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6-Benzylthioinosine analogues: Promising anti-toxoplasmic agents as inhibitors of the mammalian nucleoside transporter ENT1 (*es*)

Amol Gupte [a], *John K. Buolamwini* [a,**], *Vikas Yadav* [b], *Chung K. Chu* [b], *Fardos N.M. Naguib* [c], *Mahmoud H. el Kouni* [c,*]

[a] *Department of Pharmaceutical Sciences, College of Pharmacy, 847 Monroe Avenue, Suite 327, University of Tennessee Health Sciences Center, Memphis, TN 38163, USA*

[b] *Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA 30605, USA*

[c] *Department of Pharmacology and Toxicology, Center for AIDS Research, Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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ABSTRACT

Certain 6-benzylthioinosine analogues have been identified as potential chemotherapeutic agents against *Toxoplasma gondii* in cell culture and animal models. These compounds are selectively transported and metabolized by toxoplasma infected, but not uninfected, cells. In sharp contrast to mammalian nucleoside transporters, the toxoplasma adenosine/purine transporter (TgAT) allows the transport of these 6-benzylthioinosine analogues into infected cells. After entering the infected cell, these compounds act as subversive substrates for toxoplasma, but not the host, adenosine kinase (EC.2.7.1.20). Hence, 6-benzylthioinosine analogues become toxic to toxoplasma infected cells, but not uninfected host cells or animals. The basis for the lack of uptake of the anti-toxoplasmic 6-benzylthioinosines by uninfected host cells is currently unknown. These anti-toxoplasmic 6-benzylthioinosines may not be substrates for the mammalian nucleoside transporters or they may act as inhibitors of these transporters. Previous studies have shown that some 6-benzylthioinosines are inhibitors of the mammalian nucleoside transporter ENT1 (*es*). Therefore, we examined the efficacy of promising anti-toxoplasmic 6-benzylthioinosines as inhibitors of ENT1 (*es*) in an effort to elucidate the basis for the lack of uptake of anti-toxoplasmic 6-benzylthioinosines by uninfected host cells. The results showed that these compounds are inhibitors of ENT1 (*es*). In general, electron-withdrawing substituents at the *ortho*, *meta* or *para* positions of the benzyl ring improved binding. The most potent inhibitors identified were *m*- and *p*-nitro-6-benzylthioinosine, which had K_i values in the subnanomolar range. Therefore, anti-toxoplasmic 6-benzylthioinosines are not only selectively toxic to parasites and parasite infected cells, they are also inhibitors of nucleoside transport in host cells. This inhibition of the host nucleoside transport is an added advantage for these 6-benzylthioinosine analogues as anti-toxoplasmic agents. Inhibitors of nucleoside transport in mam-

* *Corresponding author*. Tel.: +1 205 934 1132; fax: +1 205 934 8240.
** *Corresponding author*. Tel. +1 901 448 7533; fax: +1 901 448 6828.
E-mail addresses: jbuolamwini@utmem.edu (J.K. Buolamwini), m.elkouni@ccc.uab.edu (M.H. el Kouni).

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malian cells can selectively protect the host from the toxicity of toxic purine nucleosides that may be used in future combination therapy against toxoplasmosis or from metabolites of the 6-benzylthioinosine analogues that may be released by the destruction of infected cells. These findings further advance the rationale for developing 6-benzylthioinosine analogues as selective therapeutic agents for the treatment of toxoplasmosis.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasitic protozoan that infects humans and many species of warm-blooded animals [1]. Approximately, a billion people worldwide, including 60% of the population in the US, are seropositive to *T. gondii*. Infection with *T. gondii* is asymptomatic (90% of cases) in the general population. However, Toxoplasmosis represents a major health problem for immunocompromised individuals such as AIDS patients, organ transplant recipient patients and the unborn children of infected mothers [1–4]. In such cases, toxoplasmic encephalitis is possibly the most recognized opportunistic infection of the central nervous system [2,3] and the most common cause of intracerebral mass lesions in AIDS patients. Congenital toxoplasmosis is as high as 1/1000 live births [3]. Effects range in severity from asymptomatic to stillbirth, with the most common ailments being retinochoroiditis, cerebral calcifications, psychomotor or mental retardation and severe brain damage [3].

