 3). The increase in PGI activities was clearly linked to the morphological damage caused by these two drugs, while in the DMSO treatment controls alterations of metacestode morphology were not observed (data not shown).

In order to assess this in more detail, and to show that the PGI assay could be a useful screening tool for assessing the efficacy of drugs *in vitro*, metacestodes were incubated with a set of 30 thiazolides with modifications on the thiazole and/or the salicylic acid moiety (see Figure 4 and Table 1). PGI activity was measured in parallel to a visual classification of metacestode damage assessed by eye using a scoring system as indicated (Table 1). First, none of the drugs investigated here interfered in the functional activity of PGI (data not shown). Secondly, there was a highly significant correlation between the degree of morphological damage and the PGI activity released into the supernatant (rank correlation according to Spearman, $\rho=0.917$, $P=1.5\times10^{-13}$). Interestingly, not only nitro compounds such as nitazoxanide, RM4802, RM4805 and RM4807, but also halogenated molecules such as RM4827, RM4830, RM4831, RM4835 and RM4838-RM4841 exhibited enhanced PGI activity values and morphologically detectable damage already after 5 days of treatment. Treatments with other halogenated thiazolides such as RM4804, RM4806, RM4819, RM4820, RM4822, RM4834, RM4836, RM4842 and RM4843 showed evidence of antiparasitic activities only after 10 days of treatment (data not shown).

In order to analyse structure–function relationships in more detail, the non-nitro-thiazolides were grouped with respect to the halogenations of the thiazole and the salicyl moieties of the thiazolide molecule, and the corresponding PGI activities were analysed by ANOVA (Figure 5). The analysis showed that halogenations not only of the thiazole moiety ($P=0.0056$), but also of the salicyl moiety ($P=0.0061$) were significantly correlated with metacestodicidal activity (Figure 5).

In order to investigate the most highly active thiazolides in more detail, the nitro-thiazolide RM4807 and the tribromo-thiazolide RM4841 were selected to perform a concentration

