*Biochemical Pharmacology*, Vol. 41, No. 5, pp. 815–820, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00


# EFFICACY OF COMBINATION THERAPY WITH TUBERCIDIN AND NITROBENZYLTHIOINOSINE 5'-MONOPHOSPHATE AGAINST CHRONIC AND ADVANCED STAGES OF SCHISTOSOMIASIS

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(*Received* 2 *July* 1990; *accepted* 13 *September* 1990)

**Abstract**—The efficacy of the highly selective antischistosomal combination chemotherapy with tubercidin (7-deazaadenosine) plus nitrobenzylthioinosine 5'-monophosphate (NBMPR-*P*), [el Kouni *et al.*, *Proc Natl Acad Sci USA* **80**: 6667–6670, 1983; el Kouni *et al.*, *Biochem Pharmacol* **36**: 3815–3821, 1987] was examined against chronic and advanced stages of schistosomiasis in mice. Administration of four successive daily doses of tubercidin (5 mg/kg/day) plus NBMPR-*P* (25 mg/kg/day) to *Schistosoma mansoni*-infected mice beginning 5, 6, 7 and 8 weeks post-infection and monitored for 22 weeks was very effective against the parasite. It resulted in a marked increase in survivorship of treated mice. Repetition of the dose-regimen after a 10-day rest period was even more effective. However, survivorship of infected animals decreased with the delay of therapy. Early treatment (5 weeks post-infection) resulted in 100% survival compared to 13% only for untreated animals. If therapy was instituted at 8 weeks post-infection, only 70% of the treated mice survived. Treated animals appeared healthy and were found to have less splenomegaly and hepatomegaly. Combination therapy also caused a significant reduction in the number of worms as well as the number of eggs in the liver and small intestine. However, these differences diminished as the treatment was delayed. The number of eggs in the liver was reduced from an average of 120,000 eggs per liver in untreated animals to approximately 16,000 eggs per liver when treated at 5 weeks post-infection. When treatment was delayed to 8 weeks post-infection, the reduction in liver egg count was not as dramatic (88,000 eggs per liver). Similarly, the number of eggs was reduced in the intestine from 1,759 to an average of 58 and 860 eggs per $cm^2$ of the intestine when the mice were treated at 5 and 8 weeks post-infection respectively. However, some worms survived and resumed egg production after an extended period of recuperation. Histological examination indicated that combination therapy was effective in preventing the formation of new egg granulomas but not on pre-existing granulomas.

We have demonstrated previously that nitrobenzylthioinosine 5'-monophosphate (NBMPR-*P**), a prodrug of nitrobenzylthioinosine (NBMPR) which is a potent inhibitor of nucleoside transport in mammalian cells, does not inhibit the uptake of several potential antischistosomal adenosine analogues, including tubercidin (7-deazaadenosine), by the parasite *Schistosoma mansoni* [1, 2]. Therefore, by coadministration of NBMPR-*P* (or NBMPR) with tubercidin to *S. mansoni*-, *S. japonicum*- or *S. haematobium*-infected mice, tubercidin becomes selectively toxic against the parasite [1, 3–6]. NBMPR-*P* protects the host but not the parasite from tubercidin toxicity [1, 3–5]. The combination of tubercidin plus NBMPR-*P* causes no host mortality or toxicity [1, 3–5] but results in significant worm kill, termination of oviposition, and arresting the progress of lesions associated with schistosomiasis [1, 3, 4]. Recently, it was demonstrated that the effectiveness and selectivity of combination therapy with tubercidin plus NBMPR-*P* are similar to those obtained by the use of praziquantel [6], the current drug of choice for the treatment of schistosomiasis.

In addition to schistosomiasis [1, 3–6], malaria [7–9] and trypanosomiasis [10] were also treated effectively with the combination of tubercidin plus NBMPR-*P*. Furthermore, the insensitivity to inhibitors of nucleoside transport in mammalian cells seems to be a common property of nucleoside transport among parasites or parasite-infected cells [1, 2, 7–9, 11–13]. Therefore, the mode of host-protection by nucleoside transport inhibitors in combination with purine nucleoside analogues may provide an attractive alternative for the treatment of parasitic diseases, especially in cases where drug resistance is developed and alternative effective drugs are not available. However, there is no information on the efficacy of such combinations against chronic and advanced stages of parasitic infections or its long-term effectiveness. In the present investigation, we examined the effect of the combination of tubercidin plus NBMPR-*P* on advanced stages of schistosomiasis by administering the drug combination at 5, 6, 7 and 8 weeks post-infection in heavily infected mice and monitored the effectiveness of combination therapy in the long-term progress of the disease, i.e. for 22 weeks post-infection.

