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Efficacy of atovaquone combined with clindamycin against murine infection with a cystogenic (Me49) strain of *Toxoplasma gondii*

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The efficacy of atovaquone (ATO) combined with clindamycin (CLI) against *Toxoplasma gondii* was examined in murine models of infection with a mouse-non-virulent (Me49) strain. Swiss-Webster mice inoculated by mouth with 10 or 20 cysts were treated with ATO and CLI alone or combined at dosages of ATO 5–100 and CLI 25–400 mg/kg/day for 2–4 weeks. Drug treatment was initiated (i) day 4 post-infection (acute infection), (ii) 3 months post-infection (chronic infection) and (iii) following a 2–3 week course of treatment with dexamethasone (DXM) alone or combined with cortisone-acetate (CA) introduced 3 months post-infection (reactivated toxoplasmosis). In acute infection, whereas treatment with any drug or drug combination significantly enhanced survival and reduced the brain cyst burden, in mice treated with ATO alone or combined with CLI, the cyst counts were significantly lower than in mice treated with CLI alone. In chronic infection, the decrease in the cyst burden observed 2 weeks after treatment with either drug alone was significant only in mice treated with the combined drugs. Most importantly, in reactivated toxoplasmosis, whereas an effect for the combined drugs was shown in mice suppressed with both DXM alone and combined with CA, in mice pre-treated with DXM a 3 week course of ATO ≥25 and CLI 50 mg/kg/day significantly increased survival and markedly decreased the cyst burden. The latter effect was long-term, since the cyst burdens in treated mice continued to decrease up to 3 months later, whereas they increased in the untreated mice. The results warrant clinical evaluation of the combination of ATO and CLI in the treatment of toxoplasmosis in both immunocompetent and, more importantly, immunosuppressed patients.

Introduction

The AIDS pandemic and the use of intensive immunosuppressive therapies for a variety of conditions, including before and after organ and tissue transplantation, have established a population of immunocompromised individuals prone to reactivation of opportunistic pathogens, including *Toxoplasma gondii*, particularly in geographical areas with a high exposure to this protozoan. In patients with AIDS, *T. gondii* has emerged as the leading cause of focal cerebral lesions, most commonly presenting as toxoplasmic encephalitis (TE).[1] Since available therapies are unable to eliminate the parasite from the infected host, treatment of a TE episode must be followed by life-long maintenance therapy. However, standard treatment with pyrimethamine and sulfadiazine is associated with considerable toxicity, often requiring discontinuation of the drugs.[2,3] Consequently, new therapies are critically needed. Evaluation of a number of known and newly developed drugs and drug combinations led to the concept of combination therapy as the current strategy of choice for the treatment of toxoplasmosis in this setting.[4]

The hydroxynaphthoquinone atovaquone (ATO) appears to be active not only against tachyzoites, but also against bradyzoite-containing *T. gondii* cysts.[5] Mitochondrial cytochrome *b* has recently been identified as the target for ATO in *T. gondii*.[6] ATO has been shown to be effective in animal models of infection, alone[7–10] or combined with other drugs such as pyrimethamine and sulfadiazine,[11] or rifabutin,[4,12] and

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is being used increasingly in human therapy.[13–15] We have reported on the synergic activity of ATO and clindamycin (CLI), a widely used alternative drug[16,17] with well-established anti-*T. gondii* activity,[18–21] in murine toxoplasmosis induced with the mouse-virulent (type-1) RH strain of *T. gondii*.[22] However, since type-2 strains account for 65% of cases of reactivation of toxoplasmosis in AIDS,[23] in this report we present data on the anti-*T. gondii* efficacy of ATO and CLI in murine infection induced with Me49 as a typical type-2 strain, including a model of *T. gondii* recrudescence.

Materials and methods

Mice

Female Swiss-Webster mice (Medical Military Academy Animal Research Facility, Belgrade, Yugoslavia), 6 weeks old, weighing 18–22 g at the beginning of experiments, were used. Mice were housed five or six per cage (depending on the experiment) and offered drinking water and regular mouse feed *ad libitum*, unless otherwise specified. All animal studies were performed with the approval of a local (Institute for Medical Research) ethics committee.

