Hypoxanthine derivatives in experimental infections

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C De Simone, EA Martelli, P Foresta, et al. Hypoxanthine derivatives in experimental infections. *Can J Infect Dis* 1992;3(Suppl B):106B-110B. In vivo treatment with parenterally administered hypoxanthine derivatives, notably ST 789, was able to protect cyclophosphamide-immunosuppressed mice against experimental infections with both bacterial and fungal pathogens. However, the mechanisms accounting for these effects of hypoxanthine derivatives remain to be fully established. In fact, only the treatment with ST 789 resulted in a clear enhancement of the primary antibody production as well as macrophage phagocytic activity, whereas T lymphocyte responsiveness to mitogens and both macrophage- and natural killer-dependent cytotoxicity were not significantly affected. These data, together with the recently shown ability of ST 789 to increase interleukin-6 production, suggest that monocyte/macrophages are likely to be the main cellular target of the immunomodulating activity of ST 789. Finally, in the present in vivo study, hypoxanthine derivatives did not enhance the mean survival time of tumour-bearing immunosuppressed mice.

Key Words: *Experimental infections, Hypoxanthine derivatives, ST 789*

Dérives de l'hypoxanthine dans les infections expérimentales

Le traitement in vivo à l'aide de dérivés de l'hypoxanthine administrés par voie parentérale, notamment le ST 789, a réussi à protéger des souris immunosupprimées à la cyclophosphamide contre les infections expérimentales, tant bactériennes que fongiques. Cependant, les mécanismes responsables des effets des dérivés de l'hypoxanthine demeurent méconnus. En fait, seul le traitement avec le ST 789 a donné lieu à une nette amélioration de la production d'anticorps primaires et de l'activité phagocytaire sur les macrophages, alors que la réactivité des lymphocytes T aux mitogènes et la cytotoxicité liée tant aux macrophages qu'aux cellules tueuses naturelles n'ont pas été significativement affectées. Ces données, ainsi que la capacité récemment démontrée du ST 789 à augmenter la production d'interleukine-6 suggèrent que les monocytes/macrophages sont susceptibles d'être la cible cellulaire principale de l'activité immunomodulatrice du ST 789. Finalement, dans la présente étude in vivo, les dérivés de l'hypoxanthine n'ont pas amélioré le temps de survie moyen des souris immunosupprimées porteuses d'une tumeur.

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SEVERAL RECENT REPORTS SUGGESTED THAT ARGININE AND arginine-containing hypoxanthine derivatives can strongly modulate immune functions both in vitro (1,2) and in vivo (1,3-5). ST 789, which has a pentyloxycarbonil-L-arginine residue at the N-9 position of the hypoxanthine ring, strongly enhanced in vitro interleukin-6 (IL-6) production by mitogen-driven peripheral blood mononuclear cells (6) as well as T lymphocyte responsiveness to mitogens (7,8) and natural killer- and macrophage-dependent cytotoxicity (1,4,5,9). Furthermore, cyclophosphamide-immunosuppressed mice injected with ST 789 were more resistant to experimental infections from both bacterial and fungal pathogens (10,11).

Several arginine-containing hypoxanthine derivatives are currently available. The main difference between ST 789 and its derivatives is that the derivatives, instead of having a terminal L-arg aminoacidic residue, possess an L-arg terminal peptide of varying length (two to five aminoacidic residues). However, reports evaluating both in vitro and in vivo immunomodulating properties of the derivatives are currently lacking. In contrast, ST 789 has been evaluated extensively in this respect. The present study found that, as a group, hypoxanthine derivatives other than ST 789 are not very potent immune modulators. However, after treatment with many hypoxanthine derivatives (including ST 789) experimentally infected mice were protected against challenge with several pathogens. Finally, while ST 789 had some antitumour activity, the other derivatives evaluated did not.

MATERIALS AND METHODS

Hypoxanthine derivatives: ST 789, ST 848, ST 872, ST 873, ST 897, ST 657, ST 733, ST 742, ST 754, ST 785 and ST 786, purchased from Sigma-Tau (Pomezia, Italy), were studied.