* Abbreviations: NBMPR, nitrobenzylthioinosine or 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; and NBMPR-*P*, nitrobenzylthioinosine 5'-monophosphate.

## MATERIALS AND METHODS

NBMPR-*P* was a gift from Dr. A. R. P. Paterson,

Cancer Research Unit (McEachern Laboratory), University of Alberta, Edmonton, Alberta, Canada. Tubercidin was purchased from the Sigma Chemical Co., St. Louis, MO. Female CD1 mice (20–25 g) were obtained from Charles River Laboratories, Wilmington, MA.

The life cycle of *S. mansoni* (Puerto Rico strain) is maintained using mice as the primary host and *Biomphalaria glabrata* (Puerto Rico Strain 2), as the vector snail, as previously described [1].

Mice were infected with *ca.* 250 cercariae/mouse to achieve the symptoms of severe and chronic infection in a reasonable period of time (7–8 weeks). Mice were divided into three main groups with at least 60 mice in each. The first group was the untreated controls. The other two groups, Groups A and B, received a daily injection of tubercidin (5 mg/kg/day) and NBMPR-*P* (25 mg/kg/day) for 4 successive days. Group B received a second series of four successive daily injections following a 10-day rest period. The drugs were dissolved in normal saline solution (0.9% NaCl) and injected simultaneously in a proportion of 0.2 mL/20 g body weight as described previously [1, 3, 4]. Treatment within Groups A and B began at 5, 6, 7, or 8 weeks post-infection. At each time point, a minimum of ten mice were chosen at random from each group, treated and then kept in separate cages. The 5 weeks post-infection period was chosen as the time for earliest treatment because it allows development of gametogenesis, pairing of adult worms, and oviposition [14]. The presence of mature worms was confirmed by quantitative examination of five mice before initiation of therapy. Five untreated mice were also killed and examined at 5, 6, 7 and 8 weeks post-infection as further controls for the various treatment groups (Tables 1–3).

Survivorship of mice was monitored daily up to 22 weeks post-infection, after which all surviving mice were killed. The following parameters were examined: the number, sex, copulatory status and morphology of worms recovered by portal perfusion [15] or by hook dissection of the portal and mesenteric veins [16]; the weight and histological appearance of the liver and the spleen; and the number of eggs in the liver and the number and developmental stage of the eggs in the small intestine. The number of eggs per liver was determined after overnight digestion at 37° in a solution of 1% KOH and 0.9% NaCl [17]. The number and developmental stages of eggs per $cm^2$ of the intestine were obtained by averaging the eggs counted in three 2-cm segments from the small intestine using the protocol previously described [18].

Tissues were collected from sacrificed animals and fixed immediately in 10% buffered formalin acetate at pH 7.0 to 7.1 and stored at 4° until section preparation. Tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

## RESULTS

Figure 1 and Tables 1–3 show the effects of tubercidin plus NBMPR-*P* combination administered 5, 6, 7 and 8 weeks post-infection on the different parameters of infection under investigation. Exposure to 250 cercariae is a profound challenge to the mice and only 13% of the untreated mice were able to survive the full 22 weeks. The majority of the mice died within 8 weeks after infection (Fig. 1). Treatment with tubercidin plus NBMPR-*P* dramatically increased the life span of treated mice even when administered as late as 8 weeks post-infection. The data also indicate that two treatments (Group B) were more effective than one treatment (Group A). However, the rescue of animals from schistosomiasis decreased with the delay of therapy. Early treatment (5-week) in Group B resulted in 100% survival at 22 weeks post-infection. If therapy was instituted at 8 weeks post-infection, only 70% of the treated mice survived (Table 1).

Untreated mice showed characteristic symptoms and signs of schistosomiasis such as numerous egg granulomas, enlarged liver and spleen (Table 1), and many eggs in all stages of development in the liver and small intestines (Table 3). The severity of these symptoms increased with time. Histological examination of the liver, spleen, and intestine of untreated mice, after 8 or 22 weeks of infection, showed that the normal architecture of these tissues was grossly distorted by the fibrotic response as a result of the overwhelming accumulation of eggs. After 22 weeks of infection, the normal intestinal mucosa was frequently destroyed.

