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CHEMOTHERAPY AND DRUG TARGETING IN THE TREATMENT OF LEISHMANIASIS

ANNUAL REPORT

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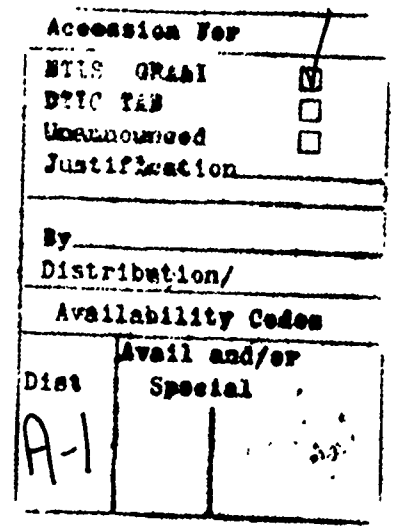

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ABSTRACT

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INTRODUCTION

Leishmaniasis is one of the major public health problems presently afflicting humanity. Leishmaniasis is estimated to affect 12 million people in Third World countries (1). It is prevalent in Southern Asia, Northern Africa, Central and South America (5). The World Health Organization estimates that worldwide there are 250,000 AIDS cases, and 5 million people infected with HIV (58). These two diseases are related in that both are caused by infectious organisms, one viral and the other protozoal, and the pathogenesis of the disease is dependent upon a deficient immune response in the host (2,4,49,50). Chemotherapeutic treatment for both these diseases is currently ineffective and/or toxic (2,59).

The purpose of this study is to develop more effective chemotherapeutic agents for leishmaniasis. In this study, both current and experimental medications for leishmaniasis and AIDS were examined to explore their toxicity to human T4 cells in vitro. These T helper white blood cells perform an essential role in mounting a normal immune response. A medication that is toxic to a patient's T4 cells would seriously impair their immune system, and thus be counterproductive in producing a cure, or attenuating the course of the illness.

Anti-infectious agents against viruses, bacteria, fungi, or protozoa, must either kill the pathogen or stop it from multiplying. This it must do without harming the infected host significantly. Generally, such drugs accomplish their

task by attacking a biochemical pathway unique to the pathogen. In the case of bacteria this is relatively easy to do because there are many differences between the structure and metabolism of bacterial cells and those of mammalian cells. In the case of protozoans and especially viruses, it is more difficult because there are fewer differences between pathogen and host.

No drug is devoid of side effects or toxicity. One must therefore always consider the balance between harm to the pathogen and harm to the host. An essential aspect of any drug is its "therapeutic index": the ratio of the toxic dose to the effective dose. This index can be used to assess the therapeutic value of drugs.

In this study, we used the growth inhibition on T4 cells caused by a drug as a measure of its toxicity to humans. The measuring point of drug toxicity on T4 cells in this study was the ID50, which is commonly reported in the literature. The ID50 is the dose of a drug that produces a fifty percent inhibition in T4 cell growth in 72 hours relative to growth in control cultures of T4 cells. These were compared with literature reports of the toxicity of the same medications on the relevant infectious organisms. With respect to the pathogens, the measuring point of drug toxicity commonly used in the literature is the ED50. i.e. the 'effective' dose of a drug that causes a 50 % decrease in the numbers of pathogenic organisms in 72 hours relative to untreated control pathogens. A therapeutic index was calculated from the ratio of toxicity of a drug on T4 cells relative to its toxicity in the infectious organism i.e. the

ID50 divided by the ED50. This therapeutic index was used as a measure of the chemotherapeutic value of the particular drug.

The first objective of this study was to determine the toxicities of antileishmanial agents on T4 cells in vitro. These drugs included the current treatments-of-choice as well as experimental agents for leishmaniasis. From these measurements, therapeutic indices were calculated to assess the chemotherapeutic value of the antileishmanial agents.