Parasites

Brain cysts of the Me49 strain of *T. gondii* (initially provided by Dr J. P. Dubey, Beltsville, MD, USA), regularly maintained by passage through Swiss-Webster mice twice a year, were used to establish chronic infection. To obtain cysts for experimental infections, mice infected at least 8 weeks previously were killed by asphyxiation in chloroform and their brains removed and homogenized in a Teflon homogenizer with 1 mL of saline each. For cyst enumeration, 25 μL of the brain suspensions was placed on slides and microscopically counted. The number of cysts per brain was calculated by multiplying the number counted in four drops by 10, giving a threshold sensitivity of 10 cysts per brain. For experimental infections, fresh mice were inoculated by intraoesophageal gavage with 250 μL brain suspensions assessed to contain 10 or 20 cysts. In our experience, inoculation of 10 cysts produces consistent infection without mortality, whereas inoculation of ≥20 cysts results in mortality;[24] thus, to allow for differences in survival after early drug treatment, inocula of 20 cysts were used to develop acute infection, whereas 10 cysts were inoculated to establish chronic infection.

Drugs

ATO (micronized powder, lot 291604A; GlaxoWellcome, Stevenage, UK, kindly provided by Dr Simon Lister) was administered at 5, 25, 50 and 100 mg/kg body weight per day.

CLI (CLI hydrochloride powder, lot 353YS; Upjohn, supplied by Yusapharm, Belgrade, Yugoslavia) was administered at 25, 50 and 400 mg/kg/day.

On the basis of the observation that mice consume 4 g of food per day,[18,20] the desired dosages were obtained by adding ATO 0.025, 0.125, 0.25 and 0.5 mg and CLI 0.125, 0.25 and 2.0 mg, respectively, per 1 g of ground mouse feed. Fresh food was supplied daily.

These dosages were selected on the basis of previous work[7,12,18,19,21] as effective (ATO 100 and CLI 400 mg/kg/day) or, to better reveal the effects of the combined therapy, as suboptimal (all lower dosages of both drugs).

To control for drug side-effects, in a preliminary experiment, separate groups of mice were given ATO (100 mg/kg/day) and CLI (400 mg/kg/day) for 3 weeks and ATO+CLI (100+400 mg/kg/day) for 2 weeks. No clinically significant toxicity (ruffled fur, lethargy, weight loss) was observed over a period of 3 months.

Dexamethasone-sodium-phosphate (DXM; ICN Galenika, Belgrade, Yugoslavia) was given at a dosage of 2.5 mg/kg/day per mouse, obtained by dissolving 5 mg of DXM per 1 L of drinking water. Treated and untreated water was changed three times a week.

Hydrocortisone-21-acetate (CA, lot. 87494; ICN Biomedicals, Aurora, OH, USA) was administered at 50 mg/kg by subcutaneous injection three times a week.

Co-amoxiclav (Panklav; Panfarma, Belgrade, Yugoslavia) was given in drinking water at 1 g/L for the period of administration of DXM and CA to prevent bacterial superinfection.

Experimental design

Acute infection model. Two separate experiments were performed. Groups of mice (n = 15–20) inoculated with 20 cysts were arbitrarily assigned to treatment with ATO and CLI, as follows: ATO 100 and CLI 400 mg/kg/day, alone and combined (experiment 1); ATO 25 and CLI 50 mg/kg/day, alone and combined (experiment 2); or left untreated to serve as controls. In both experiments, treatment was initiated day 4 post-infection and continued for 14 consecutive days. Mice were observed for 30 days after the end of treatment, at which point the survivors were killed and the brains removed and cysts counted as described above.

Chronic infection model. Groups of mice (n = 15) inoculated with 10 cysts 3 months previously were arbitrarily assigned to treatment with ATO 100, CLI 400 and ATO+CLI 100+400 mg/kg/day for 14 consecutive days or left untreated as controls. Fourteen days after the end of treatment, they were killed and processed as above.

