*J. Parasitol.*, 98(3), 2012, pp. 666–668
© American Society of Parasitologists 2012

# METRONIDAZOLE INDUCES GAMETOCYTOGENESIS IN GREGARINE ASSOCIATIONS MAINTAINED IN VITRO

**Kate Trout and Richard E. Clopton***
Department of Natural Science, Peru State College, Peru, Nebraska 68421. *e-mail: rclopton@peru.edu*

ABSTRACT: Gametocytogenesis was induced in mature associations of *Protomagalhaensia wolfi* and *Protomagalhaensia blaberae* maintained in vitro by inclusion of metronidazole in the culture medium. The response was neither strictly dosage dependent nor uniform across gregarine species. We hypothesize that metronidazole induces gregarine gametocytogenesis by disrupting *PUF2* proteins responsible for the translational control of sexual development and gametocytogenesis in apicomplexans.

Clopton and Smith (2002) and Smith and Clopton (2003) tested sulfadimethoxine, metronidazole, and potassium sorbate as potential in vivo chemotherapeutic control agents against 2 gregarine species, *Protomagalhaensia granulosae* and *Blabericola cubensis* (=*Gregarina cubensis*), infecting the discoid cockroach *Blaberus discoidalis*. They reported significant reductions in gregarine mean intensities for cockroaches treated with sulfadimethoxine and metronidazole (80% and 71%, respectively) but found no significant reduction in gametocyst production over a 7-day-long treatment period. They concluded that both compounds targeted early developmental stages in the gregarine life cycle, i.e., sporozoites and trophozoites, but had no apparent effect on late reproductive stages (gamonts). In contrast, potassium sorbate did not significantly decrease gregarine mean intensities, but it significantly increased the rate of gametocyst production (Smith and Clopton, 2003). Johny et al. (2007) reported a 90% reduction in mean intensities of the gregarine *Boliviana floridensis* in eastern Lubber grasshoppers, *Romalea microptera*, treated with metronidazole (for gregarine identification, see Johny and Whitman, 2005). Differences in parasite load reduction among these studies may reflect differences among individual gregarine species, but they are more likely the result of a much larger dosage in the latter study (0.4 mg/kg body weight per diem vs. 20 mg/kg body weight per diem, respectively). Johny et al. (2007) did not differentiate life-cycle stage specific effects or report gametocyst production.

The effects of metronidazole dosage on gregarine gamonts remain unclear. Herein, we describe a method for maintaining mature gregarine gamonts in association in vitro using a semi-defined medium and report the response of these late reproductive stages to increased concentrations of metronidazole in the medium.

## MATERIALS AND METHODS

*Protomagalhaensia blaberae* and *Protomagalhaensia wolfi* were maintained in vivo using separate breeding colonies of *Blaberus boliviensis* and *Nauphoeta cinerea*, respectively. Cockroach colonies were maintained in 22-L polycarbonate containers with coir bedding and cardboard egg-crate roosting habitat. Food (Purina® Dog Chow® brand Dog Food Complete & Balanced; Nestle Purina Pet Care Company, St. Louis, Missouri) and water were provided ad libitum.