The second objective was to test the toxicities of chemotherapeutic agents used to treat the infectious complications of AIDS patients on T4 cells and to determine if these compounds are antileishmanial. The depletion and inactivation of T4 cells is one of the hallmarks of AIDS, and without sufficient functioning T4 cells, AIDS patients are subject to opportunistic infections and malignancies (62). Many of the drugs used in the management of AIDS are based on the data available from the treatment of these infections in immunosuppressed patients without AIDS, such as cancer patients or organ transplant recipients on immunosuppressive drugs. The effect of many of these agents on the immune system of AIDS patients has not been tested (50). It is important that the chemotherapeutic agents used to treat the opportunistic infections are not toxic to the patient's T4 cells, or they will be counterproductive and worsen the prognosis of the AIDS patient.

The third objective of this study was to determine whether the therapeutic index of certain drugs could be increased by nutritional supplements. Several of the

antileishmanial drugs are purine analogs. These purine analogs get incorporated into the pathways of purine metabolism in a cell. However, because they are structurally slightly different to the parent purine, they inhibit purine metabolism, and thereby the synthesis of DNA and RNA. Such purine analogs are of chemotherapeutic value if they inhibit nucleic acid synthesis in the pathogen but not in the host. This sometimes occurs if the host enzymes are slightly different to pathogen enzymes. Two examples of nucleoside analogs that are currently used as therapeutic agents are AZT (3'-azido-2',3'-dideoxythymidine) for the treatment of AIDS, and allopurinol riboside for the treatment of leishmaniasis.

The strategy to increase the therapeutic index of purine analog drugs entailed adding a combination of the parent purine nucleoside and the analog drug to test whether this would reverse the toxicity of the drug to T4 cells, but not to leishmanial cells. An alternative mechanism of reversing the toxicity of a drug is to add to the cells the product of an enzyme which the drug is hypothesized to inhibit. This could have practical applications in that nutritional supplements given in combination with a therapeutic drug to a patient may act to decrease the toxicity of the drug to the patient while the antimicrobial effect remains unchanged.

The fourth objective of this study relates to an interesting interface between AIDS and leishmanial research. In the search for antiparasitic agents, it was found that many compounds with strong antiprotozoal activity are also effective antiviral agents (16). For example, suramin, an agent that is very effective against the protozoal parasite

causing malaria, was the first anti-HIV agent discovered (16). One of the drugs tested in this study, sinefungin, is similar to suramin in that it has strong antileishmanial properties, and also inhibits a number of viruses (22). Sinefungin is also suspected of inhibiting HIV replication in T4 cells. The anti-HIV effects of sinefungin have been pursued by our laboratory in conjunction with the Department of Virology at the University of Massachusetts Medical Center in Worcester, MA. At first, they reported that sinefungin was an extremely effective inhibitor of HIV in T4 cells at a concentration which we found to be non-toxic to uninfected T4 cells. Later, they reported contradictory results indicating that sinefungin did not exhibit strong anti-HIV effects. However, recently interest has been rekindled in this area due to the work of French scientists using sinefungin and its analogs that appear to inhibit HIV replication. Sinefungin does not exert its anti-HIV effect by inhibiting the viral enzyme, reverse transcriptase (25). One hypothesis is that sinefungin alters T4 cell metabolism so that the HIV cannot replicate in T4 cells. Consequently the metabolism of sinefungin inside uninfected T4 cells was examined. This information might provide a clue as to the relationship of the metabolic processes inside T4 cells to HIV replication. The second value of investigating the intracellular metabolism of sinefungin in T4 cells is to understand the biochemical mechanism by which sinefungin is considerably more toxic to Leishmania spp. than to human T4 cells.

In summary, the research objectives of this study were:

1)Develop a valid and reliable assay to measure the toxicity of drugs on human-derived T4 cells in vitro.

2)Determine the toxicity of experimental antileishmanial agents on T4 cells in vitro, as measured by the inhibition of cell growth.

3)Test the protective effect against the toxicity of two promising antileishmanial agents, sinefungin and SRTC, on T4 cells by the addition of adenosine and other nutritional supplements to these cells.

4)Finally, the last objective of this study was to determine the mechanism by which sinefungin inhibits T4 cell growth, and how sinefungin is metabolized inside T4 cells.

LITERATURE REVIEW

In order to provide backround information to this study, the literature review will cover four subject areas:

A) The Nature and Function of T4 Lymphocytes in the Human Immune System.