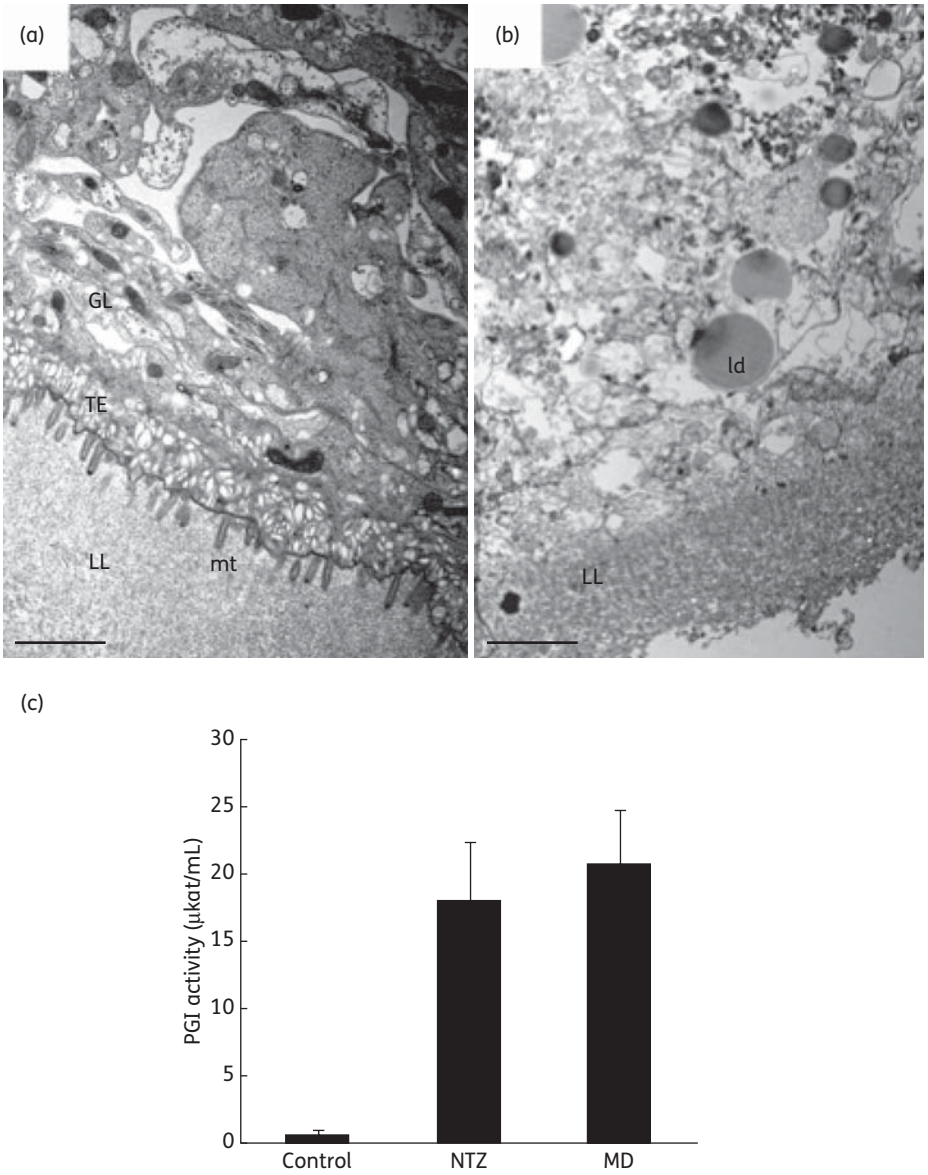

Figure 2. PGI activity correlates with drug-induced metacestode damage. (a) TEM of a control DMSO-treated metacestode. (b) Metacestode treated with nitazoxanide (5 mg/L) for 10 days. Note the significant damage induced upon nitazoxanide treatment. The bar in (a) = 1.6 μm and the bar in (b) = 1.4 μm. LL, laminated layer; TE, tegument; GL, germinal layer; mt, microtriches; ld, lipid droplets. (c) PGI activity in the medium of metacestodes grown for 10 days in the presence of nitazoxanide (NTZ; 5 mg/L), in the medium of metacestodes grown for 10 days in the presence of the corresponding amount of DMSO as a solvent control (Control) or in the medium of control metacestodes after mechanical destruction (MD). Means ± SE are given.

series experiment. Metacestodes were cultured in 24-well plates and treated with increasing concentrations (0.1–10 mg/L) of these compounds for 5 days (Figure 6). At concentrations of 5 and 10 mg/L, PGI activity levels were high for both compounds. At a concentration <5 mg/L, the nitro-thiazolide RM4807 was no longer effective, and PGI activity was not detectable at a drug concentration of 1 mg/L (Figure 6). On the other hand, the tribromo-thiazolide RM4841 did induce vesicle fluid leakage at concentrations as low as 0.5 mg/L. Given the different molecular weights of RM4807 (281) and RM4841 (416), the concentrations with half maximal activity, as calculated after logit-log (RM4807; $P = 0.008$) or log transformation (RM4841; $P = 0.003$) followed by regression analysis, were 10.6 (standard error 3.9) μM and 2.4 (1.2) μM, respectively. In the same assay, albendazole had no effect on PGI release after 5 days. After 10 days, metacestodes treated with albendazole were damaged, and released PGI with a half-maximal activity at 5.6 (0.1) mg/L corresponding to 21.1 (0.4) μM as calculated by linear regression analysis from the data ($P = 0.007$).

In order to investigate whether the screening assay presented above could be down-scaled to a 96-well format, thus allowing high- or at least medium-throughput screening

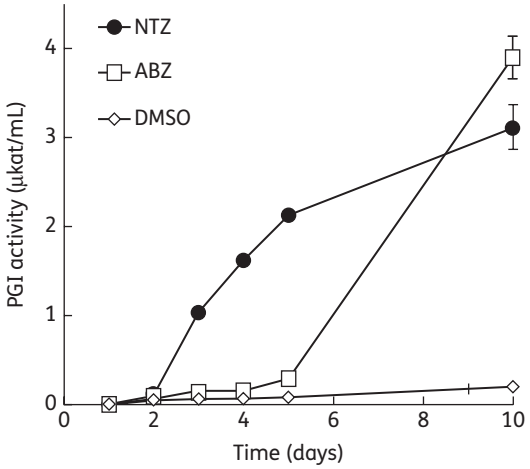

Figure 3. Release of PGI activity into the growth medium of *E. multilocularis* metacestodes is time dependent. Metacestodes were incubated for up to 10 days in the presence of nitazoxanide (NTZ; 5 mg/L), albendazole (ABZ; 5 mg/L) or the corresponding amount of DMSO as a solvent control. Means ± SE are given.