Experimental infections: Seven- to 10-week-old CD1 male mice and 12- to 16-week-old female Balb/c mice were used. *Klebsiella oxytoca* 552 (5×10^3 and 3.5×10^4), *K pneumoniae* 7823 (1.6×10^6 and 1×10^7), *Escherichia coli* ISM (1.3×10^6), *Serratia marcescens* A/1 (3.5×10^4), *Salmonella typhimurium* (8.5×10^2), *Proteus mirabilis* (5×10^3) and *Pseudomonas aeruginosa* (1×10^{10}) were inoculated intraperitoneally, and *Candida albicans* (5×10^5) and *Aspergillus fumigatus* (5×10^6) were inoculated intravenously in cyclophosphamide-immunosuppressed mice. Cyclophosphamide was administered intraperitoneally at dosages ranging from 50 to 150 mg/kg five days before the induction of infection and approximately 2 h before starting hypoxanthine derivative treatment, which was given for five consecutive days. In the *P aeruginosa* model cyclophosphamide was administered two days prior to challenge. As previously reported (12,13), host resistance to infections was estimated by the number of mice surviving after five to 15 days of observation, as well as by the mean survival time.

The protective effects of combined treatment with ST 789 and ceftazidime, and ST 789 and amphotericin B

were also evaluated in cyclophosphamide-treated mice challenged with *K oxytoca* and *C albicans*, respectively. Ceftazidime and amphotericin B were administered at per se completely ineffective dosages ranging from 0.02 to 2.0 mg/kg and 1.0 to 2.5 mg/kg, respectively.

The statistical analysis of results was performed using Fisher's test and χ^2 test.

Primary antibody production: B₆D₂F₁ mice (age six weeks) were administered hypoxanthine derivatives intraperitoneally at either 0.25, 2.5 or 25 mg/kg per day for five days. On day 3, mice were injected with 1×10^7 sheep erythrocytes in 0.2 mL. Spleen cells obtained from mice immediately following death were adjusted at a density of 1×10^7 cells/mL in Iscove's modified Dulbecco's medium (IMDM; Gibco, New York) supplemented with fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 4% polyethylene glycol (MEG; Serva, Germany) (molecular weight 6000 Da) and 50 U/mL of both penicillin and streptomycin. Antigen was added in the form of 2 mL 10% sheep red blood cells/mL of culture. Control cultures without antigen were set up in several experiments. The cultures were incubated at 37°C in 5% carbon dioxide for 90 mins. The cells were washed, resuspended in medium and assayed for plaque-forming cells by using the previously described slide modification of the hemolysis in gel method (14).

T lymphocyte proliferation to mitogens: T lymphocyte proliferative responsiveness to mitogens was assayed as previously described (15). Spleen cells were obtained from mice given five days of therapy with hypoxanthine derivatives.

Delayed T hypersensitivity reactions: Delayed T hypersensitivity reactions were assayed as previously reported (16).

Macrophage-dependent cytotoxicity: Peritoneal exudate cells were obtained by washing the abdominal cavity of B₆D₂F₁ mice with RPMI 1640. Harvested cells, were centrifuged at 400 g for 10 mins at 4°C and density adjusted to 2×10^6 cells/mL. Daudi cells were used as target cells. The cytotoxicity test was performed as previously described (5).

Phagocytic activity: The phagocytic activity of peritoneal exudate cells was assayed as previously reported (17). Briefly, peritoneal exudate cells were adjusted to a density of 4×10^6 cells/mL to which was added sheep red blood cells previously opsonized with anti-sheep erythrocyte immunoglobulins. After 1 h incubation at 37°C in 5% carbon dioxide, nonphagocytosed erythrocytes were removed using hypotonic saline solution.

Natural killer cell-dependent cytotoxicity: The assay of natural killer cell activity was performed using standard methods (18).