In contrast, combination therapy caused a striking reduction in the number and pairing of worms (Table 2) as well as the number of eggs in the liver and intestine (Table 3). Furthermore, mice treated with tubercidin plus NBMPR-*P* appeared healthy and showed less enlargement of livers and spleens than the untreated controls at 22 weeks (Table 1). These differences diminished as the treatment was delayed. The number of eggs in the liver was reduced from an average of 120,000 eggs per liver in untreated animals to approximately 16,000 eggs per liver when treated at 5 weeks post-infection. When treatment was delayed to 8 weeks post-infection, the reduction in liver egg count was not as dramatic (88,000 eggs per liver). Similarly the number of eggs was reduced in the intestine from 1,759 to an average of 58 and 860 eggs per $cm^2$ of the intestine when the mice were treated at 5 and 8 weeks post-infection respectively (Table 3). However, in contrast to our earlier studies monitored for only 10 weeks [1, 3, 4], live eggs in various stages of development were found (Table 3), indicating that oviposition had ceased immediately after treatment and then resumed as the few surviving worms recuperated.

Chemotherapy of mice at 5, 6, 7 or 8 weeks post-infection also resulted in an improved histological appearance compared to the controls. Mice receiving therapy at 5 weeks post-infection were almost totally devoid of granulomas in the liver, intestine, or spleen. Damaged worms were found in the liver parenchyma, indicating that affected parasites died *in situ*. If treatment was delayed until 8 weeks post-infection, the majority of mice still succumbed from severe organ damage which had already become advanced by week 8 post-infection and only a modest increase in percentage of survivors was observed (Fig. 1, Table 1). Nevertheless, even late treatment was partially effective; the drug combination allowed

Table 1. Effect of the time of initiation of therapy with the combination of tubercidin (5 mg/kg/day) plus NBMPR-*P* (25 mg/kg/day) on the survival and weights of liver and spleen of mice infected with *Schistosoma mansoni*

| Week post-infection | Number of mice surviving at 22 weeks | Weight* (g) | |
|---|---|---|---|
| | | Liver | Spleen |
| | Untreated controls | | |
| Killed at: | | | |
| 5-week† | | 1.74 ± 0.15 | 0.23 ± 0.06 |
| 6-week† | | 1.90 ± 0.32 | 0.31 ± 0.08 |
| 7-week† | | 2.74 ± 0.62 | 0.44 ± 0.11 |
| 8-week† | | 2.87 ± 0.97 | 0.56 ± 0.20 |
| 22-week | 4/30 | 4.53 ± 1.28 | 1.23 ± 0.47 |
| | Treatment groups‡ | | |
| Therapy initiated at: | | | |
| Group A | | | |
| 5-week | 3/10 | 2.22 ± 0.31§ | 0.24 ± 0.13§ |
| 6-week | 6/10 | 2.78 ± 0.60§ | 0.47 ± 0.22§ |
| 7-week | 5/18 | 3.26 ± 0.59 | 0.75 ± 0.28 |
| 8-week | 5/25 | 3.67 ± 0.56 | 1.04 ± 0.19 |
| Group B | | | |
| 5-week | 10/10 | 2.46 ± 0.75§ | 0.37 ± 0.26§ |
| 6-week | 7/10 | 2.06 ± 0.27§ | 0.30 ± 0.04§ |
| 7-week | 13/17 | 3.08 ± 0.57 | 0.75 ± 0.28§ |
| 8-week | 9/13 | 4.41 ± 1.35 | 0.81 ± 0.34 |

* Values are means ± SD.

† Parameters were collected from five untreated mice.

‡ Group A received daily intraperitoneal injection for 4 days. Group B received a second series of another four daily injections after a 10-day rest period. Surviving animals were killed at 22 weeks post-infection.

§ Significantly different ($P < 0.05$) from untreated controls killed at 22 weeks post-infection.