The combination of sulfonamides and pyrimethamine is widely used to treat toxoplasmosis in humans. Although these drugs are helpful in the treatment of the acute stage of the disease, they usually do not eradicate infection and as many as 50% of the patients do not respond to this therapy. The combination of sulfonamides and pyrimethamine is also ineffective against toxoplasma tissue cysts. In addition, prolonged exposure to this regimen induces serious host toxicity such as bone marrow suppression and severe skin rashes forcing the discontinuation of the therapy [2–5]. Other therapies, e.g. clindamycin or atovaquone, have met with limited success particularly in the long-term management of these patients. Furthermore, there is no effective vaccine currently available for the treatment of toxoplasmosis. Therefore, it is necessary to develop new and effective drugs with significantly low host toxicity for the treatment and management of toxoplasmosis.

The rational design of a drug depends on the exploitation of fundamental biochemical or metabolic differences between pathogens and their host. In contrast to their host, *T. gondii* is a purine auxotroph incapable of de novo purine biosynthesis and depends on its salvage pathways for vital purine requirements [6–8]. For these reasons interference in purine uptake and metabolism in *T. gondii* can be selectively detrimental to the parasite. The host cells, on the other hand, can still obtain their purine requirements by their de novo pathways [cf. 9]. Indeed, biochemical, metabolic and molecular investigations [9–14] have demonstrated that the uptake and metabolism of 6-benzylthioinosine and certain analogues are toxic against toxoplasmosis in cell culture and animal models and have no toxic-side-effects on the survival of uninfected host cells or animals. There are two main reasons for this selective toxicity. First, in contrast to mammalian nucleoside transporters, toxoplasma have an adenosine/purine transporter (TgAT) which allows the transport of such 6-benzylthioinosine analogues into infected cells [9,12,15]. Second, after entering the cell, these 6-benzylthioinosine analogues act as subversive substrates for toxoplasma, but not the host, adenosine kinase (EC.2.7.1.20) [9,11–14]. Hence, the presence of a functional toxoplasma adenosine/purine transporter and adenosine kinase are prerequisites for the selective toxicity against toxoplasma infected cells [9,11–14].

The basis for the lack of uptake of the anti-toxoplasmic 6-benzylthioinosines by uninfected host cells is currently unknown. These compounds may not be substrates for the mammalian nucleoside transporters or they could be inhibitors of these transporters. Several 6-benzylthioinosines (e.g. *p*-nitro-6-benzylthioinosine, NBMPR) have been extensively investigated as inhibitors of the mammalian equilibrative nucleoside transporter ENT1 (*es*) [16]. Therefore, in an effort to elucidate the basis for the inability of uninfected host cells to uptake the 6-benzylthioinosine analogues, we examined whether or not these compounds are inhibitors of ENT1 (*es*). The results showed that these compounds are indeed inhibitors of ENT1 (*es*). In general, electron-withdrawing substituents at the *ortho*, *meta* or *para* positions of the benzyl ring improved binding. The most potent inhibitors identified were *m*- and *p*-nitro-6-benzylthioinosine, which had K_i values in the subnanomolar range.

2. Materials and methods

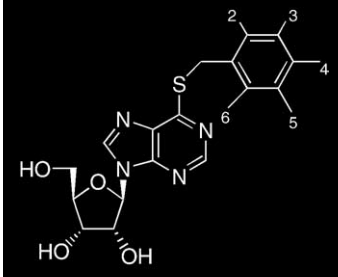
2.1. Chemicals

The 6-benzylthioinosine (6-benzyl 9-β-D-ribofuranosylpurine) analogues were synthesized as previously described [13]. The chemical structures of these compounds are shown in Table 1. All other chemicals and compounds were obtained from Sigma Chemical Co. or Fisher Scientific.

2.2. Estimation of the binding affinity ENT1 (es)

The ENT1 (*es*) nucleoside transporter binding affinity of the compounds was evaluated using a flow cytometric assay as previously described [17]. Human K562 leukemia cells grown in RPMI 1640 medium were washed once and resuspended at 1.6×10^5 cells/mL in phosphate buffered saline at pH 7.4, and incubated at room temperature for 45 min, with SAENTA (5′-*S*-(2-aminoethyl)-N^6-(4-nitrobenzyl)-5′-thioadenosine)-X8-

Table 1 – Flow cytometrically determined IC_{50} and K_i values for inhibition of SAENTA-fluorescein binding to the ENT1 transporter in K562 cells of 6-benzylthioinosine and its substituted analogues