Model of chronic infection in the immunosuppressed host (reactivated toxoplasmosis). DXM and CA were used to induce immunosuppression in chronically infected mice. The

choice of corticoid drug regimens was based on our previous work,[24] which showed that DXM, but not CA alone, induces immunosuppression leading to recrudescence of *T. gondii* in previously latent infection. However, the addition of CA to DXM potentiates the immunosuppressive effect of DXM. Clinically manifest disease followed by mortality begins after 2 weeks and continues throughout the period of corticoid administration. Thus, in experiment 1, groups of mice ($n = 12$) inoculated with 10 cysts 3 months previously were arbitrarily given DXM alone or combined with CA for 3 weeks and then treated with ATO+CLI 5+25 and 50+50 mg/kg/day for the next 4 weeks. Immediately after the end of treatment, the brain cysts were harvested from the survivors. In experiment 2, groups of chronically infected mice (n = 18) were pre-treated with DXM for 14 days and then arbitrarily given ATO+CLI 25+50, 50+50 and 100+400 mg/kg/day for 3 weeks. Five mice from each treatment group were killed immediately at the end of treatment and examined for brain cyst burdens, whereas the remaining mice were observed for the next 12 weeks, at which time (day 120 after initiation of immunosuppressive treatment) the survivors were killed and processed as above.

In all models, mice were monitored daily and deaths were recorded. In mice dying during the experiments as well as those surviving the observation period as specified in the respective model, brain parasite burdens were determined as described above.

Bioassay

If no cysts were observed in at least four samples of a brain tissue preparation, the remainder of the brain homogenate was inoculated by mouth into two fresh mice per sample (~450 μL each). Mice were killed after 6 weeks and brain tissue examined for the presence of brain cysts.

Statistical analysis

Survival rates in particular groups were estimated by the Kaplan–Meier product limit method and compared by the log rank (two curves) and multiple sample (three or more curves) tests. Differences in the numbers of cysts between groups were examined by Student's *t*-test. The level of statistical significance was $P = 0.05$.

Results

Acute infection model

The effects of a 2 week course of treatment with ATO and CLI initiated day 4 post-infection were monitored by 7 week survival, as well as by the brain cyst burdens at the time of death or by the end of the observation period. Compared with the survival in untreated mice of 60% and 65%, respectively, in experiments 1 and 2 all treated mice survived significantly better. Treatment with the lowest dosages of the drugs given alone (ATO 25 and CLI 50 mg/kg/day) protected 93% (P = 0.043 and 0.047, respectively) of mice, whereas the higher dosages of each drug alone (ATO 100 and CLI 400 mg/kg/day), as well as both combinations (ATO+CLI 25+50 and 100+400 mg/kg/day), afforded protection to virtually all mice ($P = 0.003$).

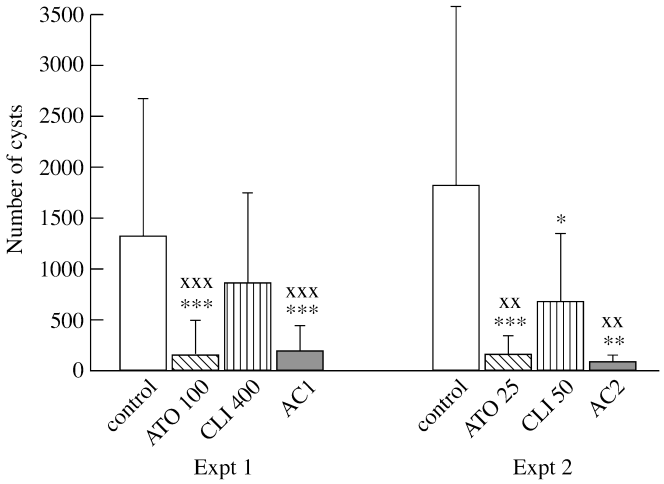

Figure 1. Brain cysts (mean ± S.D.) in mice acutely infected with 20 cysts of the Me49 strain of *T. gondii* treated with ATO and CLI alone (100 or 25 and 400 or 50 mg/kg/day, respectively) or combined (AC1 and AC2, 100+400 and 25+50 mg/kg/day, respectively) from day 4 post-infection for 14 days. Cysts were harvested 7 weeks post-infection. Control, untreated infected mice. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control mice. $^{XX}P < 0.01$ and $^{XXX}P < 0.001$ versus CLI alone.