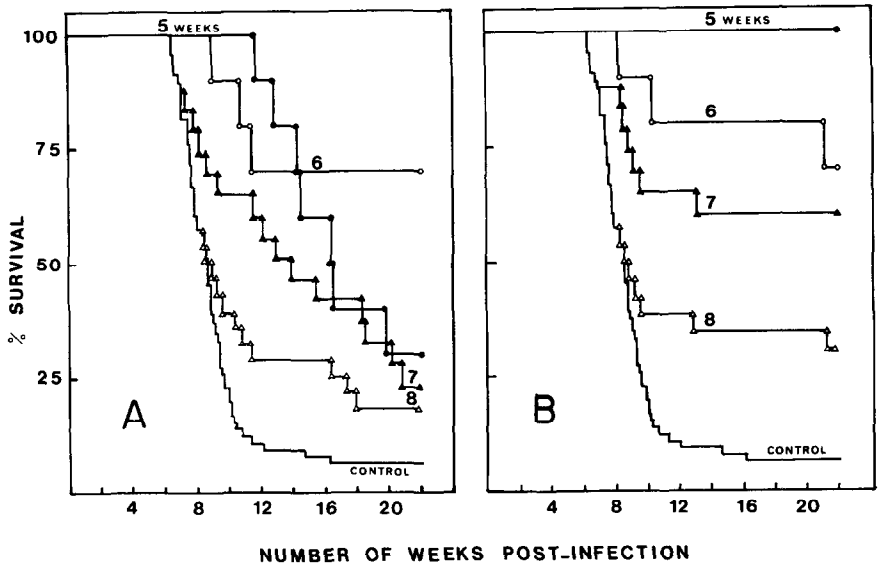


Fig. 1. Effect of combination therapy with tubercidin (5 mg/kg/day) plus NBMPR-*P* (25 mg/kg/day), initiated at 5, 6, 7 or 8 weeks post-infection, on percent survival of infected-mice monitored for 22 weeks after infection. (A) Mice received daily intraperitoneal injection for 4 successive days. (B) Mice received a second series of another four successive daily injections after a 10-day rest period. Groups A and B consisted of at least 80 mice each.

Table 2. Effect of the time of initiation of therapy with the combination of tubercidin (5 mg/kg/day) plus NBMPR-*P* (25 mg/kg/day) on the number and copulation of worms recovered, by hook dissection or by portal perfusion, from mice infected with *Schistosoma mansoni*

| Week post-infection | Recovered by hook dissection* | | | | Recovered by portal perfusion* | |
|---|---|---|---|---|---|---|
| | Worms per surviving mouse | Mesenteric veins | Portal vein | No. *in copula* | Worms per surviving mouse | No. *in copula* |
| | | | Untreated controls | | | |
| Killed at: | | | | | | |
| 5-week† | 51.0 ± 26.5 | 34.5 ± 20.1 | 16.2 ± 9.2 | 49.0 ± 26.6 | 78.0 ± 41.9 | 50.5 ± 25.8 |
| 6-week† | 80.2 ± 64.0 | 64.0 ± 19.4 | 16.2 ± 6.8 | 74.8 ± 20.4 | 126.0 ± 56.5 | 88.5 ± 54.4 |
| 7-week† | 77.4 ± 60.8 | 64.2 ± 54.8 | 13.2 ± 8.0 | 76.8 ± 60.3 | 139.0 ± 66.8 | 91.5 ± 64.8 |
| 8-week† | 87.6 ± 16.9 | 61.8 ± 15.7 | 25.8 ± 13.6 | 86.0 ± 15.6 | 85.7 ± 34.2 | 66.0 ± 31.4 |
| 22-week | 38.0 ± 21.5 | 30.5 ± 16.1 | 7.5 ± 5.7 | 36.5 ± 10.0 | ND‡ | ND |
| | | | Treatment groups§ | | | |
| Therapy started at: | | | | | | |
| Group A: | | | | | | |
| 5-week | 13.0 ± 8.5 | 9.0 ± 4.2 | 4.0 ± 4.2 | 4.0 ± 2.8 | 29.0 | 4.0 |
| 6-week | 10.0 ± 6.5 | 7.8 ± 6.2 | 2.3 ± 1.7 | 7.0 ± 6.2 | 3.0 ± 2.7 | 2.0 ± 1.4 |
| 7-week | 19.5 ± 10.5 | 13.5 ± 5.1 | 6.0 ± 5.8 | 11.0 ± 2.6 | 14.0 ± 12.3 | 10.0 ± 8.5 |
| 8-week | 22.0 ± 11.1 | 19.7 ± 9.6 | 2.3 ± 1.5 | 20.7 ± 12.2 | 33.0 ± 42.4 | 7.0 ± 7.1 |
| Group B: | | | | | | |
| 5-week | 12.6 ± 9.4 | 9.4 ± 8.5 | 3.3 ± 2.0 | 9.3 ± 10.3 | 11.5 ± 5.0 | 2.0 ± 2.8 |
| 6-week | 1.0 ± 2.0 | 0.0 | 1.0 ± 2.0 | 0.0 | 0.0 | 0.0 |
| 7-week | 14.7 ± 11.1 | 10.9 ± 10.8 | 3.8 ± 4.0 | 10.4 ± 10.2 | 14.0 ± 8.5 | 3.5 ± 3.4 |
| 8-week | 32.8 ± 13.1 | 21.5 ± 14.8 | 11.3 ± 7.7 | 21.5 ± 13.9 | 11.5 ± 10.4 | 2.5 ± 3.8 |

