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Experimental Visceral Leishmaniasis: Role of *trans*-Aconitic Acid in Combined Chemotherapy

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We previously reported the effectiveness of *trans*-aconitic acid (TAA) as an antileishmanial compound. Inhibitory effects of TAA along with other antileishmanial compounds on transformation and in vitro multiplication in macrophage cultures of *Leishmania donovani* have been assessed. The efficacy of TAA in combined chemotherapy of experimental visceral leishmaniasis has also been evaluated along with those of commonly used antileishmanial compounds such as sodium stibogluconate, pentamidine, and allopurinol. TAA (2 mM) inhibited transformation of *L. donovani* amastigotes to promastigotes by 95.2%, whereas in combination with pentamidine (5 μg/ml), allopurinol (10 μg/ml), and sodium stibogluconate (50 μg of Sb per ml), it inhibited transformation by about 100, 99, and 98.5%, respectively. Sodium stibogluconate (20 μg of Sb per ml), pentamidine (2 μg/ml), and allopurinol (5 μg/ml) suppressed the amastigote burden in peritoneal macrophage cultures from BALB/c mice by 32.6, 56.1, and 46.3%, respectively. When these three drugs were used along with TAA (5 mM), the parasite loads were reduced by 100, 100, and 88.1%, respectively. TAA (5 mM) alone suppressed the amastigote burden by 59.5%. In experimental visceral leishmaniasis in hamsters (1-month model), TAA at a dose of 200 mg/kg of body weight per day suppressed the spleen parasite load by 73.5%, and TAA in combination with sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), and allopurinol (15 mg/kg/day) inhibited the spleen parasite load by 98, 98.9, and 97%, respectively. Individually, these three drugs inhibited the parasite load by 35, 20, and 22%, respectively. TAA (400 mg/kg/day) inhibited the spleen parasite load by 99.8%, but an inhibitory effect of ~100% was noted when TAA was supplemented with an antileishmanial drug. TAA was administered in experimental animals through oral, intraperitoneal, and intramuscular routes; the intramuscular route was most effective.

A parasitic protozoan, *Leishmania donovani*, is the causative agent of visceral leishmaniasis or kala-azar. Kala-azar patients are generally treated with sodium stibogluconate, pentamidine, allopurinol, etc. The modes of action of these drugs are different (3, 10, 20). Other antileishmanial compounds are currently being tested (2, 21, 28, 30). Sodium stibogluconate, pentamidine, and allopurinol are known to have toxic effects (7, 16, 17). The number of drug-resistant and/or nonresponsive cases (2, 16) is gradually increasing, and new therapeutic regimens are needed.

Metabolic studies of leishmania have shown that fatty acids serve as an important energy source for the amastigotes, which oxidize fatty acids at about 10 times the rate by promastigotes (12). Therefore, oxidation of the short-chain fatty acids via the Krebs cycle enzymes plays an important role in the energy metabolism of the amastigotes (13). The importance of fatty acid oxidation and the inherent differences in energy metabolism between the parasite and host (24, 27, 33) prompted us to test the efficacy of *trans*-aconitic acid (TAA) as an antileishmanial agent with no known mammalian toxicity (25, 26). It has been noted previously that TAA, an inhibitor of the enzyme aconitase, inhibits the growth of *L. donovani*, the etiological agent of kala-azar (26).

In view of the toxic effects of the commonly used antileishmanial drugs and increase in the number of nonresponsive cases, combined chemotherapy has been introduced in the treatment of kala-azar; combined chemotherapy is the use of sodium stibogluconate having more toxic effects in combination with a less toxic compound, allopurinol (8). The present report deals with the efficacy of TAA in combined chemotherapy with either sodium stibogluconate, pentamidine, or allopurinol in animal models of visceral leishmaniasis.

MATERIALS AND METHODS

Parasite culture. *L. donovani*, strain MHOM/IN/86/LG, was used throughout the experiments and was obtained by culture of bone marrow of a patient in Nil Ratan Sircar Medical College, Calcutta, India (19). The strain was maintained in hamsters (*Mesocricetus auratus*, obtained from Haffkine Research Institute and bred in our animal house). The promastigote form of the strain was obtained by transformation of the spleen amastigotes and cultured in supplemented RPMI 1640 (SR) medium. SR medium was RPMI 1640 medium (GIBCO) supplemented with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO), L-glutamine (GIBCO), and 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO).

Preparation of macrophage monolayer. Macrophages prestimulated with 4% thioglycolate (26) were isolated from BALB/c mice (originally obtained from Charles River and then bred in our animal house) in SR medium by peritoneal lavage, and monolayers were prepared and incubated at 37°C for further growth as described previously (26). These monolayers were used for experimentation.

In vitro multiplication of *L. donovani* promastigotes. Freshly transformed promastigotes were centrifuged and

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washed twice in S-α-MEM (19). The growth of the promastigotes was monitored by incubating the cells in S-α-MEM supplemented with 5% heat-inactivated fetal calf serum at 24°C in the presence or absence of TAA. Aliquots were removed at different time intervals, and the number of cells was determined with a hemocytometer.

Transformation inhibition study. *L. donovani* amastigotes isolated from the spleens of infected hamsters (19) were used for transformation study in SR medium at 24°C. The effects of the different drugs in combination or alone were assessed by monitoring the number of promastigotes in the medium at different intervals.