Compound	2	3	4	5	6	IC_{50} (nM)	ENT1 K_i (nM)
1. 6-Benzylthioinosine	–H	–H	–H	–H	–H	362.3	20.1
2. *o*-Fluoro-6-benzylthioinosine	–F	–H	–H	–H	–H	350.9	19.9
3. *o*-Chloro-6-benzylthioinosine	–Cl	–H	–H	–H	–H	1757	99.5
4. *o*-Methyl-6-benzylthioinosine	$-CH_3$	–H	–H	–H	–H	285.7	16.2
5. *m*-Nitro-6-benzylthioinosine	–H	$-NO_2$	–H	–H	–H	4.81	0.27
6. *m*-Methyl-6-benzylthioinosine	–H	$-CH_3$	–H	–H	–H	1181	66.9
7. *m*-Trifluoromethyl-6-benzylthioinosine	–H	$-CF_3$	–H	–H	–H	334.5	18.9
8. *p*-Fluoro-6-benzylthioinosine	–H	–H	–F	–H	–H	159.3	9.02
9. *p*-Chloro-6-benzylthioinosine	–H	–H	–Cl	–H	–H	251.3	14.2
10. *p*-Bromo-6-benzylthioinosine	–H	–H	–Br	–H	–H	176.2	9.98
11. *p*-Cyano-6-benzylthioinosine	–H	–H	–CN	–H	–H	81.23	4.60
12. *p*-Nitro-6-benzylthioinosine	–H	–H	$-NO_2$	–H	–H	7.22	0.40
13. *p*-Methyl-6-benzylthioinosine	–H	–H	$-CH_3$	–H	–H	267.2	15.2
14. *p*-Methoxy-6-benzylthioinosine	–H	–H	$-OCH_3$	–H	–H	612.5	34.7
15. *p*-Trifluoromethoxy-6-benzylthioinosine	–H	–H	$-OCF_3$	–H	–H	124.3	7.03
16. *p*-*tert*-Butyl-6-benzylthioinosine	–H	–H	–*tert*-Butyl	–H	–H	333680	18895
17. *p*-Acetoxy-6-benzylthioinosine	–H	–H	$-COOCH_3$	–H	–H	819.1	46.4
18. 2,4-Dichloro-6-benzylthioinosine	–Cl	–H	–Cl	–H	–H	77.15	4.36
19. 3,4-Dichloro-6-benzylthioinosine	–H	–Cl	–Cl	–H	–H	222.8	12.6
20. 2-Chloro-6-fluoro-6-benzylthioinosine	–Cl	–H	–H	–H	–F	1529	86.6
21. 2,4,6-Trimethyl-6-benzylthioinosine	$-CH_3$	–H	$-CH_3$	–H	$-CH_3$	33420	1892

fluorescein, (30 nM) in the presence or absence of varying concentrations of the test compounds. Flow cytometric measurements of cell associated fluorescence were then performed with a FACSCalibur (Becton Dickinson, San Jose, CA) equipped with a 15 mW argon laser (Molecular Resources Flow Cytometry Facility, University of Tennessee Health Sciences Center). In each assay, 5000 cells were analyzed from suspensions of 5×10^5 cells/mL. The units of fluorescence were arbitrary channel numbers. Percentage (%) of control (i.e. *es* transporter-specific fluorescence in the presence of SAENTA-fluorescein without test compounds) was calculated for each sample using Eq. (1).

$$\text{control}\,(\%) = \text{SF}_\text{s} \times \frac{100}{\text{SF}_\text{f}} \quad (1)$$

where SF_s is the *es* transporter-specific fluorescence of test samples and SF_f is the *es* transporter-specific fluorescence of the SAENTA-fluorescein ligand standard in mean channel numbers.

The results obtained were entered in the PRISM program (GraphPad, San Diego, CA) to derive the concentration dependent curves (examples are shown in Fig. 1). From these curves, the IC_{50} values were computed and used to calculate inhibition constants (K_i) values from Eq. (2).

$$K_\text{i} = \text{IC}_{50}/(1 + [L]/K_\text{L}) \quad (2)$$

where [L] and K_L are the concentration and the K_d value of SAENTA-fluorescein, respectively.

The K_i values can be used to compare the abilities of the new compounds to displace the *es* transporter-specific ligand 5-(SAENTA)-X8-fluorescein, and for that matter their affinity for the *es* (ENT1) transporter.