* Values are means ± SD.

† Parameters were collected from five untreated mice.

‡ ND = not determined.

§ Animals were treated as described in Table 1. Worms were collected starting 22 weeks after infection. The average percentages of worms found *in copula* were 66 and 45% in treatment groups A and B, respectively, in contrast to 82% in the untreated controls.

Table 3. Effect of the time of initiation of therapy with the combination of tubercidin (5 mg/kg/day) plus NBMPR-*P*- (25 mg/kg/day) on the number of eggs and the stage of embryogenesis from mice infected with *Schistosoma mansoni**

| Week Post-infection | Number of eggs† | | % Eggs in stages of embryogenesis | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Per liver | Per $cm^2$ of intestine | 1 | 2 | 3 | 4 | 5 | Dead |
| | | Untreated controls | | | | | | |
| Killed at: | | | | | | | | |
| 5-week | 900 ± 1650 | 3 ± 3 | 83 | 17 | 0 | 0 | 0 | 0 |
| 6-week | 4450 ± 2400 | 133 ± 43 | 31 | 27 | 17 | 9 | 6 | 10 |
| 7-week | 8300 ± 9950 | 303 ± 357 | 17 | 32 | 16 | 8 | 15 | 12 |
| 8-week | 39,950 ± 10,800 | 849 ± 246 | 7 | 16 | 13 | 19 | 25 | 20 |
| 22-week | 120,650 ± 59,000 | 1759 ± 793 | 4 | 6 | 8 | 4 | 13 | 65 |
| | | Treatment groups‡ | | | | | | |
| Therapy started at: | | | | | | | | |
| Group A: | | | | | | | | |
| 5-week | 16,250 ± 9150§ | 58 ± 58§ | 3 | 9 | 2 | 7 | 4 | 76 |
| 6-week | 42,950 ± 26,550§ | 169 ± 143§ | 2 | 10 | 13 | 3 | 6 | 66 |
| 7-week | 77,200 ± 16,200 | 422 ± 173§ | 2 | 3 | 7 | 3 | 5 | 80 |
| 8-week | 78,000 ± 35,250 | 860 ± 355§ | 5 | 5 | 3 | 1 | 8 | 78 |
| Group B: | | | | | | | | |
| 5-week | 16,150 ± 10,850§ | 56 ± 39§ | 2 | 1 | 2 | 6 | 5 | 84 |
| 6-week | 21,150 ± 19,850§ | 246 ± 329§ | 7 | 4 | 6 | 2 | 14 | 66 |
| 7-week | 64,000 ± 31,150§ | 313 ± 281§ | 4 | 4 | 8 | 10 | 6 | 68 |
| 8-week | 88,850 ± 39,900 | 701 ± 404§ | 4 | 2 | 3 | 1 | 4 | 86 |

* The number of eggs per liver was estimated as described by Cheever [17]. Estimates of eggs per $cm^2$ of intestine were made from egg counts in three 2-cm segments of the small intestine. Stages of embryogenesis are those described by Pellegrino *et al.* [18].

† Values are means ± SD.

‡ Animals were treated as described in Table 1. Measurements were made starting 22 weeks post-infection.

§ Significantly different ($P < 0.05$) from untreated controls killed at 22 weeks post-infection.

the resolution of much of the liver and intestine pathology. Following treatment at 8 weeks post-infection, numerous worm fragments were found in liver portal veins. Eggs found in the 8-week-treated mice were generally dead and condensed into marginal aggregates. Such changes were seen in the liver, spleen and intestine. In a few surviving mice, the intestine was essentially free of eggs, or egg tubercles, while in others evidence of resumption of egg laying was seen, along with dead egg clusters. Some worms were seen still alive in the liver 13 weeks after chemotherapy; however, their appearance was not normal (e.g. absent integumentary tubercles in males, and rudimentary vitelline gland in females).