In vitro multiplication of *L. donovani* amastigotes within macrophages. Macrophage monolayers prepared as described above were washed once with SR medium and used in this experiment. *L. donovani* promastigotes two subpassages after transformation were added to the macrophage monolayers (macrophage/parasite ratio of ~1:10) and incubated in the same medium with or without TAA (synthesized in our laboratory [5, 26] by a standard method). TAA was also used in combination with an antileishmanial drug like sodium stibogluconate (Wellcome Foundation), pentamidine (May & Baker), and allopurinol (Sigma, St. Louis, Mo.) at 37°C for 5 days, with the incubation medium changed every 2 days. The monolayers were washed, dried, fixed with methanol, and Giemsa stained, and the number of amastigotes per infected macrophage was counted by microscopic observations.

Antileishmanial effect of TAA after administration through different routes. Male Syrian golden hamsters were infected intracardially with virulent promastigotes (2×10^7 promastigotes per animal). After 5 days of infection, TAA was administered by either oral or intraperitoneal or intramuscular route at doses up to 400 mg/kg of body weight per day for 20 consecutive days. After a 4-day interval, the animals were then sacrificed, liver and spleen weights were noted, and impression smears of the spleen were prepared, fixed, and stained. The total number of parasites in the spleen was determined microscopically (26, 34).

In vivo antileishmanial activity of TAA alone and in combination with other antileishmanial drugs. Antileishmanial activity of TAA in infected hamsters was tested by two methods. The first was the 8-day method (34). TAA alone and in combination with other antileishmanial drugs was administered to infected hamsters. After 1 day of infection with virulent parasites (5×10^7 promastigotes per animal), TAA was administered orally for 5 consecutive days at different doses. Sodium stibogluconate was administered intraperitoneally 1 day after infection and for three alternate days (50 mg of Sb per kg per day). Pentamidine (8 mg/kg/day) was also administered by the same schedule as for sodium stibogluconate. The schedule of allopurinol (15-mg/kg/day) administration was the same as for TAA. Animals were sacrificed on the 8th day of infection. Liver and spleen weights were noted, and the parasite burden of the spleen was assessed from stained impression smears (26, 34). A similar control experiment without any drug treatment was done.

To assess the efficacy of TAA in combined chemotherapy in long-established infections, we devised the 30-day model. Male golden hamsters were infected intracardially with virulent promastigotes (10^7 promastigotes per animal). After 5 days of infection, TAA alone and in combination with other antileishmanial drugs was administered to the animals. TAA was administered orally for 20 consecutive days at different doses. Sodium stibogluconate was injected intraperitoneally for 5 consecutive days at doses up to 100 mg of Sb per kg per day. This treatment was then repeated after 10 days. Allopurinol (15 mg/kg/day) was administered orally for 15 consecutive days. Pentamidine was injected intraperitoneally for 10 alternate days at doses of 8 mg/kg/day. Animals were sacrificed on the 30th day of infection. Liver and spleen weights were noted, impression smears of the spleen were prepared, fixed, and Giemsa stained, and the total parasite burden in the spleen was determined microscopically (26, 34). Similar control experiments were done with infected and uninfected animals with no drug treatment. For further confirmation, in vitro transformation was studied at 24°C with spleen pieces taken from animals in all experimental groups. Aliquots were withdrawn at intervals, and the presence of promastigotes was noted microscopically.

Statistical methods. The data were initially subjected to simple analysis of variance techniques. Two-way analysis of variance was done for the data in Table 2 in two experimental designs; in the 2×3 design, there were two levels of TAA dose and three levels of administration route and no control (no dose), whereas in the 3×3 design, the control (no-dose) level was the third level of TAA administered through the three different routes. The effect of dose is found highly significant (at 0.05% level) for both experimental designs. The effect of administration route is found to be significant at the 2.5% level but insignificant at the 1% level in the 2×3 design, whereas it is found to be insignificant at the 2.5% level in the 3×3 design. The interaction effect is absent at the 10% level of significance in both 2×3 and 3×3 designs. Dunnett's test for multiple comparison (36) was used to compare all the treated groups with a single control (no-drug) group (Tables 1 to 4). Tukey's test for multiple comparison (37) was performed for comparisons between the treated groups as indicated in Tables 1, 3, and 4.

RESULTS

In vitro growth inhibition of *L. donovani* promastigotes by TAA. Multiplication *of L. donovani* promastigotes in vitro was inhibited by TAA in a dose-dependent manner, which was reversed by the addition of an equimolar amount of *cis*-aconitic acid (CAA) (Sigma) or citrate (Sigma). In the controls, where either CAA or citrate was added, enhanced multiplication of the parasite was observed (Fig. 1).

Role of TAA in combination with other antileishmanial compounds on in vitro transformation of *L. donovani* amastigotes to promastigotes. TAA inhibited transformation of *L. donovani* amastigotes significantly (95.2%) at a dose of 2 mM. TAA at a dose of 10 mM completely inhibited the process. Slight positivity in transformation was observed after 96 h of incubation by TAA at 5 mM. Sodium stibogluconate (50 μg of Sb per ml) did not inhibit transformation, but in combination with 5 mM TAA, it completely inhibited the process. Pentamidine (5 μg/ml) and allopurinol (10 μg/ml) inhibited of transformation of amastigotes by 79 and 82%, respectively, at 120 h. Complete inhibition was brought about by these two compounds in combination with 5 mM TAA.

Inhibition of multiplication of *L. donovani* within peritoneal macrophages in vitro. TAA at a dose of 5 mM reduced the number of amastigotes per infected macrophage by about 60%, but in combination with sodium stibogluconate at a dose of 20 μg of Sb per ml, it completely inhibited the process. Allopurinol at a dose of 5 μg/ml reduced the number of amastigotes by about 46%, but in combination with TAA (5 mM), it reduced the number of amastigotes by 88%.