Moreover, the treatment clearly reduced the number of cysts (Figure 1). However, important differences between the effects of ATO and CLI were observed. Although, compared with untreated mice, the cyst burdens were reduced in mice treated with CLI, the difference reached significance ($P = 0.027$) only in experiment 2. On the other hand, in both experiments, the cyst burdens in mice treated with ATO both alone and combined with CLI were significantly lower than in both untreated mice ($P < 0.0001$) and mice treated with CLI alone ($P = 0.0002$ and 0.0008, respectively, in experiment 1; and P = 0.0044 and 0.005, respectively, in experiment 2). However, there were no differences in either experiment between the effects of ATO both alone and combined with CLI ($P > 0.05$).

Interestingly, no brain cysts were observed on direct examination of the brain tissue in eight treated mice (two treated with ATO 25, one treated with ATO 100, one treated with CLI 400, two treated with ATO+CLI 25+50 and two treated with ATO+CLI 100+400 mg/kg/day). However, sub-inoculation of these brain homogenates into fresh mice gave rise to *T. gondii* cysts in virtually all instances, indicating that no drug regimen was able to eradicate the infection.

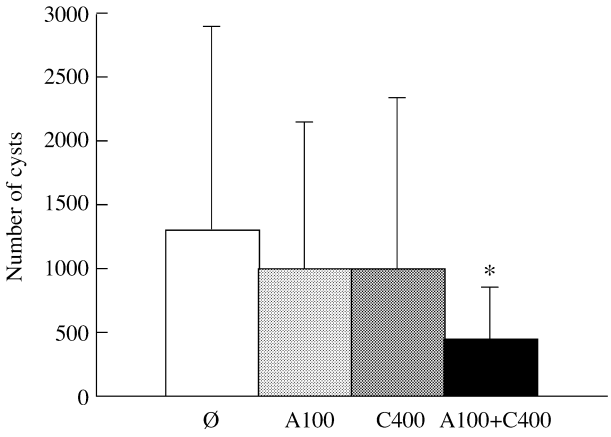

Figure 2. Brain cysts (mean ± S.D.) in mice chronically infected with 10 cysts of the Me49 strain of *T. gondii* 3 months previously, treated with ATO and CLI alone (100 and 400 mg/kg/day, respectively) or combined (100+400 mg/kg/day) for 14 days. Cysts were harvested 2 weeks after the end of treatment. Ø, control (untreated infected) mice. *$P < 0.05$ versus control mice.

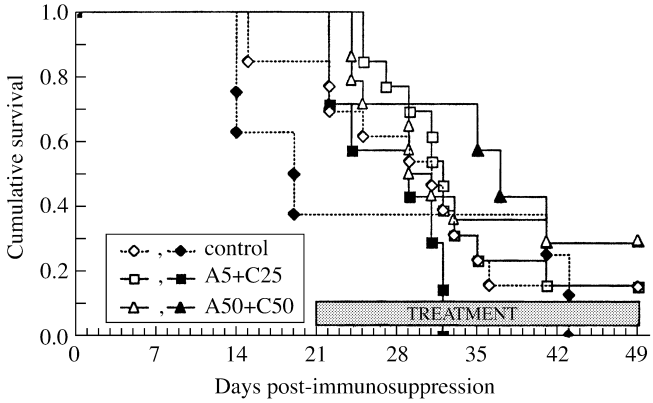

Figure 3. Effect of a 4 week course of treatment with combinations of ATO and CLI (5+25 and 50+50 mg/kg/day) on the survival (Kaplan–Meier estimates) of chronically infected mice immunosuppressed with DXM alone (open symbols) or combined with CA (filled symbols) for 3 weeks. Chronic infection was established by inoculation of 10 cysts of the Me49 strain of *T. gondii* 3 months before immunosuppressive treatment.

Chronic infection model

In chronically infected mice, which all survived throughout the experiment whether treated or not, 2 weeks after the end of a 2 week course of treatment with ATO 100 and CLI 400 mg/kg/day alone, the cyst burdens were insignificantly ($P > 0.05$) decreased, i.e. to 75.9% and 76.4%, respectively, of those in untreated mice (Figure 2). However, compared with untreated mice, treatment with ATO combined with CLI reduced the cyst burden significantly ($P = 0.033$). Compared with mice treated with ATO and CLI alone, the cyst burden in mice treated with the combined drugs was lowered to only 43%; although this difference did not reach significance, it came close ($P = 0.055$ and 0.078, respectively).

