Induction of sulfadiazine resistance in vitro in Toxoplasma gondii

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HIGHLIGHTS
- Adaptation of a specific Toxoplasma enzyme immunoassay on Vero cells.
- Determination of IC50 values on several T. gondii strains.
- Confirmation of sulfadiazine susceptibilities on T. gondii strains.
- Development in vitro of two sulfadiazine resistant strains: RH-RSDZ and ME-49-RSDZ.

ABSTRACT
We induced sulfadiazine resistance in two sulfadiazine sensitive strains of Toxoplasma gondii, RH (Type I) and ME-49 (Type II) in vitro by using drug pressure. At first, sulfadiazine susceptibility of the two sensitive strains and two naturally resistant strains of T. gondii was evaluated on Vero cells using an enzyme-linked immunosorbent assay (ELISA). The IC50 values of sulfadiazine were 77 µg/mL for RH, 51 µg/mL for ME-49 and higher than 1000 µg/mL for the two natural resistant strains. Secondly, induced resistance of the strains by gradually increase sulfadiazine concentration was verified by this test, which resulted IC50 values at higher than 1000 µg/mL. In conclusion we developed in vitro two sulfadiazine resistant strains called RH-RSDZ and ME-49-RSDZ. These strains resistant to sulfadiazine would be useful to characterize resistance mechanisms to sulfadiazine.

1. Introduction
Prophylaxis and treatment for toxoplasmosis are based on a combination of pyrimethamine and sulfonamide. These two drugs act synergistically to block the folate biosynthesis pathway by inhibiting dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), which are essential for parasite survival and replication. Nevertheless several treatment failures have been reported for the treatment of toxoplasmic encephalitis, chorioretinitis, and congenital toxoplasmosis (Baatz et al., 2006; Dannemann et al., 1992; Katlama et al., 1996; Petersen, 2007; Torres et al., 1997). Several pharmacological factors may contribute to these failures: malabsorption, drug intolerance, compliance or tissue diffusion. However, given the target of drugs used in the treatment of toxoplasmosis, it is suspected that factors of resistance or lower susceptibility depending on the parasite strain may exist.

Recently, in vitro sulfadiazine (SDZ) susceptibilities of 17 Toxoplasma gondii strains were evaluated by Meneceur et al. (2008) by calculating the 50% inhibitory concentration (IC50)
according to a method developed by Derouin and Chastang (1988) on MRC-5 cells. The existence of three strains naturally resistant to sulfadiazine was observed: TgA 103001 (previously described as B1, type I strain), TgH 32006, (previously described as RMS-1995-ABE, type II strain) and TgH 32045 (previously described as RMS-2001-MAU, type II variant strain). These three strains have shown IC50 values for sulfadiazine >50 µg/mL on MRC-5 cells in comparison to the other strains studied which had an IC50 ranging from 3 to 18.8 µg/mL and therefore considered as sensitive (e.g., IC50 of the RH strain = 6.52 µg/mL and IC50 of the ME-49 strain = 6.55 µg/mL of sulfadiazine) (Meneceur et al., 2008). No apparent correlation with strain genotype or mutations in known drug target genes was found in these three strains (Meneceur et al., 2008). Thus, the mechanism of resistance to sulfadiazine in T. gondii remains unclear.

Until now, the only data on the selection of sulfonamide resistance in T. gondii are obtained experimentally. Firstly, a sulphonamide resistant strain, called SR strain, was developed in mice after 290 days exposure to sulphamethoxazole (Sander and Midvedt, 1971). Then, Pfefferkorn et al. (1992), induced resistance to the wild-type RH by the use of chemical mutagenesis and growth in gradually increased sulfadiazine concentrations. This mutant parasite (R-SulR5) showed to be 300-fold more resistant than the parental strain RH. Further study on R-SulR5 confirmed previous observations that growth of this strain is sulfonamide resistant with an IC50 value to be nearly 5 mM (equivalent to 1360 µg/mL) like another sulfadiazine resistant strain Swa-20 isolated from patient with clinical toxoplasmosis (Aspinall et al., 2002).

In our present study, we induced sulfadiazine resistance on two sensitive strains of T. gondii in vitro and in order to verify this resistance, we performed an in vitro quantitative sulfadiazine resistance study on parasites by using enzyme-linked immunosorbent assay (ELISA).