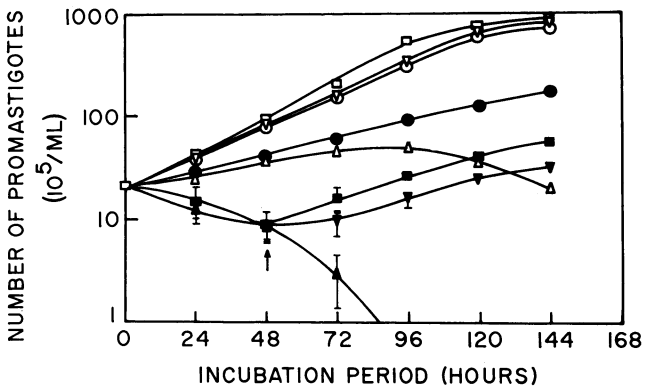

FIG. 1. Effect of TAA on in vitro multiplication of *L. donovani* promastigotes. The arrow indicates when CAA or citrate was added. Each point shows the mean ± standard deviation. Each experiment was done twice, with three replicates for each system. Symbols: ○, control (no drug); ●, 5 mM TAA; △, 10 mM TAA; ▲, 20 mM TAA; □, 20 mM CAA; ■, 20 mM TAA and 20 mM CAA (added after 48 h of culture); ▽, 20 mM citrate; ▼, 20 mM TAA and 20 mM citrate (added after 48 h of culture).

Pentamidine at a dose of 2 μg/ml reduced the amastigote count by about 56%, while in combination with 5 mM TAA, it completely eliminated the parasites. Inhibition in the amastigote count caused by TAA (5 mM) increased significantly when TAA was supplemented with either sodium stibogluconate (20 μg of Sb per ml), pentamidine (2 μg/ml), or allopurinol (5 μg/ml). TAA (5 mM), pentamidine (2 μg/ml), allopurinol (5 μg/ml), and sodium stibogluconate (20 μg of Sb per ml) significantly reduced the percentage of infected macrophages by 25, 29.2, 22, and 15.7%, respectively. Reduction in the percentage of infected macrophages caused by TAA was enhanced significantly by the addition of other clinical agents (Table 1).

Efficacy of route of administration of TAA in *L. donovani*-infected hamsters. TAA at two different doses was administered in infected hamsters through oral, intraperitoneal, and intramuscular routes. Spleen parasite burden was significantly reduced by all routes. The percentage of suppression of the parasite burden was more or less the same when TAA was administered either orally or intraperitoneally, but a slight increase was noted when TAA was administered intramuscularly. The increased liver and spleen weights of the animals caused by infection dropped to near-control (uninfected) values after administration of TAA (200 or 400 mg/kg/day) (Table 2).

Role of TAA in combination chemotherapy against visceral leishmaniasis in hamsters. Sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), and allopurinol (15 mg/kg/day) reduced the parasite load of spleen by 30, 7, and 18%, respectively, in the 8-day infected-hamster model. These three drugs in combination with TAA (200 mg/kg/day) reduced the parasite load by 87, 90, and 79.2%, respectively. TAA at doses of 200 and 400 mg/kg/day reduced the parasite load by 62.5 and 98.5%, respectively. Suppression of the spleen parasite load by TAA (200 mg/kg/day) was significantly increased by the addition of either sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), or allopurinol (15 mg/kg/day). The addition of either sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), or allopurinol (15 mg/kg/day) did not significantly increase the suppression of the parasite load caused by TAA (400 mg/kg/day) (Table 3).

In the 1-month model, TAA reduced the spleen parasite burden by 73.5 and 99.8% at doses of 200 and 400 mg/kg/day, respectively. Sodium stibogluconate at 50 mg of Sb per kg per day reduced the spleen parasite burden by 35%, while the reduction was 72% by sodium stibogluconate at 100 mg of Sb per kg per day. Allopurinol (15 mg/kg/day) and pentamidine (8 mg/kg/day) reduced the total spleen parasite burden by 22 and 20%, respectively. Sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), and allopurinol (15 mg/kg/day) in combination with TAA (200 mg/kg/day) reduced the spleen parasite load by 98, 98.9, and 97%, respectively. Allopurinol (15 mg/kg/day) in combination with sodium stibogluconate at doses of 50 and 100 mg of Sb per kg per day reduced the load by 45 and 89%, respectively. Sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), and allopurinol (15 mg/kg/day) in combination with TAA (400 mg/kg/day) completely destroyed the spleen parasite population in infected hamsters (Table 4). Reduction in the spleen parasite load caused by TAA (200 mg/kg/day) increased significantly when TAA was used in combination with either sodium stibogluconate (50 mg of Sb per kg per day), pentamidine (8 mg/kg/day), or allopurinol (15 mg/kg/day). Suppression of parasite burden by TAA (400 mg/kg/day) was too high to assess statistical significance for combined suppression caused by supplementation with the clinical agents. Promastigotes could be de-

TABLE 1. Effects of TAA and TAA in combination with other antileishmanial compounds on the multiplication of *L. donovani* amastigotes within BALB/c mouse peritoneal macrophages in vitro[a]