Model of reactivated toxoplasmosis

In experiment 1, chronically infected mice pre-treated with DXM alone or combined with CA for 3 weeks were submitted to a 4 week course of treatment with combinations of ATO+CLI 5+25 and 50+50 mg/kg/day. All untreated mice immunosuppressed with both DXM and CA succumbed to the infection by day 43, whereas 18% of those suppressed with DXM alone survived the observation period (Figure 3). Compared with these, mice treated with ATO+CLI 5+25 mg/kg/day did not survive any better (0% and 18% survival, respectively, for the DXM+CA and DXM pre-treated groups). In contrast, treatment at 50+50 mg/kg/day enhanced the survival of mice on suppressive regimen to 30%. However, the expected probabilities of survival did not vary significantly either among all groups taken together ($P = 0.2919$, multiple sample test) or between any two groups ($P > 0.05$). Similarly, although the cyst burdens (in all mice dying during the treatment/observation period or survivors killed at the end of the experiment) (Figure 4) were decreased, compared with untreated mice on either suppressive regimen, in mice treated with ATO+CLI 5+25 mg/kg/day by two-fold (to 59.2% and 46.4% of the control values) and even by four-fold in mice treated with ATO+CLI 50+50 mg/kg/day (to 27.2% and 23.1% of the control values), these differences were not significant, obviously due to high standard deviations in the untreated mice. Still, the cyst reduction induced by the higher dosage combination (50+50 mg/kg/day) in mice on either suppressive regimen was close to significant compared with the respective controls (DXM+CA, $P = 0.07$; DXM, $P = 0.058$).

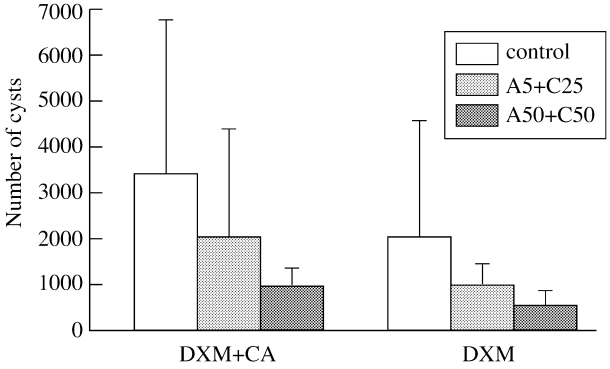

Figure 4. Brain cysts (mean ± S.D.) in chronically infected mice immunosuppressed with DXM alone or combined with CA for 3 weeks and treated with combinations of ATO and CLI (5+25 and 50+50 mg/kg/day) for 4 weeks. Chronic infection was established by inoculation of 10 cysts of the Me49 strain of *T. gondii* 3 months before immunosuppressive treatment. Control, untreated infected immunosuppressed mice.

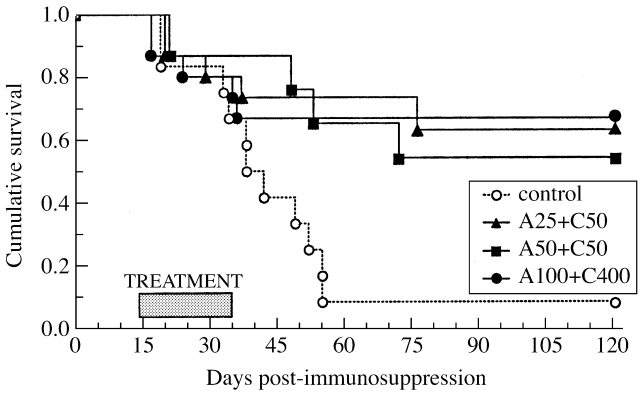

Figure 5. Effect of a 3 week course of treatment with combinations of ATO and CLI (25+50, 50+50 and 100+400 mg/kg/day) on the survival (Kaplan–Meier estimates) of chronically infected mice immunosuppressed with DXM for 2 weeks. Chronic infection was established by inoculation of 10 *T. gondii* cysts of the Me49 strain 3 months before immunosuppressive treatment.