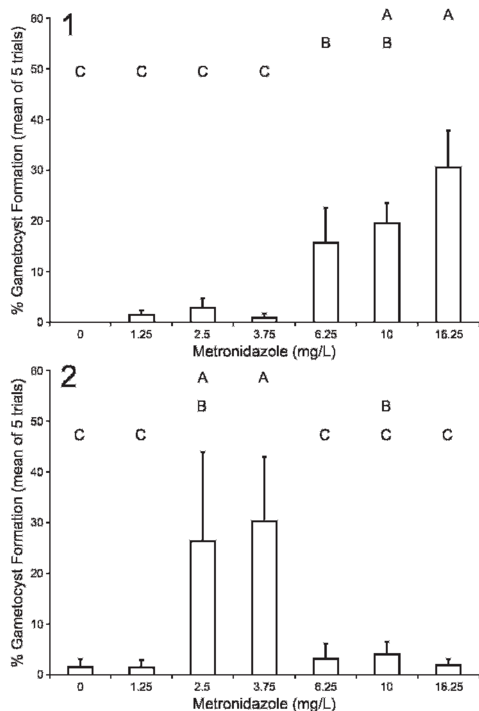
Gregarine associations for in vitro maintenance and metronidazole testing were obtained by dissection. Cockroaches were eviscerated, their alimentary canals were dissected in dilute cockroach saline (NaCl, 120 mM; KCl, 1.35 mM; $CaCl_2$, 1.8 mM; $NaHCO_3$, 1.2 mM; modified from Griffiths and Tauber, 1943), and mature gregarine associations were isolated, triple rinsed in dilute cockroach saline, and transferred by pipette for in vitro culture. Dissections were conducted using ambient light to reduce heat transfer to the dissection dish. Associations were maintained in a semi-defined media (Griffiths and Tauber with albumin [GTA]). Fresh GTA medium was prepared by combining 3 volumes of aqueous cockroach saline (NaCl, 240 mM; KCl, 2.7 mM; $CaCl_2$ [supplied as $CaCl_2 \cdot 2H_2O$], 3.6 mM; $NaHCO_3$, 2.4 mM; ACS-grade reagents [Spectrum Chemical Manufacturing Corporation, New Brunswick, New Jersey]), syringe sterilized by passage through an Acrodisc® 25-mm PF syringe filter with 0.8/0.2-μm Supor® low-protein-binding membrane (Pall Corporation, Ann Arbor, Michigan), and 1 part fresh chicken egg albumin harvested from locally obtained commercial eggs and agitating the medium overnight on a slow shaker table at room temperature to allow the albumin to disassociate and enter solution. Gregarines were maintained in vitro in groups of 20–30 mature gregarine associations in GTA contained in 1-ml polycarbonate micro-observation cells (Ward's Natural Science, Rochester, New York), which were sealed with a pressure-fit cover and stored at room temperature. Gregarines maintained in this manner retain their gliding mobility and plasmalemmal integrity for up to 21 days, but they rarely enter syzygy or form gametocysts. To test the effects of metronidazole on syzygy and gametocyst formation, 1 micro-observation cell culture was used for each replicate of 6 metronidazole treatments and a control (GTA). Treatments consisted of GTA medium with 1.25, 2.5, 3.75, 6.25, 10, or 16.25 mg/L metronidazole. Each treatment was replicated 5 times for associations of *P. blaberae* and *P. wolfi*, respectively. The effect of metronidazole was quantified by counting the gametocysts formed by culture day 12. Differences in arcsine-transformed proportions among treatments were analyzed using ANOVA ($\alpha = 0.05$). Fisher's least significant difference tests (95% confidence interval) were used to make post-hoc pairwise comparisons among treatments.

## RESULTS

Metronidazole treatment induced a significant increase in syzygy and gametocyst formation for both gregarine species tested. For associations of *P. wolfi*, the percentage of syzygy and gametocyst formation differed significantly across 6 levels of metronidazole treatment and the untreated control (ANOVA, $F$ [6, 28] = 11.332, $P < 0.0001$). For associations of *P. blaberae*, the percentage of syzygy and gametocyst formation differed significantly across 6 levels of metronidazole treatment and the untreated control (ANOVA, $F$ [6, 28] = 2.828, $P < 0.028$). However, the effect among treatments was not a correlated dosage response.

Figures 1 and 2 present the mean percentage of gametocysts formed over 5 trials of an untreated control and 6 levels of metronidazole treatment for *P. wolfi* and *P. blaberae*, respectively. Very few untreated associations entered syzygy to produce gametocysts. Of 108 and 95 associations in the control groups for *P. wolfi* and *P. blaberae*, respectively, only 2 associations of *P. blaberae* formed gametocysts. No control association of *P. wolfi* entered syzygy in vitro. For associations of *P. wolfi* (Fig. 1), low levels of metronidazole induced an insignificant amount of syzygy and gametocyst formation (2, 3, and 1 gametocysts from 151, 102, and 120 associations tested, respectively, for 1.25, 2.5, and

Received 24 October 2011; revised 11 January 2012; accepted 20 January 2012.
* To whom correspondence should be addressed.
**DOI: 10.1645/GE-3020.1**




FIGURES 1–2. Mean percentage of gametocysts formed over 5 trials of an untreated control and 6 levels of metronidazole treatment by mature gregarine associations in vitro. Error bars indicate 95% confidence interval. Bars underlying identical letters indicate treatment groups that are not significantly different (Fisher's least significant difference test, 95% confidence interval). (**1**) Response of mature associations of *Protomagalhaensia wolfi.* (**2**) Response of mature associations of *Protomagalhaensia blaberae.*

3.75 mg/L metronidazole). Significant increases in syzygy and gametocyst production were observed only at higher treatment levels (20, 27, and 46 gametocysts from 133, 139, and 152 associations tested, respectively, for 6.25, 10, and 16.25 mg/L metronidazole). For associations of *P. blaberae* (Fig. 2), neither low nor high levels of metronidazole induced a significant change in syzygy and gametocyst formation (2, 4, 5, and 2 gametocysts from 105, 149, 137, and 132 associations tested, respectively, for 1.25, 6.25, 10, and 16.25 mg/L metronidazole). Significant increases in syzygy and gametocyst production were observed only at moderate treatment levels (42 and 34 gametocysts from 127 and 122 associations tested, respectively, for 2.5 and 3.75 mg/L metronidazole). The response of gregarine associations to metronidazole in vitro over the dosage range tested is observed as a threshold response that is saturated and inhibited in *P. blaberae* but not in *P. wolfi.*

## DISCUSSION

Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) is a synthetic pharmaceutical compound with demonstrated action against both pathogenic bacterial, e.g., species of *Bacteroides*, *Clostridium*, and *Helicobacter*, and protistan, e.g., species of *Entamoeba*, *Giardia*, and *Trichomonas*, targets (Johnson, 1993). Capable of providing significant reduction in infection intensities of young gregarines at sufficiently high doses, the results presented herein demonstrate that metronidazole can also induce syzygy and gametocyst formation in mature gregarine stages.