Drug (concn)	No. of amastigotes/macrophage (% inhibition)	% of infected macrophages (% inhibition)
None (control)	13.55 ± 2.35	96.0 ± 7.65
TAA (5 mM)	5.50 ± 0.66[b] (59.5)	72.0 ± 5.85[b] (25)
TAA (10 mM)	2.20 ± 0.25[b] (85.1)	56.0 ± 4.60[b] (41.7)
Sod-sb[c] (20 μg of Sb/ml)	9.14 ± 1.45[b] (32.6)	81.0 ± 6.55[b] (15.7)
TAA (5 mM) + Sod-sb (20 μg of Sb/ml)	0 (100.0)	0 (100.0)
Pentamidine (2 μg/ml)	5.95 ± 0.64[b] (56.1)	68.0 ± 3.22[b] (29.2)
TAA (5 mM) + pentamidine (2 μg/ml)	0 (100.0)	0 (100.0)
Allopurinol (5 μg/ml)	7.28 ± 0.86[b] (46.3)	75.0 ± 4.55[b] (22)
TAA (5 mM) + allopurinol (5 μg/ml)	1.62 ± 0.25[b,d] (88.1)	52.0 ± 5.25[b,d] (46)

[a] Values are means ± standard deviations (n = 6). Experiments were done twice with three replicates for each system.
[b] Significantly different from values obtained with control group (no drug) ($P < 0.01$ by Dunnett's test).
[c] Sod-sb, sodium stibogluconate.
[d] Significantly different from values obtained with TAA treatment (5 mM) ($P < 0.001$ by Tukey's test).

TABLE 2. Effects of administration of TAA by three different routes on *L. donovani*-infected hamsters (1-month model)[a]

Experimental group or treatment	Route of administration[b]	Body wt (g)	Liver wt (g)	Spleen wt (mg)	Spleen parasite burden (no. of cells [10^9])	% Suppression
Uninfected control		90.20 ± 1.99	2.65 ± 0.10	140.0 ± 28.0		
Control (no drug)		84.14 ± 1.94	4.27 ± 0.22	802.0 ± 9.0	1.380 ± 0.210	
TAA (200 mg/kg/day)	p.o.	64.78 ± 2.84	3.38 ± 0.10[c]	174.0 ± 13.0[c]	0.370 ± 0.025[c]	73.2
TAA (400 mg/kg/day)	p.o.	81.28 ± 1.81	2.80 ± 0.14[c]	136.0 ± 12.0[c]	0.025 ± 0.006[c]	98.2
TAA (200 mg/kg/day)	i.p.	75.95 ± 2.68	3.22 ± 0.16[c]	181.0 ± 13.0[c]	0.388 ± 0.050[c]	71.9
TAA (400 mg/kg/day)	i.p.	84.00 ± 2.77	2.95 ± 0.09[c]	147.0 ± 8.0[c]	0.017 ± 0.003[c]	98.7
TAA (200 mg/kg/day)	i.m.	76.71 ± 1.80	3.09 ± 0.13[c]	177.0 ± 21.0[c]	0.318 ± 0.070[c]	77.0
TAA (400 mg/kg/day)	i.m.	86.11 ± 2.67	2.46 ± 0.09[c]	138.0 ± 14.0[c]	0.005 ± 0.001[c]	99.6

[a] Values are means ± standard deviations ($n = 6$). Experiments were done twice with three animals in each group.
[b] p.o., oral; i.p., intraperitoneal; i.m., intramuscular.
[c] Significantly different from values obtained with control group (no drug) ($P < 0.01$ by Dunnett's test).

tected after 10 days in culture with spleen pieces taken from infected hamsters treated with TAA at 400 mg/kg/day and after 3 to 5 days in culture with spleen pieces taken from infected control animal with no drug treatment. No promastigote was noted in the culture with spleen pieces taken from infected hamsters treated with TAA (400 mg/kg/day) in combination with sodium stibogluconate (50 mg of Sb per kg per day) or pentamidine (8 mg/kg/day) or allopurinol (15 mg/kg/day).

DISCUSSION

The commonly used antileishmanial drugs such as sodium stibogluconate, pentamidine, and allopurinol are reported to have toxic effects and pentamidine is the most toxic (2, 16). Treatment with sodium stibogluconate has been reported to be associated with electrocardiographic abnormalities, generally of the T wave flattening or inverting (7). It was further observed that the T-wave abnormality correlated linearly with daily dose and duration of Sb treatment (2, 7). Generally, in visceral leishmaniasis patients, a daily dose of 20 mg of Sb per kg of body weight for 30 days is used for effective results (2). In many cases, this drug must be used for a longer period. Still, Sb nonresponsiveness is now very common in kala-azar patients, and such patients are primarily treated with pentamidine, a second-line drug. Patients treated with pentamidine suffer from different side effects like cardiotoxicity, gastrointestinal toxicity, disturbances of the central nervous system, albuminurea, allergic manifestation, metabolic complications, etc. (16). The slow excretion and cumulative toxicity of pentamidine limit its use to short courses. Short (10 to 20 days) courses of treatment of visceral leishmaniasis resulted in relapse rates of about 20% in the Mediterranean (31), 40% in India (14), and 100% in Kenya (22). A subsequent course of treatment after an interval of 10

TABLE 3. Effects of TAA and TAA in combination with other antileishmanial compounds on *L. donovani*-infected hamsters (8-day model)[a]