2. Material and methods

2.1. Parasites

Four strains of T. gondii tachyzoites have been used in this study: RH (type I, sensitive strain), TgA 103001 (type I, natural resistant strain), ME-49 (type II, sensitive strain) and TgH 32006 (type II, natural resistant strain). All strains studied were provided by the French Biological Toxoplasma Resource Centre (BRC Toxoplasma, France) and are previously described for their sulfadiazine susceptibilities in MRC-5 cell culture (Meneceur et al., 2008).

2.2. Parasites growth

Tachyzoites were maintained on Vero cell monolayers (ATCC, CCL-81) at 37°C in a 5% CO2 humidified incubator. Cells and parasites were grown in complete medium: Iscove’s Modified Dulbecco’s Medium/Glutamax (IMDM; Invitrogen, France) supplemented with 2% (v/v) fetal calf serum (Biowest, France) and antibiotics (100 IU/ mL penicillin and 0.1 mg/mL streptomycin) (GIBCO). Parasite growth rates varied depending on strain genotypes (Meneceur et al., 2008); host cells were infected at different parasite to cell ratio (1:1 for the type I strains, 4:1 for the type II strains). For all the study, cells and tachyzoites were counted using a Kova Slide counting chamber in the presence of Trypan blue (v/v). The parasites were routinely checked for Mycoplasma spp. contamination and found to be negative using a Mycoplasma spp. real-time PCR (Ishikawa et al., 2006).

2.3. In vitro studies

The assessment of the in vitro inhibitory effects of antimicrobial agent on Toxoplasma growth was determine on Vero cell cultures using 96-well plates. Briefly, 200 µL of a suspension containing 15,000 cells were placed into each well of 96-well plates and incubated for 3 h at 37°C to allow cells to adhere. Then, each well of the plate, except eight control wells, was inoculated with 50 µL of tachyzoites suspension (i.e., 7500 tachyzoites in 50 µL for type I strains and 30000 tachyzoites in 50 µL for type II strains). After 3 h at 37°C, SDZ (Sigma-Aldrich, France) at various concentrations (10, 25, 50, 75, 100, 250, 500, 750 and 1000 µg/mL) was added to the wells. After 72 h, the cultures were fixed with cold methanol. Each concentration was tested in eight replicate wells. Toxoplasma growth was evaluated by an enzyme-linked immunosorbent assay (ELISA) performed directly on the infected cultures, with an anti-T. gondii SAG-1-HRP conjugated monoclonal antibody (Argene Biotech, France): culture plates were rehydrated 10 min in PBS 1×,
then 60 \mu L per well of the monoclonal antibody (1.01 mg/mL) at a
titration of 1:3000 diluted in antibody buffer [Tris/NaCl (pH 7.3),
0.1% (v/v) IGEPAL CA-630 (Sigma–Aldrich, France), 0.2 mM Ethyl-
mercuriothiol-Benzoësaüre Natrium Sals (Merck Millipore, France), 25% (v/v) fetal calf serum (Biowest), 50 mM Potassium
hexacyanoferrate (Sigma–Aldrich)] was added and incubated for
1 h at 37 °C. After four washing with 1X washing buffer [0.14 M So-
dium chloride (Merck Millipore), 2.5 mM Potassium chloride (Merck Millipore), 2.5 mM Potassium chloride (Merck Millipore), 2.5 mM Di-potassium hydrogen phosphate dihydrate (Merck Millipore), 10 mM Di-sodium hydrogen phospho-
dehydrate (Merck Millipore), 0.02 mM Ethylmercuriothiol-
Benzoësaüre Natrium Sals (Merck Millipore), 0.1% (v/v) Tween 20
(Merck Millipore)], o-phenylenediamine dihydrochloride (OPD, Sigma) substrate was added (200 \mu L per well), one tablet of OPD
was dissolved in 10 mL of substrate buffer [40 mM citric acid monohydrate (Merck Millipore, France), 120 mM Di-sodium hydrogen phosphate dihydrate (Merck Millipore), 0.2 mM Ethyl-
mercuriothiol-Benzoësaüre Natrium Sals (Merck Millipore), 0.02% (v/v) hydrogen peroxide 30%].

The reaction was stopped by the
addition of 100 \mu L per well of 1 N HCl, 200 \mu L were taken from
each well and transferred into new 96-well plates for spectrophotometric readings at 450 nm corrected at 630 nm; blank readings
were made on the mean value of the eight negative control wells.