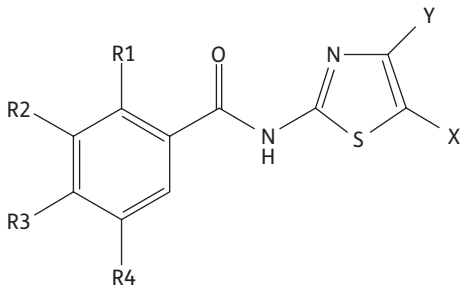

Figure 4. Core structure of the thiazolides. R1–R4, X and Y of the compounds used in this study are indicated in Table 1.

for metacestodicidal activities, 2–4 mm size class metacestodes were transferred to a 24-well plate and to a 96-well plate in the presence of nitazoxanide (5 mg/L) as described. After 5 days, PGI activity in the supernatants of 7 randomly selected wells of the 24-well plate and of 12 randomly selected wells of the 96-well plate was measured, the mean values and the variances were determined and the coefficient of variation was calculated. The mean values in both formats were not significantly different ($P > 0.05$) and the coefficients of variation were similar, namely ~0.25. Thus, this screen can easily be adapted to 96-well formats suitable for high-throughput screenings (Table 2). When the screen described above was repeated in 96-well plates, the results obtained were correlated to the results obtained in 24-well plates with a high significance (regression according to Pearson, $P = 3.3 \times 10^{-8}$).

Discussion

PGI activity is a marker that indicates drug-induced metacestode damage in vitro

Previous studies have shown that *in vitro* drug treatment of *E. multilocularis* metacestodes could represent a valuable tool for the investigation of chemotherapeutically interesting compounds with respect to their antiparasitic activities including drug uptake studies by HPLC, NMR studies of metabolic alterations induced through drug treatment and the investigation of ultrastructural changes imposed through drugs.[4–8,14,15] However, when it comes to performing drug testing assays with a multitude of chemotherapeutically interesting reagents, these techniques suffer from their complexity or require large amounts of parasite material. TEM is helpful but time consuming, and only a small portion of the metacestode can be investigated using the electron microscope. Thus, a practical and reliable assay for monitoring parasite damage is required for monitoring the effects of drugs on parasite structural integrity and viability.

Here, we present such an assay that allows assessment of the antiparasitic efficacy of chemotherapeutically interesting compounds against *E. multilocularis* metacestodes. PGI is an abundant component of the metacestode vesicle fluid, but it is not secreted into the medium by intact parasites. Once damage occurs and the parasites suffer from structural impairment, metacestodes lose turgor, leakage of vesicle fluid into the medium supernatant occurs and PGI activity is thus readily detected. The levels of enzymatic activity upon drug treatment correlate well with the morphological alterations observed visually (inspection by eye, SEM and TEM) and activity levels rose in a dose- and time-dependent manner. This assay can be performed in 24- and 96-well plates, yielding essentially identical results, and is thus suitable for reliably screening larger numbers of compounds for metacestodicidal activity. Moreover, this assay can be used for the investigation of time- and concentration-dependent drug effects in a more quantifiable and standardizable way than methods based on morphological investigations.

Structure–activity relationship of thiazolides

With the PGI assay described here, we have investigated the structure–activity relationship of a group of anti-infective thiazolides, a drug family with activities against a broad spectrum of parasites and tumour cells.[13,23–28] For helminths, as well as for anaerobic bacteria and protozoa, the mode of action of nitazoxanide, the mother compound of the family,[12] has been proposed to be related to the presence of the thiazole-associated nitro group (see, for example, Hemphill *et al.*[27]), but detailed investigations have been lacking. For anaerobic microorganisms such as *Campylobacter jejuni*, *Helicobacter pylori* or *Giardia lamblia*, the mode of action is thought to be linked to the inhibition of pyruvate ferredoxin oxidoreductase (PFOR) activity through interference with its co-factor thiamine pyrophosphate.[29] Moreover, Pankuch and Appelbaum[30] compared the activities of nitazoxanide and tizoxanide with five other thiazolides against anaerobic bacteria and showed that only compounds that carried a nitro-thiazole were active *in vitro*, while bromo derivatives were not. Other studies in *G. lamblia* also demonstrated that only nitro-thiazolides exhibited profound antigiardial activity *in vitro*,[31] and identified a novel nitroreductase (GLNR1) as a potential target.[32] PFOR and NR are, however, lacking in *E. multilocularis*. In contrast, investigations on intracellular parasites such as *Neospora caninum* showed that the activity of nitazoxanide was independent of the nitro-thiazole group, and bromo compounds were also effective against this parasite *in vitro*.[23–25] In addition, nitazoxanide and several bromo derivatives affect the