Experimental group or treatment	Body wt (g)	Liver wt (g)	Spleen wt (mg)	Spleen parasite burden (no. of cells [10^8])	% Suppression
Uninfected control	57.80 ± 0.86	2.26 ± 0.18	124.0 ± 13.0		
Control (no drug)	54.05 ± 1.13	3.90 ± 0.60	405.0 ± 57.0	4.020 ± 0.610	
TAA (200 mg/kg/day)	52.55 ± 1.19	2.99 ± 0.17[c]	282.0 ± 16.0[c]	1.510 ± 0.290[c]	62.5
TAA (400 mg/kg/day)	57.56 ± 1.29	2.86 ± 0.43[c]	172.0 ± 26.0[c]	0.063 ± 0.013[c]	98.5
Sod-sb[b] (50 mg of Sb/kg/day)	54.43 ± 1.56	3.65 ± 0.73[d]	401.0 ± 43.0[d]	2.840 ± 0.390[c]	30.0
Pentamidine (8 mg/kg/day)	51.61 ± 0.94	3.11 ± 0.47[e]	417.0 ± 49.0[d]	3.770 ± 0.620[d]	7.0
Allopurinol (15 mg/kg/day)	52.50 ± 1.12	3.71 ± 0.45[d]	413.0 ± 36.0[d]	3.300 ± 0.440[d]	18.0
TAA (200 mg/kg/day) + Sod-sb (50 mg of Sb/kg/day)	54.58 ± 1.97	2.95 ± 0.49[c]	255.0 ± 14.0[c]	0.515 ± 0.061[c,f]	87.0
TAA (400 mg/kg/day) + Sod-sb (50 mg of Sb/kg/day)	57.01 ± 0.78	2.23 ± 0.27[c]	134.0 ± 23.0[c]	0.051 ± 0.004[c,g]	98.8
TAA (200 mg/kg/day) + pentamidine (8 mg/kg/day)	51.81 ± 1.40	2.88 ± 0.36[c]	243.0 ± 24.0[c]	0.407 ± 0.034[c,f]	90.0
TAA (400 mg/kg/day) + pentamidine (8 mg/kg/day)	60.30 ± 1.74	2.56 ± 0.26[c]	160.0 ± 19.0[c]	0.013 ± 0.003[c,g]	99.7
TAA (200 mg/kg/day) + allopurinol (15 mg/kg/day)	54.55 ± 0.88	2.96 ± 0.17[c]	262.0 ± 12.0[c]	0.840 ± 0.055[c,h]	79.2
TAA (400 mg/kg/day) + allopurinol (15 mg/kg/day)	53.32 ± 1.10	2.68 ± 0.22[c]	170.0 ± 20.0[c]	0.040 ± 0.009[c,g]	99.0

[a] Values are means ± standard deviations ($n = 6$). Experiments were done twice with three animals in each group.
[b] Sod-sb, sodium stibogluconate.
[c] Significantly different from values obtained with control group (no drug) ($P < 0.01$ by Dunnett's test).
[d] Not significantly different from values obtained with control group (no drug) ($P > 0.05$ by Dunnett's test).
[e] Significantly different from values obtained with control group (no drug) ($0.05 > P > 0.01$ by Dunnett's test).
[f] Significantly different from values obtained with TAA treatment (200 mg/kg/day) ($P < 0.001$ by Tukey's test).
[g] Not significantly different from values obtained with TAA treatment (400 mg/kg/day) ($P > 0.5$ by Tukey's test).
[h] Significantly different from values obtained with TAA treatment (200 mg/kg/day) ($0.025 > P > 0.01$ by Tukey's test).

TABLE 4. Effects of TAA and TAA in combination with other antileishmanial compounds on *L. donovani*-infected hamsters (1-month model)[a]

Experimental group or treatment	Body wt (g)	Liver wt (g)	Spleen wt (mg)	Spleen parasite burden (no. of cells [10^8])	% Suppression
Uninfected control	84.00 ± 2.16	2.59 ± 0.40	142.0 ± 14.0		
Control (no drug)	70.00 ± 3.00	4.06 ± 0.53	784.0 ± 31.0	8.860 ± 1.670	
TAA (200 mg/kg/day)	80.00 ± 3.20	3.08 ± 0.17[c]	162.0 ± 28.0[c]	2.350 ± 0.780[c]	73.5
TAA (400 mg/kg/day)	84.60 ± 2.40	2.98 ± 0.25[c]	138.0 ± 36.0[c]	0.015 ± 0.013[c]	99.8
Sod-sb[b] (50 mg of Sb/kg/day)	79.00 ± 3.70	4.13 ± 0.31[d]	758.0 ± 16.0[c]	5.760 ± 1.530[c]	35.0
Sod-sb (100 mg of Sb/kg/day)	70.40 ± 4.68	3.80 ± 0.79[d]	582.0 ± 35.0[c]	2.420 ± 0.620[c]	72.0
Allopurinol (15 mg/kg/day)	81.30 ± 2.63	4.23 ± 0.25[d]	709.0 ± 18.0[c]	6.940 ± 0.640[c]	22.0
Pentamidine (8 mg/kg/day)	72.22 ± 3.15	4.00 ± 0.30[d]	715.0 ± 22.0[c]	7.080 ± 1.120[c]	20.0
TAA (200 mg/kg/day) + Sod-sb (50 mg of Sb/kg/day)	77.90 ± 3.74	2.77 ± 0.41[c]	147.0 ± 18.0[c]	0.167 ± 0.021[c,e]	98.0
TAA (400 mg/kg/day) + Sod-sb (50 mg of Sb/kg/day)	87.80 ± 5.97	2.80 ± 0.32[c]	141.0 ± 26.0[c]	0	~100.0
Sod-sb (50 mg of Sb/kg/day) + allopurinol (15 mg/kg/day)	76.20 ± 4.46	3.95 ± 0.17[d]	464.0 ± 23.0[c]	4.870 ± 1.050[c]	45.0
Sod-sb (100 mg of Sb/kg/day) + allopurinol (15 mg/kg/day)	73.30 ± 3.33	3.89 ± 0.18[d]	458.0 ± 10.0[c]	0.980 ± 0.170[c]	89.0
TAA (200 mg/kg/day) + allopurinol (15 mg/kg/day)	79.10 ± 2.17	3.02 ± 0.29[c]	151.0 ± 19.0[c]	0.267 ± 0.086[c,e]	97.0
TAA (400 mg/kg/day) + allopurinol (15 mg/kg/day)	79.55 ± 4.10	2.90 ± 0.47[c]	149.0 ± 11.0[c]	0	~100.0
TAA (200 mg/kg/day) + pentamidine (8 mg/kg/day)	81.12 ± 2.23	2.95 ± 0.42[c]	146.0 ± 15.0[c]	0.090 ± 0.008[c,e]	98.9
TAA (400 mg/kg/day) + pentamidine (8 mg/kg/day)	78.00 ± 3.16	2.50 ± 0.35[c]	143.0 ± 18.0[c]	0	~100.0