In the same time, the culture plates were immediately washed
with distilled water, stained with kit RAL 555 (RAL Diagnostics, France) and examined microscopically (Axiovert 200 M, Zeiss, France) at magnification 20× to observe large foci of parasitized
cells on the positive-control cultures and sulfadiazine effects on
the other wells. For each tested strains, the data reported here
are representative of two experiments performed in eight repli-
cates at each drug concentration.

2.4. Sulfadiazine resistance induction

To induce SDZ resistance, parental strains RH and ME-49 were
cultivated on Vero cells (as previously described in Section 2.3) with
gradient increase in sulfadiazine concentration (Sigma–Aldrich, France). SDZ was initially dissolved in 50% NaOH 2 N-50% complete
medium at a concentration of 100 mg/mL. Cultures were started at a
concentration of SDZ closed to the IC50 value of parental strains. SDZ
concentration was increased every three–five passages from 50 to
800 \mu g/mL of SDZ, when tachyzoites displayed reproducible growth and infectivity. The duration between each passage varied from 4 to
25 days for the mutant RH strain and 4 to 30 days for the mutant ME-

![Fig. 2. Optical microscopic observation of *T. gondii* growth for Type I strains in presence of SDZ for 72 h. Strains of *T. gondii*, RH (a–c), TgA 103001 (d–f) and RH-RSDZ (g–i) were cultured on Vero cells with SDZ at 0 \mu g/mL (a, d, g), 75 \mu g/mL (b, e, h) and 1000 \mu g/mL (c, f, i) for 72 h. Cultures were then fixed with methanol, stained with kit RAL 555 and microscopically inspected (magnification: 20×, bars represent 40 \mu m). Tachyzoites were indicated by arrows.](image-url)
49 strain. Strains required 23 passages for the mutant RH and 25 passages for the mutant ME-49. The RH and ME-49 strains resistant to sulfadiazine were called RH-RSDZ and ME-49-RSDZ, respectively.

2.5. \textit{IC}_{50} data analysis

The effect of SDZ at various concentrations was described by data plotting as previously described (Derouin and Chastang, 1989) with minor modifications to the y axis (optical density for Derouin and Chastang were turned into percentage of parasites growth in our study). Results were averaged, OD values for cultures without drug treatment was used at 100\% value of parasites growth and plotted in function of the logarithm of SDZ concentration. Briefly, curves displayed a sigmoid shape with three linear regression models. This allowed us to describe each drug effect as a sequence of three lines: line 1 indicated the absence of inhibitory effect, line 2 indicated a marked increase of inhibition, and line 3 indicated a residual effect for higher concentrations. For ELISA assay, values are expressed as mean ± SD.

3. Results

In order to develop a laboratory-induced \textit{in vitro} resistance on two strains by gradually increase sulfadiazine concentration, several aspects were examined: (i) to adapt the specific \textit{Toxoplasma} enzyme immunoassay developed by Derouin and Chastang (1988) to Vero cells, (ii) to determine IC\textsubscript{50} values on previously studied strains (RH, ME-49, TgA 103001 and TgH 32006) (Meneceur et al., 2008) and to confirm their SDZ-susceptibility and (iii) to verify the SDZ-resistance of the two strains RH-RSDZ and ME-49-RSDZ.

Preliminary studies indicate that final concentrations of NaOH 2 N in dilution of SDZ, that is to say 0.1 N at maximum SDZ concentration, did not inhibit the growth of \textit{T. gondii} (\textit{data not shown}).

3.1. RH strain (sulfadiazine sensitive)

For the RH strain, an inhibitory effect was observed for SDZ concentration greater than 50 \mu{g}/mL. Between 50 and 100 \mu{g}/mL, this effect was summarized by regression line L2: \( y = 522.34 - 108.65 \ln(C) \) where C is the concentration. The IC\textsubscript{50} was estimated to be 77 \mu{g}/mL of SDZ (Fig. 1a). The microscopic observation showed a decreased of the tachyzoite rosettes number at a concentration close to the IC\textsubscript{50} (75 \mu{g}/mL) (Fig. 2b) in comparison to the control (Fig. 2a) and a complete inhibition of \textit{T. gondii} growth at 1000 \mu{g}/mL (Fig. 2c).