The molecular mechanism inducing gametocytogenesis in gregarines is unknown, but it is probably similar to that demonstrated for other apicomplexans, particularly *Plasmodium falciparum*. Gametocytogenesis in *P. falciparum* is regulated by the RNA-binding protein *PfPUF2*, which provides translational control of the mRNA products that induce sexual development and gametocytogenesis (Miao et al., 2010). Disruption of *PfPUF2* induces gametocytogenesis, and Maio et al. (2010) suggest that the PUF-family RNA-binding proteins are highly conserved and probably at work across Apicomplexa. If so, a similar RNA-binding protein may control gametocytogenesis in gregarines, and the results described herein may be the result of the interaction of gregarine *PUF2* proteins and metronidazole.

Metronidazole is administered orally as an inactive prodrug that enters target and non-target cells via diffusion. In affected cells, the nitro group of the prodrug is reduced to a nitroso intermediate that will form sulfinamides and thioester linkages with cystine-bearing enzymes, deactivating these critical enzymes and halting normal cellular metabolism. Reduction of the prodrug nitro group requires single electron transfers from a metabolic pathway with a very low redox potential, usually pyruvate:ferredoxin oxioreductase. Reduction of the prodrug to its cytotoxic form and subsequent enzyme linkage maintain a diffusion gradient for additional prodrug uptake from the environment. Metronidazole is ineffective in aerobic cells because they lack electron-transport proteins with sufficiently negative redox potential to reduce the prodrug to an active form (Land and Johnson, 1999).

We suggest that the same mode of action can induce gregarine gametocytogenesis. Cui et al. (2002) elucidated the primary structure of *PfPUF2* (GenBank AAM44411.1), describing a 514 amino acid protein with an embedded 259 amino acid PUF RNA-binding region. The binding region itself includes 11 cysteines, suggesting that sulfur bridges underpin the conformational shape and function of the protein. We suggest that formation of sulfinamides and thioester linkages with cystines by metronidazole nitroso intermediates disrupts *PUF2* mRNA binding and eliminates translational inhibition of gametocytogenesis.

## ACKNOWLEDGMENTS

This material is based upon work supported in part by the National Science Foundation through grant NSF DEB-1019419. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

## LITERATURE CITED

CLOPTON, R. E., AND A. SMITH. 2002. Efficacy of oral sulfadimethoxine against two gregarine parasites, *Protomagalhaensia granulosae* and *Gregarina cubensis* (Apicomplexa: Eugregarinida) infecting the death's head cockroach, *Blaberus discoidalis*. Journal of Parasitology **88:** 786–789.

CUI, L., Q. FAN, AND J. LI. 2002. The malaria parasite *Plasmodium falciparum* encodes members of the PUF RNA-binding protein family with conserved RNA binding activity. Nucleic Acids Research **30:** 4607–4617.

GRIFFITHS, J. T., AND O. E. TAUBER. 1943. The effects of pH and of various concentrations of sodium, potassium, and calcium chloride on muscular activity in the isolated crop of *Periplaneta americana* (Orthoptera). Journal of General Physiology **26:** 541–558.

JOHNSON, P. J. 1993. Metronidazole and drug resistance. Parasitology Today **9:** 183–186.

JOHNY, S., A. MERISKO, AND D. W. WHITMAN. 2007. Efficacy of eleven antimicrobials against a gregarine parasite (Apicomplexa: Protozoa).

Annals of Clinical Microbiology and Antimicrobials **6:** 15. doi:10.1186/1476-0711-6-15.

———, AND D. W. WHITMAN. 2005. Description and laboratory biology of *Boliviana floridensis* n. sp. (Apicomplexa: Eugregarinida) parasitizing the eastern Lubber grasshopper, *Romalea microptera* (Orthoptera: Romalidae), from Florida, U.S.A. Comparative Parasitology **72:** 150–156.

LAND, K. M., AND P. J. JOHNSON. 1999. Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa. Drug Resistance Updates **2:** 289–294.

MIAO, J., J. LI, Q. FAN, X. LI, AND L. CUI. 2010. The PUF-family RNA-binding protein *PfPuf2* regulates sexual development and sex differentiation in the malaria parasite *Plasmodium falciparum*. Journal of Cell Science **123:** 1039–1049.

SMITH, A., AND R. E. CLOPTON. 2003. Efficacy of oral metronidazole and potassium sorbate against two gregarine parasites, *Protomagalhaensia granulosae* and *Gregarina cubensis* (Apicomplexa: Eugregarinida) infecting the death's head cockroach, *Blaberus discoidalis*. Comparative Parasitology **70:** 196–199.