3. Results and discussion

The 6-benzylthioinosine analogues were tested as *es* transporter binding ligands by a facile competitive binding flow cytometric assay using the K562 chronic myelogenous leukemia cell line. The high-affinity *es* transporter fluorescent ligand, SAENTA-X8-fluorescein, was used as the competitive ligand to be displaced by the test compounds. The data presented in Fig. 1 show that the 6-benzylthioinosine analogues exhibited a wide range of binding affinities at the *es* transporter as measured by their ability to displace the *es* nucleoside transporter-specific ligand. The data in Table 1 show the binding affinities (K_i values) of the different 6-benzylthioinosine analogues ranging from subnanomolar concentrations for compound **5** (K_i = 0.27 nM) to low micromolar concentrations for compound **16** (K_i = 18.0 μM). This represents an approximately 70,000-fold difference.

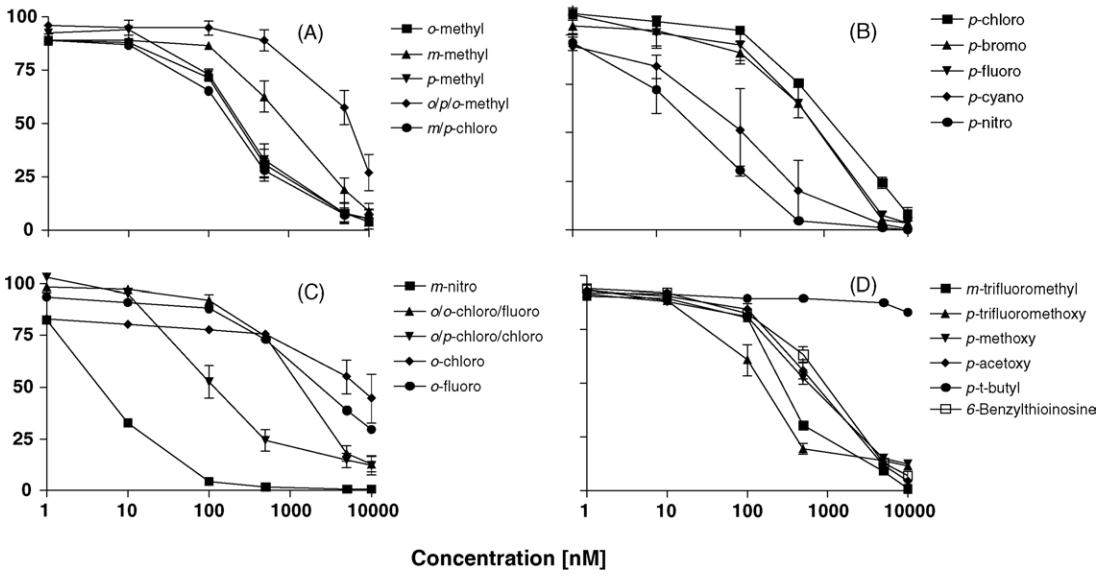


Fig. 1 – Competitive displacement of the ENT1 nucleoside transporter ligand SAENTA-X8-fluorescein from K562 cells by benzylthioinosine analogues. Cells were harvested and incubated with 30 nM SAENTA-fluorescein in the presence or absence of varying concentration of the inhibitor for 45 min at room temperature, and analyzed by flow cytometry. Data analysis was performed on 5000 cells per sample, and data points are the mean and ranges for duplicate samples. The graphs have been divided into four sets, A, B, C and D for the purpose of clarity.

Although the data are too limited to elucidate a structure-activity relationship for the binding of these compounds, some observations can be made. In general, electron-withdrawing substituents at the *ortho*, *meta* or *para* positions of the benzyl ring improved binding. The greatest improvement was seen when a nitro group was added at either the *para* (**12**) or *meta* (**5**) position, which resulted in an increase in binding by approximately 50- and 75-fold, respectively, as compared to the reference compound (**1**). However, it may not be the electron-withdrawing property of the nitro group alone that is improving binding. Fluorine is very electronegative and yet *p*-fluoro-6-benzylthioinosine (**8**) bound only two-fold better than the reference compound (**1**). One feature of the nitro group that differentiates it from the other electron-withdrawing substituents examined is its negative charge (due to electrons dispersed over the nitrogen and oxygen atoms). It is possible that this negative charge interacts with a positively charged moiety in the binding site of the transporter. In addition, the fact that both the *para* and the *meta* substituted compounds (**12**, **5**) bind strongly suggests that the benzyl group can rotate around the thio linkage allowing the negatively charged nitro group at either position to interact with the transporter binding site.