3.2. ME-49 strain (sulfadiazine sensitive)

An inhibitory effect was observed for SDZ concentration greater than 25 \mu{g}/mL for the ME-49 strain. Between 25 and 100 \mu{g}/mL, this effect was summarized by regression line L2: \( y = 276.26 - 57.484 \ln(C) \). The IC\textsubscript{50} was estimated to be 51 \mu{g}/mL of SDZ (Fig. 3a). This inhibitory effect appeared on microscopic observation at 50 \mu{g}/mL, which halved parasite growth rate (Fig. 4b) comparing to the control (Fig. 4a). \textit{T. gondii} growth was totally inhibited at 1000 \mu{g}/mL (Fig. 4c).

3.3. TgA 103001 strain (sulfadiazine resistant)

SDZ had no effect on the TgA 103001 strain. This was summarized by regression line L1 indicating the absence of inhibitory effect. In this case, the IC\textsubscript{50} value for SDZ was higher than 1000 \mu{g}/mL of SDZ (Fig. 1b). Microscopic examination of the stained-culture showed no inhibitory effect for the SDZ concentrations up to 1000 \mu{g}/mL, with an important cell lysis due to parasites (Fig. 2d–f).

3.4. TgH 32006 strain (sulfadiazine resistant)

SDZ had no effect on the TgH 32006 strain. This was summarized by regression line L1 indicating the absence of inhibitory effect. In this case, the IC\textsubscript{50} value for SDZ was higher than 1000 \mu{g}/mL (Fig. 1b). Microscopic observation (Fig. 2d–f). This effect was confirmed by microscopic observation (Fig. 4d–f).

3.5. Sulfadiazine resistance induction

RH and ME-49 strains have IC\textsubscript{50} values at ~75 \mu{g}/mL and 50 \mu{g}/mL, respectively (Figs. 1a and 3a). To develop sulfadiazine resistant strains \textit{in vitro}, the two strains cultures were started at the same concentration of SDZ (50 \mu{g}/mL of SDZ). Over a period of 8 months,
RH and ME-49 strains were grown in the presence of increasing SDZ concentration (50, 100, 200, 400 and 800 µg/mL of SDZ) to obtain two strains that are able to grow in the presence of 800 µg/mL of SDZ. These two strains were called RH-RSDZ and ME-49-RSDZ.

3.6. Susceptibilities of RH-RSDZ and ME-49-RSDZ

SDZ had no effect on the RH-RSDZ and ME-49-RSDZ strains. Their growth curves (Figs. 1c and 3c, respectively) showed the same profile as those of natural resistant strains: TgA 103001 and TgH 32006 (Figs. 1b and 3b, respectively). This was summarized by regression line L1 on the two graphs (Figs. 1c and 3c) indicating the absence of inhibitory effect. In this case, for the two strains RH-RSDZ and ME-49-RSDZ, IC50 values for SDZ were higher than 1000 µg/mL.

Microscopic observations of the stained cultures showed no inhibition of parasites growth with SDZ up to 1000 µg/mL for RH-RSDZ (Fig. 2g–i) and ME-49-RSDZ (Fig. 4g–i).

The resistance of RH-RSDZ and ME-49-RSDZ strains is consistently observed after several passages in cell culture without sulfadiazine and the same IC50 values were observed after thawing of these strains (data not shown). Moreover, tachyzoites of these strains displayed reproducible growth and infectivity as parental strains RH and ME-49.

4. Discussion

In this study, the first step was to adapt, on Vero cells, the specific Toxoplasma enzyme immunoassay developed by Derouin and Chastang (1988) and used by Meneceur et al. on MRC-5 cells to evaluate the sulfadiazine susceptibilities of 17 T. gondii strains. Similar to all primary cell lines, using MRC-5 cells have limitations such as availability and limited life in cell culture. On contrary, T. gondii culture on Vero cell has significant advantages such as yield, purity and viability (Saadatnia et al., 2010) which make it a good candidate for standardization. Actually, numerous human strains of T. gondii are available at the BRC Toxoplasma (France) (more than 600 isolates) and we want to develop an easy-to-use test to assess the susceptibilities to sulfadiazine. We induced resistance by gradually increasing sulfadiazine concentration on two strains (RH, Type I and ME-49, Type II) in order to have the same genotype as two of the three strains described as naturally resistant by Meneceur et al. (2008).