Clearly, a 3 week suppression with DXM both alone and combined with CA induced recrudescence of *T. gondii* that was hard to treat, particularly with low dosages of ATO+CLI (5+25 mg/kg/day) even if administered for as long as 4 weeks. Since in the acute infection model a good effect of ATO combined with CLI was shown when the dosage of ATO was at least 25 mg/kg/day, a final experiment was performed in which mice pre-treated with DXM for only 2 weeks were treated with ATO+CLI 25+50, 50+50 and 100+400 mg/kg/day for 3 weeks. Mice were observed for 120 days after the beginning of immunosuppressive treatment. Compared with the 9% survival rate in untreated immunosuppressed mice (Figure 5), treatment significantly enhanced survival, with protection rates ranging from 55% to 67%, depending on the regimen ($P < 0.05$ for all groups, log rank test). On the other hand, there were no significant variations either among different treatment groups taken together ($P = 0.939$, multiple sample test) or between any two groups ($P > 0.05$).

Increased survival in mice treated with any of the ATO+CLI 25+50, 50+50 and 100+400 mg/kg/day regimens was associated with a significant reduction of the cyst burdens (taking into account all mice dying during the treatment/observation period or killed at the end of the experiment) (Figure 6a) compared with untreated mice ($P = 0.017$, 0.0001 and 0.0001, respectively). However, comparison of the cyst burdens between the end of the treatment (day 36) and the end of the 120 day observation period (Figure 6b) showed that in mice on each course of treatment the cyst burdens continued to decrease over time; this decrease was highly significant with the highest dosage regimen ($P = 0.0003$). In contrast, the cyst burdens in the untreated mice increased over time ($P < 0.0001$). As a result, the cyst counts in all treated mice, already reduced at the end of treatment, were seven- to 14-fold lower than in untreated mice by day 120 ($P < 0.0001$). This indicates that a short-course (3 week) treatment with combinations of ATO and CLI has a long-term (at least 3 month) effect on renewed cyst formation in immunosuppressed mice.

Discussion

The results presented here clearly demonstrate the anti-*T. gondii* activity of the combination of ATO and CLI in murine infection induced with a mouse-non-virulent (type-2) parasite

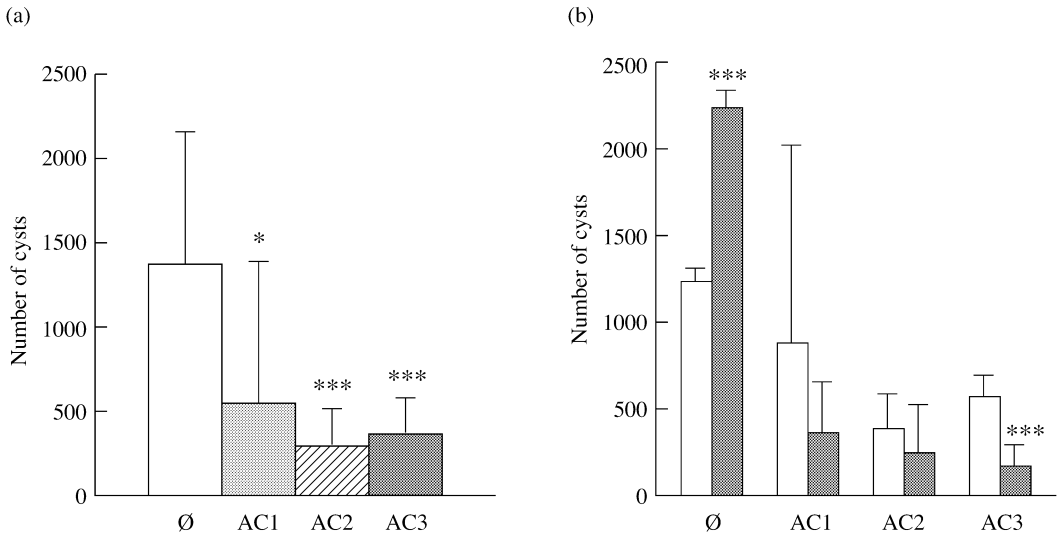

Figure 6. Brain cysts (mean ± S.D.) in chronically infected mice immunosuppressed with DXM for 2 weeks and treated with combinations of ATO and CLI (AC1, AC2 and AC3, 25+50, 50+50 and 100+400 mg/kg/day, respectively) for 3 weeks in (a) all mice irrespective of the time of death/sacrifice (*$P < 0.05$ and ***$P < 0.001$ versus control mice) and (b) mice killed immediately after treatment (day 36) (white bars) and at the end of the experiment (day 120) (black bars) (***$P < 0.001$ between time points). Chronic infection was established by inoculation of 10 cysts of the Me49 strain of *T. gondii* 3 months before immunosuppressive treatment. Ø, control (untreated infected immunosuppressed) mice.

strain. In all models, i.e. in acute, chronic and reactivated toxoplasmosis, the combined drugs were effective in terms of both significantly increased survival and decreased brain cyst burdens compared with no treatment.