Antitumoral activity: Antitumoral activity was assayed as previously described (19). Briefly, inbred C57B1/6 (age eight weeks) and DBA/2 (age six weeks) male mice were used in the experiments with both carcinoma and leukemia models. Outbred CD1 male

TABLE 1
Protective effects of ST 789 in cyclophosphamide-immunosuppressed mice infected with *Klebsiella oxytoca* 552

Treatment (mg/kg/day)	Number of deaths/total number of animals tested (%)	
	5x10 ³ bacteria	3.5x10 ⁴ bacteria
Controls	9/143 (6.3)	32/69 (46.4)
Controls (immunosuppressed)	90/153 (58.8)	52/70 (74.3)
ST 789 (0.2)	4/60 (6.7)**	17/40 (42.5)*
ST 789 (2)	1/25 (4.0)**	2/20 (10.0)**
ST 789 (25)	6/108 (5.6)**	10/50 (20.0)**

ST 789 administered daily by intraperitoneal injection for five consecutive days 2 h after cyclophosphamide immunosuppression. Mice were infected 24 h after the last ST 789 administration. *P<0.05; **P<0.001 (χ^2 test)

mice (age six weeks) were used in the experiments for sarcoma model. Lewis lung carcinoma cells were injected at a density of 1x10⁵ cells/mL in 0.1 mL TC 199 medium; P388 and P1210 leukemia cell lines were injected intraperitoneally at a density of 1x10⁵ cells/mL in 0.1 mL of Hank's medium; 180 sarcoma was injected intraperitoneally at a density of 1x10³ cells/mL in 0.1 mL Hank's medium. The injections were performed on day zero. Hypoxanthine derivatives were administered intraperitoneally at concentrations of 0.25 and 25 mg/kg per day, respectively, throughout days 1 to 10 in leukemia- and sarcoma-bearing animals, whereas in Lewis lung carcinoma model the drugs were administered on days 1 to 20.

The statistical analysis of differences in mean survival time was performed by using the Mann-Whitney U test.

RESULTS

Experimental infections: All hypoxanthine derivatives tested were able to protect cyclophosphamide-treated mice against experimental infections with both bacterial and fungal pathogens as shown by improvement in both percentage mortality and mean survival time. In the *K. oxytoca*, *S. marcescens* and *S. typhimurium* infective models, ST 789 was most effective when administered intraperitoneally, as it proved active at concentrations as low as 0.25 mg/kg (Tables 1,2). Similar results were obtained in *K. pneumoniae*, *E. coli* and *Ps. mirabilis* models (data not shown).

In contrast, subcutaneous administrations of the immunomodulator were able to protect mice against only *P. aeruginosa* and *C. albicans* infections, the former occurring when ST 789 was administered alone or in the association with gentamicin (data not shown), and the latter occurring when ST 789 was combined with an 'ineffective' dosage of amphotericin B (Table 3). Oral ST 789 administration was completely ineffective.

Dosages per se completely ineffective of ST 789 and ceftazidime significantly protected, in terms of survival, immunosuppressed mice from experimental infection

TABLE 2
Protective effects of ST 789 in cyclophosphamide-immunosuppressed mice infected with *Serratia marcescens* A/1 and *Salmonella typhimurium*

Treatment (mg/kg/day)	Number of deaths/total number of animals tested (%)	
	3.5x10 ⁴ <i>S. marcescens</i>	8.5x10 ² <i>S. typhimurium</i>
Controls	6/20 (30)	3/20 (15)
Controls (immuno- suppressed)	16/20 (80)	11/20 (55)
ST 789 (0.25)	ND	1/10 (10)*
ST 789 (25)	10/20 (50)*	3/20 (15)**

ST 789 administered daily by intraperitoneal injection for five consecutive days 2 h after cyclophosphamide immunosuppression. Mice were infected 24 h after the last ST 789 administration. ND Not done *P<0.05; **P<0.01 (Fisher's test)

TABLE 3
Protective effects of combined treatment with ST 789 and amphotericin B in cyclophosphamide-immunosuppressed mice challenged with *Candida albicans*

ST 789 (mg/kg/day)	Amphotericin B (mg/kg)	Number of deaths/ total number of animals tested (%)
Control*	0	15/15 (100)
0	1	7/7 (100)
0	2.5	9/9 (100)
50	1	1/10 (10)**
50	2.5	2/10 (20)**