In accordance with Meneceur et al. (2008), our results showed that the RH and ME-49 strains were sensitive to SDZ with an IC50 value at 77 µg/mL for the RH strain and 51 µg/mL for the ME-49 strain, whereas IC50 was determined to be 6.5 µg/mL on MRC-5 cells for both strains (Meneceur et al., 2008). Using the same approach, we confirmed that the strains TgA 103001 and TgH 32006 were resistant to sulfadiazine with IC50 values higher than 1000 µg/mL of SDZ on
Vero cells whereas the IC50 values for theses resistant strains were shown to be higher than 50 μg/mL on MRC-5 cells (Meneceur et al., 2008). Moreover, several other strains studied by Meneceur et al. (2008) were examined by our test that showed similar results. Strain TgH 32045, described as resistant on MRC5 cells, was also found to be resistant on Vero cells by our team with IC50 values higher than 1000 μg/mL of SDZ (data not shown). Indeed, numerous studies have shown that the drug susceptibility for parasites depended on the host cells used: using RH strain as model, IC50 value was found to be 6.5 μg/mL of SDZ on HEP2 cells (Van Der Ven et al., 1996) and MRC-5 cells (Meneceur et al., 2008) while IC50 was found to be 70 μg/mL on HFF cells (De Oliveira et al., 2009) and 1800 μg/mL on Hela cells (Jin et al., 2009). In comparison to MRC-5 cells, Vero cells showed a broader range to study toxicity of sulfadiazine: IC50 values for SDZ varied between 50–75 μg/mL and 1000 μg/mL on Vero cells while IC50 values varied only between 6 μg/mL and 50 μg/mL on MRC-5 cells.

Until now, relevant studies investigating mutant resistant to sulfonamides in T. gondii are focused on the R-Su1R-5 strain obtained by mutagenesis (Pfefferkorn et al., 1992). This mutant parasite was selected by a combination of chemical mutagenesis and growth in gradually increased sulfadiazine concentrations and has been shown to be 300 times more resistant to sulfonamides than the wild-type RH strain. Aspinall et al. (2002) studied the R-Su1R-5 strain and confirmed that this strain was sulfonamide resistant with an IC50 value to be nearly 5 mM.

When exposed for a long period to sulfonamides, T. gondii can develop resistance in vitro as we demonstrated it in this present work, as well as in vivo (Sander and Midtvedt, 1971), where SR strain (sulfonamide resistant strain originated from RH strain) was transferred on mice for 290 days receiving additional 0.5 mg sulfamethoxazole daily in their diet. These results raise the question already asked by Aspinall et al., 2002: was the resistance acquired following parasite exposure to drugs, or was it innate? Among the 17 Toxoplasma strains studied by Meneceur et al., 2008, three were determined to be sulfadiazine resistant, which was also confirmed by our test. These strains included TgA 32006, which was coming from a congenital infection with a resulting treatment consisting of pyrimethamine and sulfadoxine for 12 weeks; TgA 103001, which was coming from an aborted fetus of bovine without drug exposure before strain isolation and TgH 32045, which was coming from a congenital toxoplasmosis without sulfonamides used during treatment (Meneceur et al., 2008). Aspinall et al., 2002 studied a SDZ-resistant clinical isolate (Swa-20), which was isolated without sulfonamide treatment. These SDZ-resistant clinical isolates, except the TgH 32006 strain, suggested that parasites already carrying sulfonamide resistance before infection.

In the present study, the RH-RSDZ and ME-49-RSDZ strains developed in our laboratory are stable after several passages in cell culture without drug pressure (more than 5 months) and also after thawing (data not shown). This indicates that resistance was acquired and continues without affecting phenotypic traits of parasite.

To conclude, we have developed two SDZ-resistant strains (Type I and II) in vitro named RH-RSDZ and ME-49-RSDZ and we have confirmed their susceptibility to SDZ on Vero cells. These resistant strains could establish a good approach to study and understand the mechanism of sulfadiazine resistance in T. gondii by using several techniques such as proteomics techniques (comparison of proteomes between sensitive and in vitro resistant strains) or transcriptomics analysis (comparison of mRNA levels between sensitive and in vitro resistant strains). The development of this new ELISA test will be useful to test strains included in BRC Toxoplasma and to evaluate the prevalence among these resistant strains. In the case of treatment failures of toxoplasmosis, ELISA test could also be carried out to verify strains susceptibilities to the drugs used. Moreover, further studies of these strains could make connection between naturally resistant strains and induced resistant strains to better understand resistance mechanisms.

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References