In acutely infected mice, however, treatment with combinations of ATO and CLI, in addition to protecting 100% of the mice, significantly reduced the cyst burden compared with treatment with CLI alone, but not with ATO alone, indicating that in this model the most effective component of the combination was ATO. ATO both alone and combined with CLI greatly reduced the brain cyst burden, which remained extremely low weeks after treatment, even below 10 in some, as shown by their apparent absence on direct examination. However, positive bioassays in all such cases indicate that the drugs appear, at least under the experimental conditions used (duration of treatment, drug dosages), incapable of eradicating the parasite.

On the other hand, in chronically infected mice, ATO combined with CLI significantly reduced the brain cyst load compared not only with no treatment but also with either drug alone. A stronger effect of the combined drugs as opposed to a similarly insignificant reduction of the parasite burden by each drug alone, in view of the known activity of ATO against cysts, probably reflects the natural history of infection with the Me49 strain; this strain tends continuously to give rise to new cyst formation, presumably preceded by cyst rupture and proliferation of tachyzoites that are then converted into bradyzoites; CLI is active against these tachyzoites, reducing their number and hence reducing the number of newly formed cysts.

The discrepancy between the effect of ATO in acute infection, where it reduced the brain cyst burden comparably to the combined drugs, and its lower efficacy in 3-month-old chronic infections, may be explained by ATO being more effective against metabolically active immature bradyzoites than against mature ones.[8]

Most importantly, ATO combined with CLI showed activity in models of corticoid drug-induced recrudescence of *T. gondii*, as shown by significantly increased brain cyst burdens in immunosuppressed mice. This activity was dependent directly on the drug dosage and indirectly on the level of immunosuppression. In immunosuppression induced by pre-treatment with both DXM and CA, survival was lower and brain cyst burdens higher than in mice suppressed with DXM alone. The lowest dosages of the drugs required to achieve a protective effect were ATO 25 and CLI 50 mg/kg/day, which is similar to what we had shown in infection induced with the RH strain of *T. gondii*.[22] Duration of corticoid drug administration constituted an important variable of the immunosuppressive protocol. Thus, in mice pretreated with DXM for 2 and 3 weeks, courses of treatment with ATO+CLI 50+50 mg/kg/day protected 67% and 30%, respectively. Therefore, treatment was more likely to reduce the parasite load below critical levels when initiated after 2 rather than 3 weeks of DXM administration. This is consistent with the reported DXM-induced release of parasites from pre-existing cysts and new cyst formation as early as after 6–12 days of administration.[25] Another important observation was that, when given at effective dosages, the effects of combinations of ATO and CLI were long-term, as shown by the tendency of the brain cyst loads, which increase over time in untreated mice, to continue decreasing for months after the end of treatment. Without attempting to speculate on the underlying reasons, this finding may be of the greatest significance from a clinical standpoint.

Even several-fold lower cyst burdens were not always statistically significant, due, as mentioned, to the wide standard deviations, which probably reflect the variations in the number of bradyzoites within initial cysts (between a few and several hundred),[26] and subsequent variability in new cyst formation.

In conclusion, the demonstrated efficacy of ATO combined with CLI in murine infection with a cystogenic strain of *T. gondii*, taken together with our previous findings of synergic activity of the same drug combination against infection induced with the RH strain, clearly shows the anti-*T. gondii* potential of this drug combination. In as much as findings in animal models may be extrapolated to the human situation, given that ATO is generally well tolerated[13,14] and, moreover, that in combination the drugs may be used at dosages lower than when used alone, which may reduce the rare adverse side-effects of CLI,[16,17] the combination of ATO and CLI may offer an efficient yet safe alternative for the treatment of toxoplasmosis in both immunocompetent and immunosuppressed individuals, something that warrants clinical evaluation.

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