B) The Nature of the Disease Leishmaniasis, and the Current Chemotherapeutic Agents used in the Treatment of this Disease.

C) The Biological Role of Purines and the Chemotherapeutic Value of Purine Analogs.

D) Description of the Chemotherapeutic Agents Tested in this Study.

A. The Nature and Function of T4 Lymphocytes.

The white blood cells that are central to a normal human immune response fall into two classes: the B cells, so named because they develop in the bone marrow, and the T cells, which originate in the bone marrow but complete their development in the thymus gland. B cells are the source of antibodies, the proteins that bind to antigens and aid in their removal or destuction. When a B cell recognizes an antigen, which may be circulating in the blood or the lymph or displayed on the surface of an infected cell, it becomes activated. It divides to produce an enlarged clone of B

cells, all bearing antibody molecules on their membrane that act as specific receptors for the antigen. Some of the cells, known as plasma cells, actively secrete antibody. Other longer-lived B cells are one root of the immunity that forestalls recurrence of many infections. These fmemory cells remain in circulation for years, ready to mount a response to the antigen if it challenges the body again (62).

The T cells are more complex in classification and function. They make up four subsets on the basis of function. Only one kind of T cell actively defends the body, namely the cytotoxic T cells. These destroy infected, foreign or malignant cells by lysing them. The other kinds of T cells modulate the immune response of both cytotoxic T cells and B cells by either secreting messenger proteins or by direct contact with the participating cells.

Inducer T cells trigger the maturation of T lymphocytes from precursor forms into functionally distinct cells. Helper cells are a precondition for the action of other T cells and most B cells - once they recognize a specific antigen, they enable cytotoxic T lymphocytes to destroy cells bearing the antigen and B cells to secrete appropriate antibody. The fourth kind of T cells, suppressor T cells, dampen the immune response of B and T cells, in effect shutting down the immune defenses several weeks after an infection activates them. Helper and suppressor cells thus have opposite effects on cytotoxic T cells and antibody- producing B cells. Suppressor

T cells also turn off helper cells when shutting down the immune response (63).

On the basis of biochemical markers on their surface, T cells are divided into two main kinds: T4 cells, which have helper and inducer roles, and T8 cells, with suppressor and cytotoxic functions.

T4 and T8 cells are also set apart by the type of cell surface protein they are able to recognize. Most T cells cannot recognize free antigen circulating in the blood or lymph, and they can respond to antigen on a cell surface only under particular conditions. The foreign substance must be displayed in conjunction with one of the host cell's own proteins: molecules coded for by the segments of DNA making up the Major Histocompatability Complex (MHC). For an immune response to ensue, the antigen receptor on the surface of a T cell must simultaneously recognize the antigen and the MHC protein.

T8 cells recognize antigen in the context in the Class I MHC proteins, a kind of molecule that is present on the surface of all nucleated cells. Hence a cytotoxic T8 cell ordinarily can kill any infected cell that carries an antigen for which the T8 cell is specific. T4 cells, in contrast, respond to antigen that is associated with Class II MHC proteins, which are found primarily on the surface of specialized cells known as antigen-presenting cells. The main antigen-presenting cell is the macrophage, found in the skin

and other tissues. Others include the Langerhans cells of the skin and the dendritic cells of the blood, lymph nodes and spleen. Macrophages function by engulfing a virus or other intruder, enzymatically breaking down its proteins in a highly specific way and displaying the antigenic protein fragments on the cell membrane together with Class II MHC proteins. Macrophages thereby prepare the antigen for recognition by T4 cells.

T4 cells play a central role in the normal function of the immune system. Once activated by appropriate antigen-presenting cells, they secrete soluble protein factors called interleukin-2 and gamma-interferon. Under the influence of interleukin-2, cytotoxic, suppressor and helper T cells proliferate into enlarged clones of mature cells. Gamma-interferon stimulates macrophages in their role of engulfing virus and presenting antigen. T4 cells are also vital to the production of antibody. B cells require a signal from the helper T cells, in the form either of interleukin-2 or direct contact, in order to respond to antigen. Once they have received this signal, the antigen-specific B cells multiply into an enlarged clone of antibody-secreting plasma cells and a population of memory B cells.