ST 789 administered subcutaneously daily, five days prior to infection. Amphotericin B administered by single intraperitoneal injection two days after infection. *Untreated, cyclophosphamide-immunosuppressed (150 mg/kg) five days before infection) mice infected with 5x10⁵ *C. albicans* cells. **P<0.001 (Fisher's test)

TABLE 4
Protective effects of combined treatment with ST 789 and ceftazidime in cyclophosphamide-immunosuppressed mice challenged with *Klebsiella oxytoca* 552

ST 789 (mg/kg/day)	Ceftazidime (mg/kg)	Number of deaths/ total number of animals tested (%)
Control	0	8/10 (80)
0.05	0	8/10 (80)
Control	0.02	9/10 (90)
Control	0.2	2/10 (20)
Control	2.0	0/10 (0)
0.05	0.02	3/10 (30)*
0.05	0.2	0/10 (0)
0.05	2.0	0/10 (0)

ST 789 administered intraperitoneally daily five days prior to infection. Ceftazidime administered intraperitoneally 1 h after infection. Control Untreated, cyclophosphamide-immunosuppressed (100 mg/kg five days before infection) mice infected with 3.5x10⁴ *K. oxytoca* cells. *P<0.05 (Fisher's test)

with *K. oxytoca* (Table 4). The same was true in the *A. fumigatus* model since ST 789 and amphotericin B administered at dosages per se ineffective were synergistic (data not shown).

Primary antibody production: A significant increase in the number of plaque-forming cells was found in mice administered with ST 657 at dosages of 2.5 and 25 mg/kg. ST 789 resulted in a strong increase of plaque-forming cells when administered only at 25 mg/kg. On the other hand, both ST 783 and ST 733 resulted in a significant decrease in the number of plaque-forming cells (data not shown). Finally, ST 848, ST 872, ST 897, ST 742, ST 754, ST 785 and ST 786 did not significantly affect the number of plaque-forming cells.

T lymphocyte responsiveness to mitogens: None of the hypoxanthine derivatives affected lymphocyte proliferation following stimulation with mitogens, such as phytohemagglutinin, lipopolysaccharide and concanavalin A. A slight but not significant reduction of mitogen-driven lymphocyte proliferation was found in mice administered ST 657. Overall, these data support previous experiments performed in the authors' laboratory indicating that ST 789 is not able to enhance *in vitro* the surface expression of activation antigens, such as IL-2 and transferrin receptors, as well as IL-2 and interferon- γ production by phytohemagglutinin-stimulated peripheral blood mononuclear cells (6,20).

Delayed T cell hyperreactivity reactions: None of the hypoxanthine derivatives investigated significantly affected delayed T cell hyperreactivity reactions.

Macrophage-dependent cytotoxicity: Treatment with hypoxanthine derivatives did not result in any significant change of macrophage cytotoxicity.

Phagocytic activity of peritoneal exudate cells: ST 789 strongly increased the phagocytic capacity of peritoneal exudate cells, as demonstrated by the increased number of phagocytosed sheep erythrocytes per single cell as well as per 100 exudate cells (data not shown). However, the percentage of macrophages was not affected by administering ST 789. ST 657, ST 733, ST 754 and ST 785 were not as effective as ST 789 in enhancing phagocytosis.

Natural killer cell-dependent cytotoxicity: ST 789 administration resulted in a slight reduction of natural killer cell cytotoxicity. However, natural killer cell activity was not affected by administering hypoxanthine derivatives other than ST 789 (data not shown).

Antitumour activity: In these experimental models, hypoxanthine derivatives did not exert any antitumour activity, demonstrated by the inability to detect any increase in mean survival time of tumour-bearing mice following *in vivo* treatment (data not shown).