The present investigation clearly establishes that the promising anti-toxoplasmic agents, 6-benzylthioinosine and its analogues, are inhibitors of the human ENT1 (*es*) nucleoside transporter (Fig. 1; Table 1). In addition, our previous studies [9], as well as the voluminous literature on nucleoside transporters, have demonstrated that 6-benzylthioinosines are not taken up by mammalian cells. Thus, these compounds are not permeants of other known mammalian nucleoside transporters. These findings are in contrast to the studies with TgAT1, the adenosine/purine nucleosides carrier of *T. gondii*, where 6-benzylthioinosines and certain analogues were found to be permeants, but not inhibitors, of the parasite nucleoside transporter [9,12]. This is further evidence that the human ENT1 (*es*) nucleoside transporter, as well as other mammalian equilibrative and concentrative transporters, is different from the parasite TgAT1 in its substrate specificity. Furthermore, the fact that 6-benzylthioinosine and its analogues are inhibitors of mammalian, but not parasite, nucleoside transport is an added advantage to the 6-benzylthioinosines as highly selective anti-toxoplasmic agents. Several inhibitors of nucleoside transport in mammalian cells were shown to selectively protect the host from the toxicity of anti-parasitic purine nucleosides [18–27].

In conclusion, our present (Table 1; Fig. 1) and previous results [9,11–14] demonstrate that there are at least two advantages for anti-toxoplasmic 6-benzylthioinosines. First, these 6-benzylthioinosines are selectively permeated and metabolized by parasite-infected cells, whereas these compounds do not enter or are subject to metabolism by uninfected host cells. Second, as inhibitors of the host nucleoside transport, 6-benzylthioinosines can protect uninfected host cells from other toxic nucleosides that may be used in future combination therapy or from possible metabolites of the 6-benzylthioinosine analogues that may be released upon the destruction of infected cells.

These findings further advance the rationale for developing 6-benzylthioinosine analogues as promising, selective therapies for the treatment of toxoplasmosis. In this regard, it is encouraging that 6-benzylthioinosines are apparently safe for in vivo administration. Studies with compound **5** in animals showed no evidence of host toxicity [14,19–22,25–28]. Compound **5** was non-toxic to animals as judged by blood chemistry, hematological studies and gross and histological

examinations [26,28]. No evidence for injury to the liver, kidney, spleen, pancreas, mesentery or peritoneal mesothelium was observed [26,28].