[a] Values are means ± standard deviations (n = 6). Experiments were done twice with three animals in each group.
[b] Sod-sb, sodium stibogluconate.
[c] Significantly different from values obtained with control group (no drug) ($P < 0.01$ by Dunnett's test).
[d] Not significantly different from values obtained with control group (no drug) ($P > 0.05$ by Dunnett's test).
[e] Significantly different from values obtained with TAA treatment (200 mg/kg/day) ($P < 0.001$ by Tukey's test).

to 15 days reduced the relapse rate from 54 to 18% in another series of patients in India (32). Moreover, pentamidine has been found to be ineffective against East African kala-azar patients (22, 35). It has been found that treatment with allopurinol at doses of 11 to 36 mg/kg/day for 14 to 31 days cured 8 of 16 Indian kala-azar patients on the basis of negative bone marrow cultures after therapy, but 13 of 16 patients have been reported to relapse (15). Although allopurinol inhibits the growth of *Leishmania* promatigotes in vitro, the inhibitory effect of the drug has been reported to be insufficient to resist growth of the parasites in visceral leishmaniasis cases (23) and particularly in previously untreated patients (18), possibly because the amastigotes are exposed to high concentrations of host cell ATP and GTP (20), which are utilized by the parasite after cleavage to their corresponding ribonucleosides (1, 4). In spite of the theoretical activity of allopurinol against the purine scavenger pathway of *Leishmania* spp., it has been reported not to be so effective in cutaneous leishmaniasis caused by *Leishmania panamensis*, *Leishmania guanensis*, and *Leishmania mexicana* (11). The adverse reaction associated with allopurinol is skin rash (17).

Kala-azar patients who have not responded to sodium stibogluconate therapy have been successfully treated with allopurinol along with sodium stibogluconate, indicating that these two drugs may act synergistically (8). However, combined therapy is not always effective. A Brazilian patient who did not respond to combined chemotherapy died after treatment with Glucantime, pentostam plus allopurinol, amphotericin B, and pentamidine (9). Other possible combined chemotherapeutic regimens are yet to be studied to reduce the toxic effects of commonly used drugs and to effectively treat nonresponsive cases.

TAA effectively inhibited the growth of virulent *L. donovani* promastigotes in vitro and the transformation of amastigotes to promastigotes. The multiplication of amastigote stage in vivo in hamsters and in vitro in macrophage cultures is suppressed significantly by TAA. The effective doses of commonly used antileishmanial drugs have been found to be reduced, if administered with TAA. Effectiveness of Sb treatment in experimental visceral leishmaniasis with doses of 50 to 125 mg/kg/day has been reported (29). A dose of 50 mg of Sb per kg per day has been used in the present study and reduced about 30% of the total parasite burden in the spleens of infected hamsters, but the same dose along with TAA (200 mg/kg/day) drastically reduced the parasite load in the spleens, indicating a synergistic effect of these two drugs. In our preliminary experiments in which pentamidine was administered at doses of 15 and 25 mg/kg/day for five alternate days, severe toxicity leading to death was found. Therefore, a reduced dose of pentamidine of 8 mg/kg/day which had no lethal effect and did not produce apparent signs and symptoms of toxicity was selected. Pentamidine at this dose had no significant antileishmanial effect but exhibited a marked inhibitory effect on the parasite load when supplemented with TAA (200 mg/kg/day). A slight inhibitory effect (about 20%) by allopurinol (15 mg/kg/day) alone has been noted. A marked enhancement of the antileishmanial activity was observed when the drug was administered in combination with TAA (200 mg/kg/day). TAA at a dose of 400 mg/kg/day in combination with low doses of either sodium stibogluconate, pentamidine, or allopurinol suppressed the spleen parasite burden by ~100%, as evident from negative results in spleen culture. However, viable parasites may remain elsewhere in the reticuloendothelial system of the animals.

Although a high dose of TAA is required for significant suppression of the parasite burden, TAA can be administered orally effectively and used successfully with low doses of other antileishmanial compounds without any apparent toxicity in the experimental animals even when it (400 mg/kg of body weight) was given for a longer period (Tables 2 and 4). Our previous study also showed that the mice did not die when TAA (2 g/kg of body weight) was administered intraperitoneally (26).