DISCUSSION

The results clearly show that hypoxanthine derivatives, most notably ST 789, exhibit immunomodulating properties. In fact, this group of molecules protected cyclophosphamide-immunosuppressed mice against experimental infections with bacterial and fungal pathogens, confirming previous reports (10,11). Furthermore, the combined administration of ST 789 and

antimicrobial agents, such as ceftazidime, amphotericin B and gentamicin, at doses *per se* completely ineffective, strongly protected cyclophosphamide-treated mice in several models of experimental infections. Therefore, ST 789 either alone or in combination with antibiotics enhances host resistance to pathogens.

The mechanisms responsible for these effects of hypoxanthine derivatives are unclear. The reported ability of ST 789 to restore mitogen responsiveness of lymphocytes from cyclophosphamide-treated mice is likely to play a major role (21). However, other cells could mediate the immunomodulating activity of ST 789. In fact, ST 789 treatment expanded bone marrow natural killer progenitor cells *in vivo*, thus strongly enhancing the regeneration of mouse natural killer cell activity (9). Furthermore, as cyclophosphamide treatment severely reduces the number of circulating granulocytes (22), the capacity of hypoxanthine derivatives to protect cyclophosphamide-administered mice against pathogens might – at least in part – depend on their myelorestorative ability. However, a role for colony stimulating factors in mediating the immunomodulation of hypoxanthine derivatives is still unproven.

Data from the authors' laboratory showing an increased *in vitro* production of IL-6 by peripheral blood mononuclear cells cultured in the presence of both mitogens and ST 789 compared with control cultures suggest that hypoxanthine derivatives could act *in vivo* by modulating cytokine production (6). Furthermore, the present report shows that *in vivo* administered ST 789, as well as other hypoxanthine derivatives, strongly enhanced the phagocytic activity of peritoneal exudate macrophages. Since macrophages are the main cellular source of IL-6 (23) this suggests that hypoxanthine derivatives exert an action on monocytes/macrophages. Overall, these findings suggest that hypoxanthine derivatives could modulate infections with intracellular pathogens such as *listeria*, *legionella*, *brucella*, *salmonella* and *mycobacteria*.

A temporal relationship in cytokine production has been recently demonstrated for tumour necrosis factor, IL-1 and IL-6 production (24). After a bacterial challenge, the appearance of IL-6 follows that of tumour necrosis factor and IL-2 during endotoxemia (25,26). Thus, the most likely scheme of activation leading to cytokine production involves the early release of tumour necrosis factor and IL-1 from monocytes/macrophages which subsequently induces the secretion of IL-6 and other cytokines from both monocytes and structural cells, such as fibroblasts and endothelial cells. This cascade is the basis for the finding of sequential elevations of cytokines in plasma in experimental models of inflammation (25-27).

The role of tumour necrosis factor in mediating the ST 789-dependent increase of IL-6 production remains to be established. Furthermore, there is no report on the ability of hypoxanthine derivatives other than ST

789 to modulate cytokine release *in vivo* or *in vitro*. In this study, these compounds exhibited immunomodulating activity *in vivo*. However, the *in vitro* lymphocyte responsiveness to mitogens, natural killer cell activity and delayed T cell hyperreactivity reactions were not significantly affected. Only ST 789 was able to increase the primary *in vitro* antibody production compared with other hypoxanthine derivatives, possibly by increasing IL-6 production (6). Therefore, the mechanisms involved in determining enhanced host resistance by hypoxanthine derivatives are still unresolved.

Finally, in the present study hypoxanthine derivatives did not show any *in vivo* antitumour activity. The results are in contradiction with previous reports in-

dicating that mice injected with ST 789 exhibited significant resistance against two synergistic tumours (7). Furthermore, the arginine residue of ST 789 has been suggested to play a pivotal role in mediating the anti-tumour activity of ST 789 by increasing T lymphocyte cytotoxicity (3,4) as well as natural killer cell and macrophage cytotoxicity (3-5). The enhancement of T helper function could also be involved, as suggested by the absolute increase of CD4+ T cells observed in cancer patients following treatment with arginine-lysine combination (unpublished data). The inability to find any increase in mean survival time of tumour-bearing mice following *in vivo* administration of hypoxanthine derivatives may be related to peculiarities of the experimental protocol.

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