Table 1. Chemical structures of thiazolides used in this study

Compound	X	Y	R1	R2	R3	R4	Damage	PGI
nitazoxanide	NO_2	H	$OCOCH_3$	H	H	H	1	1.6
RM4802	NO_2	H	$OCOCH_3$	CH_3	H	H	2	2.8
RM4803	Br	H	$OCOCH_3$	CH_3	H	H	0	0.1
RM4804	Cl	H	$OCOCH_3$	CH_3	H	H	1	0.6
RM4805	NO_2	H	OH	OCH_3	H	H	2	1.9
RM4806	Br	H	OH	OCH_3	H	H	1	0.2
RM4807	NO_2	H	OH	H	H	CH_3	2	1.9
RM4808	-SO_2-phenyl-NO_2	H	$OCOCH_3$	H	H	H	0	0.1
RM4819	Br	H	OH	CH_3	H	H	1	1.1
RM4820	Br	H	$OCOCH_3$	H	H	H	1	0.7
RM4821	Br	H	$OCOCH_3$	H	H	CH_3	0	0.1
RM4822	Br	H	$OCOCH_3$	H	CH_3	H	1	0.9
RM4824	H	$CO_2CH_2CH_3$	$OCOCH_3$	H	H	H	0	0.1
RM4827	Br	H	OH	Cl	H	H	2	2.2
RM4828	$NHCOCH_3$	H	$OCOCH_3$	H	H	H	0	0.3
RM4829	H	Ph	OH	CH3	H	H	0	0.2
RM4830	H	Ph	OH	Cl	H	H	2	1.5
RM4831	Br	H	OH	H	F	H	2	1.5
RM4833	Br	H	OH	H	OCH_3	H	2	0.7
RM4834	Br	H	OH	H	H	OCH_3	0	0.1
RM4835	Cl	H	OH	H	OCH_3	H	3	2.2
RM4836	Cl	H	OH	H	H	OCH_3	0	0.1
RM4837	H	CH_2CH_2OH	OH	H	H	H	0	0.1
RM4838	Br	H	OH	H	Cl	H	2	2.1
RM4839	Br	H	OH	H	H	Cl	2	1.4
RM4840	Br	H	OH	H	H	Br	2	1.9
RM4841	Br	H	OH	Br	H	Br	3	2.4
RM4842	H	Ph	OH	H	H	Br	1	0.3
RM4843	H	Ph	OH	H	H	H	0	0.1
RM4844	$NHCOCH_3$	H	OH	H	H	H	0	0.2
RM4845	$NHCOCH_3$	$OCOCH_3$	$OCOCH_3$	H	H	H	0	0.1

The thiazolide core structure is shown in Figure 4. R1–R4 are substituents of the salicyl moiety and X and Y are substituents of the thiazole moiety. Effects on *E. multilocularis* metacestodes incubated with the compounds (at 5 mg/L) in 24-well plates for 5 days are given as visual damage, namely 0, <10%; 1, 10%–50%; 2, 50%–90%; and 3, >90% of damaged metacestodes, and activity of PGI released into the medium (μkat/mL).

proliferation of normal human cells such as foreskin fibroblasts *in vitro*, and are toxic towards colon cancer cells.[28]

Our screen has revealed that not only nitro-thiazolides, but also halogenated thiazolides, are effective against metacestodes. The presence of halogens, both on the thiazole and on the salicyl moieties of the thiazolide backbone is significantly correlated with high activity. The most promising compound for further investigations on antiechinococcocal activity is RM4841, a tribromo-thiazolide. When compared with albendazole, RM4841 affects metacestode integrity much more rapidly and at a lower concentration range.

Is the mode of action of thiazolides in E. multilocularis similar to that postulated for tumour cells?

Not only nitro- but also bromo-thiazolides inhibit the proliferation of human Caco2 cells, whereas thiazolides with other substitutions have no effects. In a pull-down study, human glutathione-*S*-transferase P1 (hGSTP1) has been identified as a thiazolide-binding protein, and the activity of recombinant GSTP1 is inhibited by thiazolides that also inhibit cellular proliferation. Moreover, knock-down of hGSTP1 in 293T cells correlated with lower sensitivity, and overexpression of hGSTP1 led to higher sensitivity of the cells to thiazolides.[28] In *E. multilocularis*, a GST has been characterized that shares catalytic properties with hGSTP1,[33] and, based on the present results, *Echinococcus* GST as a potential target should be investigated. In any case, these findings stress the similarities between the intrinsic biological properties of *E. multilocularis* metacestodes and cancer cells, as proposed by others.[34] Thus, in principle, a wide range of anticancer drugs could be tested for metacestodicidal activity. The fact that the PGI assay can also be performed in a 96-well format suggests that this could be done at a significantly increased rate.