The AIDS virus alters and slows the growth of infected T4 cells, while other kinds of T cells continue to multiply normally. Ordinarily T4 cells make up from 60 to 80 percent

of the circulating T-cell population; in AIDS patients they can become too rare to be detected (62).

This reduction of the T4-cell population in AIDS has consequences that reflect the the cell's central place in the immune system. Lacking T4-cell help, B cells are unable to produce adequate quantities of specific antibody to the AIDS virus or to any other infection. The cytotoxic T-cell response is similarly hampered. Suppressor T cells cannot fulfill their role either. The B cells of AIDS patients, for example, continuously secrete large amounts of non-specific immunoglobulin because they never receive the T suppressor cell signal that ordinarily would shut them down. In addition, with the loss of T4 cells the level of interleukin-2 falls, slowing the clonal expansion of mature T cells. The reduced production of interleukin-2 and gamma-interferon depresses the activity of natural killer cells and macrophages, which these proteins normally stimulate (63).

B. The Nature of the Disease Leishmaniasis, and the Current Chemotherapeutic Agents used in the Treatment of this Disease.

> "It is remarkable that a disease as widespread and as serious as leishmaniasis has defied our efforts at chemotherapy. At this writing, there is no wholly satisfactory treatment for any of the forms of leishmaniasis and the possibilities on the horizon are relatively few." J. Joseph Marr, 1983 (6).

Leishmaniasis is caused by protozoan parasites belonging to the genus Leishmania (Phylum-protozoa: Order-Kinetoplastida: Family- Trypanosomatidae). The lifecycle of the organism occurs in two hosts, a vertebrate and an insect (64). The vertebrate hosts are most commonly rodents and canines, but also include humans, marsupials, sloths and anteaters. The disease is spread when female sandflies of the genus Phlebotomous (old world) or Lutzomyia (new world) feed off the blood of an infected animal, become infected with Leishmania, and transfer the parasite to new vertebrate hosts. Leishmanial parasites exist in two forms: 1) a flagellated promastigote in the insect vector. The promastigote lives in the sandfly's gut, migrates to the probiscus, and is inoculated into the skin of a further host when the sandfly takes another blood meal. 2) an amastigote in the vetebrate host. The amastigote form lives in the macrophages of the skin, viscera or blood, and is about 2x5 µm in size. The pathogenic form of this protozoan is the amastigote. Since it resides within the hosts' macrophages, it is particularly resistant to chemotherapeutic treatment. In most vertebrate hosts, Leishmania spp. cause few or no pathological effects. Animals such as the dog and rat form a reservoir of the parasite. Humans are an unusual host (except for L. donavani), and often present severe pathological reactions to the organism (6).

One of the biggest difficulties in treating leishmaniasis is the diversity of species of Leishmania. Each species has a specific geographical location, and a tropism to particular organs or tissues within the vertebrate host. Consequently, three clinical syndromes of leishmaniasis are distinguished, and each clinical type is associated with specific species of the organism (5). The three syndromes are:

1. Visceral Leishmaniasis: or kala-azar ('black fever' in Hindi) affects the internal organs, especially the spleen and liver. It is associated with L. donovani. Anemia, portal hypertension and ascites are symptoms of advanced infection. The major complications are bacteremia due to the inability of the reticuloendothelial system to remove these organisms from the blood and bleeding secondary to portal hypertension. Currently there are major epidemics in India and Kenya. Figure 1 shows the distribution of the disease around the world. Visceral leishmaniasis is generally fatal unless treated. In some areas, the parasite is resistant to available drugs (5).

2. Mucocutaneous Leishmaniasis: (espundia) presents most commonly as ulcers of the palatal and nasal mucosa which progress to destroy much of the cartilaginous structure of these areas. At times the laryngeal and tracheal cartilages may be involved. It is found in Central and South America, especially the Amazon basin and the forested areas east of

the Andes. This form of the disease is associated with L. braziliensis (5,6).