The exact mechanism of action of TAA on the host and parasite is not known. Growth inhibition of *Leishmania* promastigotes suggests that energy metabolism of the parasite is highly susceptible to TAA. It is also evident from the strong inhibition of transformation that the biogenesis of mitochondria is also related to energy metabolism of the parasite. However, the inhibitory effect of TAA was greater in amastigotes than in promastigotes. The in vitro transformation of *Trypanosoma brucei* bloodstream forms to procyclic forms is reported to be stimulated by citrate or *cis*-aconitate, which suggests the involvement of the Krebs cycle in morphogenesis of *T. brucei* (6). The amastigote form of *Leishmania* spp. depends mostly on fatty acid oxidation via the Krebs cycle for its energy supply (12).

The enzyme activities of the Krebs cycle for the host and the parasite are different. Citrate synthase is present in extremely low levels in *Leishmania* spp. (24, 27), which limits the production of citrate. Reversal of the inhibitory action of TAA on aconitase of the parasite may be drastically hampered because of citrate limitation. On the other hand, a high level of citrate synthase is present in host cell mitochondria and may increase the supply of citrate that can reverse the inhibitory effect of TAA. There might be a difference in the mechanism of inhibition of the enzyme aconitase in the parasite and host. Isocitrate is formed from citrate by the action of aconitase, and it can also reverse the inhibitory action of TAA on aconitase. Isocitrate-mediated reversal of the inhibitory effect seems to be limited in the parasite because of retarded formation of isocitrate. Briefly, because of the limited supply of citrate and isocitrate, the reversal of aconitase inhibition by TAA may be much less important in the parasite. Another remarkable difference in the two metabolic pathways is that the glyoxalate cycle is operative in *Leishmania* spp. (33) and absent in the host. The glyoxalate cycle functions through an aldol cleavage of isocitrate to succinate and glyoxalate by the action of isocitrate liase (33). Retardation of isocitrate formation caused by inhibition of aconitase by TAA may also affect the operation of the cycle, which plays a pivotal role in the replenishment of dicarboxylic acid intermediates of the tricarboxylic acid cycle from two-carbon substrates and synthesis of polysaccharide from acetate (33). All of these reasons may cause the different adverse effects of TAA in the host and parasite.

In brief, it is found that the adverse effects of TAA are manifested primarily on the parasite, and no apparent signs and symptoms of toxicity in the animals were noted. However, to further demonstrate the efficacy and lack of toxicity of TAA, various studies must be done. These studies include the following: investigation of respiration of the parasite and host liver slices and determination of the P/O ratio; incorporation of radiolabelled glucose and acetate into metabolic intermediates of tricarboxylic acid and glyoxalate cycles and into cellular components of the host and parasite in the presence and absence of TAA; determination of K_i, using TAA, for the enzyme aconitase of the host and parasite; and long-term toxicity of the drug in animals, etc.

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REFERENCES

1. **Akiyama, H. J., and R. D. Haight.** 1971. Interaction of *Leishmania donovani* and hamster peritoneal macrophages. Am. J. Trop. Med. Hyg. **20**:539–545.
2. **Berman, J. D.** 1988. Chemotherapy for leishmaniasis: biochemical mechanisms, clinical efficacy, and future strategies. Rev. Infect. Dis. **10**:560–586.
3. **Berman, J. D., J. V. Gallalee, and J. M. Best.** 1987. Sodium stibogluconate (pentostam) inhibition of glucose catabolism via the glycolytic pathway, and fatty acid β-oxidation in *Leishmania mexicana* amastigotes. Biochem. Pharmacol. **36**:197–201.
4. **Bhattacharya, A., and J. Janovy, Jr.** 1975. *Leishmania donovani*: autoradiographic evidence for molecular exchanges between parasites and host cells. Exp. Parasitol. **37**:353–360.
5. **Bruce, W. F.** 1959. Methods of preparation, p. 12–15. *In* A. H. Blatt (ed.), Organic syntheses collective volumes, vol. II. Wiley, New York.
6. **Brun, R., and M. Schonenberger.** 1981. Stimulating effect of citrate and *cis*-aconitate on the transformation of *Trypanosoma brucei* blood stream forms to procyclic forms *in vitro*. Z. Parasitenkd. **66**:17–24.
7. **Chulay, J. D., H. C. Spencer, and M. Mugambi.** 1985. Electrocardiographic changes during treatment of leishmaniasis with pentavalent antimony (sodium-stibogluconate). Am. J. Trop. Med. Hyg. **34**:702–709.
8. **Chunge, C. N., G. Gachihi, R. Muigai, K. Wasuna, J. R. Rashid, J. D. Chulay, G. Anabwani, C. N. Oster, and A. D. M. Bryceson.** 1985. Visceral leishmaniasis unresponsive to antimonial drugs. III. Successful treatment using a combination of sodium stibogluconate plus allopurinol. Trans. R. Soc. Trop. Med. Hyg. **79**:715–718.
9. **Dietz, R., R. C. Araujo, M. L. R. Lima, J. A. Vexenat, P. D. Marsden, and A. C. Barreto.** 1985. Ensaio terapeutico con Glucantime em saquis (*Callithrox jacchus*) infectados com una cepa de *Leishmania donovani* aparentemente resistente as tratamouto. Rev. Soc. Bras. Med. Trop. **18**:39–42.
10. **Ghosh, A. K., F. K. Bhattacharyya, and D. K. Ghosh.** 1985. *Leishmania donovani*: amastigote inhibition and mode of action of berberine. Exp. Parasitol. **60**:404–413.
11. **Guderian, R. H., M. E. Chico, M. D. Rogers, K. M. Pattishal, M. Grogl, and J. D. Berman.** 1991. Placebo controlled treatment of Ecuadorian cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. **45**:92–97.
12. **Hart, D. T., and G. H. Coombs.** 1982. *Leishmania mexicana*: energy metabolism of amastigotes and promastigotes. Exp. Parasitol. **54**:397–409.
13. **Hart, D. T., K. Vickerman, and G. H. Coombs.** 1981. Respiration of *Leishmania mexicana* amastigotes and promastigotes. Mol. Biochem. Parasitol. **4**:39–51.
14. **Hazarika, A. N.** 1949. Treatment of kala-azar with pentamidine isethionate: a study of 55 cases. Ind. Med. Gaz. **84**:140–145.
15. **Jha, T. K.** 1983. Evaluation of allopurinol in the treatment of kala-azar occurring in North Bihar, India. Trans. R. Soc. Trop. Med. Hyg. **77**:204–207.
16. **Jha, T. K.** 1983. Evaluation of diamidine compounds (pentamidine isethionate) in the treatment of resistant cases of kala-azar occurring in North Bihar, India. Trans. R. Soc. Trop. Med. Hyg. **77**:167–170.
17. **Kager, P. A., P. H. Rees, F. M. Manguyu, K. M. Bhatt, B. T. Wellde, W. T. Hockmeyer, and W. H. Lyerly, Jr.** 1984. Clinical haematological and parasitological response to treatment of visceral leishmaniasis in Kenya. Trop. Geogr. Med. **36**:21–35.