AE is not regarded as one of the major parasitic diseases, and the number of patients acquiring this infection is relatively low. However, the consequences for the individual patient are extremely severe and the disease leads to death in those patients

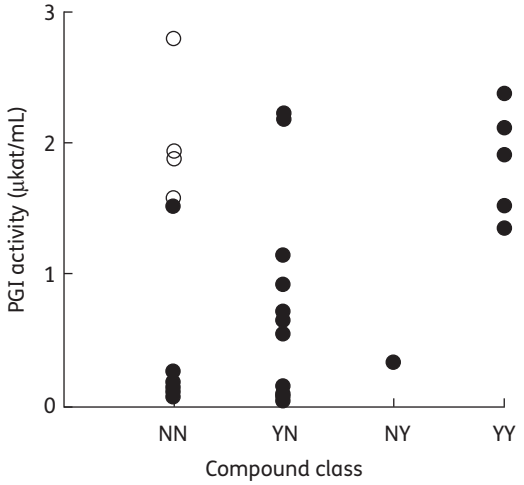

Figure 5. Release of PGI activity into the growth medium of *E. multilocularis* metacestodes treated with thiazolides (5 mg/L; 5 days) as described in Table 1. Thiazolides were classed with respect to the presence or absence of halogen groups. YY, halogens on both the thiazole moiety and the salicyl moiety; YN, halogens only on the thiazole moiety; NY, halogens only on the salicyl moiety (only one compound); NN, no halogens, nitro compounds within this group are depicted with open circles.

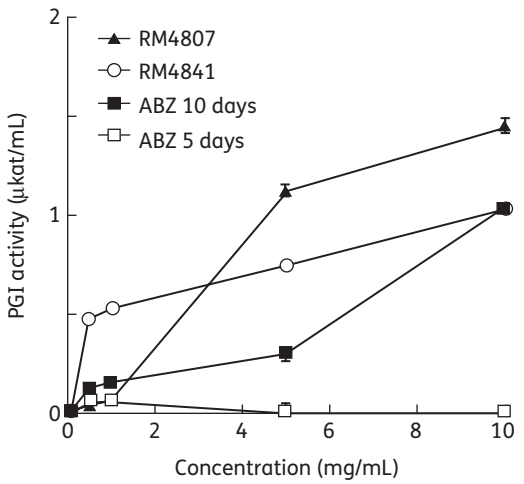

Figure 6. Release of PGI activity into the growth medium of *E. multilocularis* metacestodes depends on drug concentration. Metacestodes were incubated for 5 days in the presence of the nitro-thiazolide RM4807, the tribromo-thiazolide RM4841, albendazole (ABZ; 5 and 10 days; 0–10 mg/L) or the corresponding amount of DMSO as a solvent control (values subtracted). Activities were measured in triplicate. Means ± SE are given.

for whom chemotherapy is unsuccessful in halting parasite growth. In addition, any drug that would improve the chemotherapeutic treatment of AE could also produce a significant improvement in cystic echinococcosis caused by the closely related *Echinococcus granulosus*, which affects many more patients worldwide with a significantly higher impact, similar to what has been found for African trypanosomiasis.[35] A first step in the improvement of current therapies will be the *in vitro* screening of novel reagents, and the test system based on monitoring PGI activity appears to be an ideal tool for studies involving large numbers of compounds. Moreover, besides PGI and the previously described AP, other vesicle fluid constituents could be exploited as viability markers in a similar way.

Table 2. Comparison between PGI assays carried out in 24- and 96-well formats

	Format	
	96-well	24-well
Mean value	1.71	1.34
Variance	0.18	0.10
Standard deviation	0.43	0.32
Coefficient of variation	0.25	0.24

E. multilocularis metacestodes were grown for 5 days in the presence of nitazoxanide in 96- and 24-well plates. Supernatants of 7 wells (24-well plate) or 12 wells (96-well plate) were randomly selected, and PGI activity was measured in the supernatant. Mean values and parameters estimating the variation between samples are given.

Acknowledgements

Many thanks go to Klaus Brehm and Markus Spiliotis who have provided crucial help in the culture of *E. multilocularis* metacestodes.

Funding

This work was supported by grants obtained through the Karl Enigk Foundation (TiHo Hannover, Germany), the Swiss National Science Foundation (31-111780 and 3100A0-112532/1), the Novartis Research Foundation, Novartis Animal Health, Swiss Life Jubiläumsstiftung, Helvetia Sana and the OPO Foundation.

Transparency declarations

None to declare.

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