## DISCUSSION

The present results demonstrate that the combination of tubercidin plus NBMPR-*P* was effective against chronic and advanced stages of schistosomiasis as well as against heavy infection in mice. It significantly increased the life span and reduced the worm and egg burden of the treated animals. Previous studies showed that chemotherapy with tubercidin plus NBMPR-*P* is highly selective against early and moderate *S. mansoni*-, *S. japonicum*- and *S. haematobium* infections in mice [1, 3–6]. As in the present study, it produced high worm lethality, terminated oviposition and significantly reduced the total number of eggs in the liver and small intestine [1, 3, 4]. In that respect, the combination of tubercidin plus NBMPR-*P* is comparable to praziquantel [6], the drug of choice for the treatment of this disease.

The selectivity of the tubercidin plus NBMPR-*P* combination is attributed to the fact that NBMPR-*P* is a prodrug of NBMPR which inhibits nucleoside transport in most mammalian cells [19] but not in schistosomes [1, 2]. Thus, it protects the host but not the parasite from the lethal effects of tubercidin and produces the high selective toxicity against the parasite. The lack of host toxicity was confirmed by histological examination, hematological studies and blood chemistry [5].

Histological studies indicate that although combination therapy reduces the diffuse inflammatory process and the number of granulomas, it does not reverse or prevent the progression of the inflammatory and fibrotic tissue changes already underway before treatment is initiated. This is in agreement with the findings of Sadun *et al.* [20]. However, Warren [21] showed that treatment with stibophen results in rapid disappearance of inflammatory cells within 5 weeks, followed by resorption of fibrous tissues within 15–20 weeks, and disappearance of most of the granulomas by 1 year, at which time only occasional, small areas of fibrosis are noted. The presence of typical egg granulomas in the spleen, liver, intestines and kidney suggests that in the heavy-infected state no organ is spared and that the presence or absence of pathology in any one organ may simply be a reflection of the degree of infection rather than of the lack of vulnerability of that organ.

Although the present study shows that a two-course regimen with a 10-day interval in between cycles effectively killed the worms or reduced their oviposition through week 22, a few surviving worms eventually recovered and resumed oviposition. Therefore, in order to achieve more effective worm killing, the total number of courses of treatment with 10-day rest periods in between could be increased. At least three courses of such therapy have been given to non-infected mice without any lethality (unpublished results). How individual schistosomes avoid the lethal effect of tubercidin is presently unknown. The residual worm survival after treatment (21%) is not unique to the combination therapy with tubercidin plus NBMPR-*P*. Treatment with praziquantel, the current drug of choice for the treatment of schistosomiasis, results in 65–80% kill of adult worms in *S. mansoni*-infected mice [22] compared to an average of 79% kill in the present study (Table 2). In fact, a recent study reported that there is no significant difference between the efficacy of praziquantel and tubercidin plus NBMPR-*P* in the treatment of *S. mansoni*- or *S. haematobium*-infected mice [6].

In conclusion, the present results indicate that combination therapy with tubercidin plus NBMPR-*P* is effective against chronic and advanced schistosomiasis as evidenced by worm kill and reduction of the numbers of granulomatous lesions in the various host organs as well as the prevention of new granulomas in mice. A two-course regimen was more efficacious than a one-course regimen. However, combination therapy was unable to induce regression or prevent the evolution of egg granulomas already initiated when treatment commenced. Finally, the success and simplicity of host protection from the toxicity of potential antiparasitic nucleoside analogues by nucleoside transport inhibitors for the treatment of schistosomiasis [1, 3–6] as well as of *Plasmodium falciparum* [8, 9], *P. yoelii* [7] and *Trypanosoma gambiense* [10] infections suggest that this chemotherapeutic approach for the treatment of parasitic diseases may be useful especially in cases where drug resistance is encountered and no satisfactory alternative drugs are available.

*Acknowledgements*—The author thanks Dr. Alfred W. Senft for many helpful discussions and Norma J. Messier for excellent technical assistance. This research was supported by Grant AI-22219 from the NIAID, DHHS and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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