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REFERENCES

[1] Dubey JP, Beattie CP. Toxoplasmosis of animals and man. Boca Raton, FL: CRC Press, 1988.
[2] Luft BJ. In: Walzer PD, Genta RM, editors. *Toxoplasma gondii*. Parasitic infections in the compromised host. New York: Marcel Dekker; 1989. p. 179–279.
[3] Remington JS, McLeond R, Desmonts G. Toxoplasmosis. In: Remington JS, Klein JO, editors. Infectious diseases of the fetus and newborn infant. Philadelphia: W.B. Saunders Company; 1995. p. 140–267.
[4] Subauste CS, Remington JS. Immunity to *Toxoplasma gondii*. Curr Opin Immunol 1993;5:532–7.
[5] Wong S, Remington JS. Biology of *Toxoplasma gondii*. AIDS 1993;7:299–316.
[6] Perrotto J, Keister DB, Gelderman AH. Incorporation of precursors into *toxoplasma* DNA. J Protozool 1971;18: 470–3.
[7] Schwartzman JD, Pfefferkorn ER. *Toxoplasma gondii*: purine synthesis and salvage in mutant host cells and parasites. Exp Parasitol 1982;53:77–86.
[8] Krug EC, Marr JJ, Berens RL. Purine metabolism in *Toxoplasma gondii*. J Biol Chem 1989;264:10601–7.
[9] el Kouni MH. Potential chemotherapeutic targets in the purine metabolism of parasites. Pharmacol Ther 2003;99:283–309.
[10] Iltzsch MH, Uber SS, Tankersley KO, el Kouni MH. Structure activity relationship for the binding of nucleoside ligands to adenosine kinase from *Toxoplasma gondii*. Biochem Pharmacol 1995;49:1501–12.
[11] el Kouni MH, Guarcello V, Al Safarjalani ON, Naguib FNM. Metabolism and selective toxicity of 6-nitrobenzylthioinosine in *Toxoplasma gondii*. Antimicrob Agents Chemother 1999;43:2437–43.
[12] Al Safarjalani ON, Naguib FNM, el Kouni MH. Uptake of nitrobenzylthioinosine and purine β-L-nucleosides by intracellular *Toxoplasma gondii*. Antimicrob Agents Chemother 2003;47:3247–51.
[13] Yadav V, Chu CK, Rais RH, Al Safarjalani ON, Guarcello V, Naguib FNM, et al. Synthesis, biological activity and molecular modeling of 6-benzylthioinosine analogues as subversive substrates of *Toxoplasma gondii* adenosine kinase. J Med Chem 2004;47:1987–96.
[14] Rais RH, Al Safarjalani ON, Yadav V, Guarcello V, Kirk M, Chu CK, et al. 6-Benzylthioinosine analogues as subversive substrate of *Toxoplasma gondii* adenosine kinase: activities and selective toxicities. Biochem Pharmacol 2005;69:1409–19.
[15] Chiang C-W, Carter N, Sullivan Jr WE, Donald RGB, Roos DS, Naguib FNM, et al. The adenosine transporter of *Toxoplasma gondii*. Identification by insertional mutagenesis, cloning, and recombinant expression. J Biol Chem 1999;274:35255–61.
[16] Brajeswar P, Chen Mf, Paterson ARP. Inhibitors of nucleoside transport. A structure–activity study using human erythrocytes. J Med Chem 1975;18:968–73.
[17] Zhu Z, Furr F, Buolamwini JK. Synthesis and flow cytometric evaluation of novel 1,2,3,4-tetrahydroisoquinoline conformationally constrained analogues of nitrobenzylmercaptopurine riboside (NBMPR) designed for probing its conformation when bound to the *es* nucleoside transporter. J Med Chem 2003;46:831–7.
[18] Ogbunude POJ, Ikediobi CO. Effects of nitrobenzylthioinosinate on the toxicity of tubercidin and ethidium against *Trypanosoma gambiense*. Acta Trop 1982;39:219–24.
[19] el Kouni MH, Diop D, Cha S. Combination therapy of schistosomiasis by tubercidin and nitrobenzylthioinosine 5′-monophosphate. Proc Natl Acad Sci USA 1983;80:6667–70.
[20] el Kouni MH, Knopf PM, Cha S. Combination therapy of *Schistosoma japonicum* by tubercidin and nitrobenzylthioinosine 5′-monophosphate. Biochem Pharmacol 1985;34:3921–3.
[21] el Kouni MH, Cha S. Metabolism of adenosine analogues by *Schistosoma mansoni* and the effect of nucleoside transport inhibitors. Biochem Pharmacol 1987;36:1099–106.
[22] el Kouni MH, Messier NJ, Cha S. Treatment of schistosomiasis by purine nucleoside analogues in combination with nucleoside transport inhibitors. Biochem Pharmacol 1987;36:3815–21.
[23] Gati W, Stoyke AFW, Gero AM, Paterson ARP. Nucleoside permeation in mouse erythrocytes infected with *Plasmodium yoelii*. Biochem Biophys Res Commun 1987;145:1134–41.
[24] Gero AM, Bugledich EMA, Paterson ARP, Jamieson GP. Stage-specific alteration of nucleoside membrane permeability and nitrobenzylthioinosine insensitivity in *Plasmodium falciparum* infected erythrocytes. Mol Biochem Parasitol 1988;27:159–70.
[25] Baer HP, El-Soofi A, Selim A. Treatment of *Schistosoma mansoni*- and *Schistosoma haematobium*-infected mice with a combination of tubercidin and nucleoside transport inhibitor. Med Sci Res 1988;16. 919.
[26] el Kouni MH, Diop D, O'Shea P, Carlisle R, Sommadossi J-P. Prevention of tubercidin host toxicity by nitrobenzylthioinosine 5′-monophosphate for the treatment of schistosomiasis. Antimicrob Agents Chemother 1989;33:824–7.
[27] el Kouni MH. Efficacy of combination therapy with tubercidin and nitrobenzylthioinosine 5′-monophosphate against chronic and advanced stages of schistosomiasis. Biochem Pharmacol 1991;41:815–20.
[28] Kolassa N, Jakobs ES, Buzzell GR, Paterson ARP. Manipulation of toxicity and tissue distribution of tubercidin in mice by nitrobenzylthioinosine 5′-monophosphate. Biochem Pharmacol 1982;31:1863–74.