18. **Kager, P. A., P. H. Rees, B. T. Wellde, W. T. Hockmeyer, and W. H. Lyerly.** 1981. Allopurinol in the treatment of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. **75**:556–559.
19. **Kar, K., K. Mukherji, K. Naskar, A. Bhattacharya, and D. K. Ghosh.** 1990. *Leishmania donovani*: a chemically defined medium suitable for cultivation and cloning of promastigotes and transformation of amastigotes to promastigotes. J. Protozool. **37**:227–279.
20. **Looker, D. L., J. J. Marr, and R. L. Berens.** 1984. Mechanisms of action of pyrazolo pyrimidines in *Leishmania donovani*. J. Biol. Chem. **261**:9412–9415.
21. **Mahato, S. B., and T. Ghosal.** 1987. Advances in the chemotherapy of leishmaniasis. J. Sci. Ind. Res. **46**:456–464.
22. **Manson-Bahr, P. E. C.** 1959. East African kala-azar with special reference to the pathology, prophylaxis and treatment. Trans. R. Soc. Trop. Med. Hyg. **53**:123–137.
23. **Marr, J. J., R. L. Berens, D. J. Nelson, T. A. Krenitsky, T. Spector, S. W. Lafon, and G. B. Elion.** 1982. Antileishmanial action of 4-thiopyrazolo (3,4-d) pyrimidine and its ribonucleoside. Biochem. Pharmacol. **31**:143–148.
24. **Meade, J. C., T. A. Glaser, P. F. Bonventre, and A. J. Mukkada.** 1984. Enzymes of carbohydrate metabolism in *Leishmania donovani* amastigotes. J. Protozool. **31**:156–161.
25. **Miller, R. E., and S. M. Cantor.** 1951. Aconitic acid, a by-product in the manufacture of sugar. Adv. Carbohydr. Chem. **1951**:231–249.
26. **Misra, S., T. Sanyal, D. Sarkar, P. K. Bhattacharya, and D. K. Ghosh.** 1989. Evaluation of antileishmanial activity of *trans*-aconitic acid. Biochem. Med. Metab. Biol. **42**:171–178.
27. **Mukkada, A. J.** 1985. Energy metabolism in *Leishmania*, p. 49–64. *In* K.-P. Chang and R. S. Bray (ed.), Leishmaniasis. Elsevier, Amsterdam.
28. **Neal, R. A.** 1987. Experimental chemotherapy, p. 793–837. *In* W. Peters and R. Killick-Kendrick (ed.), Leishmaniases I: biology and medicine, vol. II. Academic Press, Inc. (London), Ltd., London.
29. **Raether, W., H. Seidenath, and H. Loewe.** 1978. Action of p(4-amidinophenoxy) benzaldehyde-p-amidino phenylhydrazone dihydrochloride on *Leishmania donovani* infections on the golden hamster. Ann. Trop. Med. Parasitol. **72**:543–547.
30. **Sarkar, S., T. Mukherjee, P. Patra, and A. Bhaduri.** 1992. Antileishmanial activity of hamycin: a polyene antibiotic. Biochem. Biophys. Res. Commun. **182**:86–91.
31. **Sarrouy, C.** 1948. Traitement moderne du kala-azar infantile. Sem. Hop. Paris **24**:1862–1864.
32. **Sengupta, P. C.** 1953. Chemotherapy of leishmanial diseases: a resume of recent researches. Ind. Med. Gaz. **88**:20–35.
33. **Simon, M. W., E. Martin, and A. J. Mukkada.** 1978. Evidence for a functional glyoxalate cycle in the leishmaniae. J. Bacteriol. **135**:895–899.
34. **Stauber, L. A., E. M. Franchino, and J. Grun.** 1958. An eight-day method for screening compounds against *Leishmania donovani* in the golden hamster. J. Protozool. **5**:269–273.
35. **Wijers, D. J. B.** 1971. A ten year study of kala-azar in Tharaka (Meru district, Kenya). II. Relapses. East Afr. Med. J. **48**:550–558.
36. **Winer, B. J.** 1971. Statistical principles in experimental design, p. 201–204. McGraw-Hill, New York.
37. **Zar, J. H.** 1984. Biostatistical analysis, 2nd ed., p. 186–190. Prentice-Hall, Inc., Englewood Cliffs, N.J.