3. Cutaneous Leishmaniasis: presents as open, ulcerated lesions on the skin. This form generally heals spontaneously over a period of months. Figure 2 illustrates the global distribution of the syndrome. It is associated with L. braziliensis and L. mexicana in the new world, L. tropica, L. major and L. aethiopica in the old world (6). In people with defective cell-mediated responses to leishmanial antigens, the disease can progress to the incurable 'diffuse cutaneous leishmaniasis', a condition similar to leprosy (4).

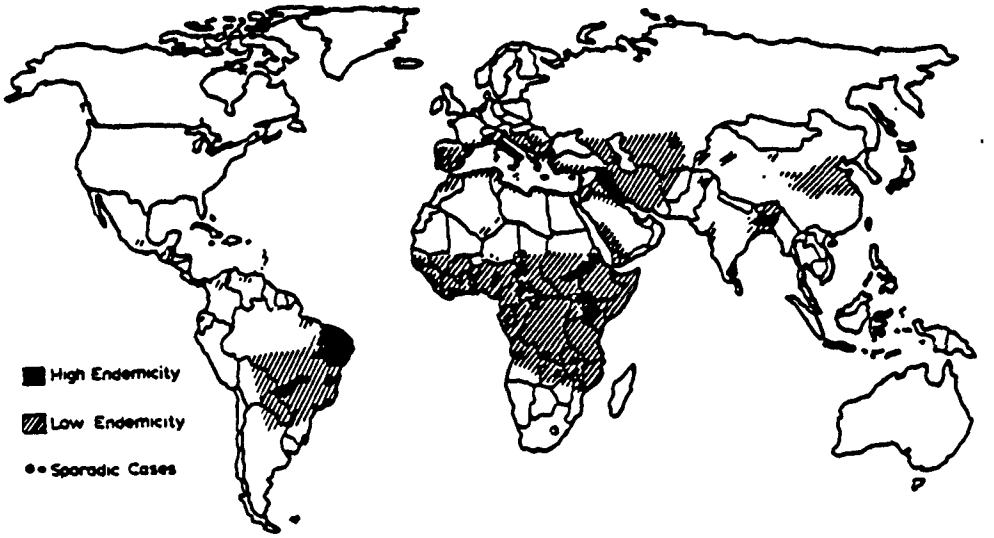


Figure 1. Distribution of Visceral Leishmaniasis. From the 6th Programme Report of the WHO Special Programme for Research and Training in Tropical Diseases on Leishmaniasis (5).

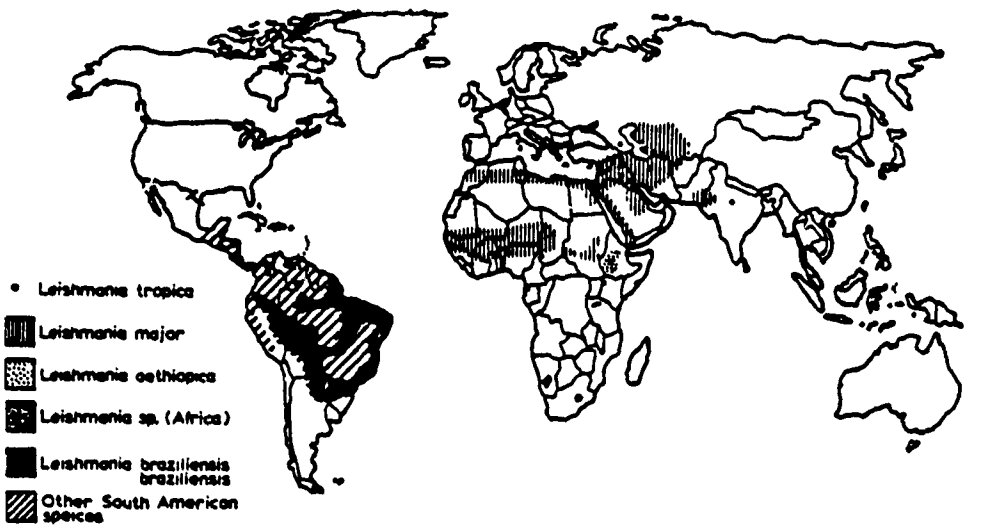


Figure 2. Distribution of Cutaneous and Mucocutaneous Leishmaniasis (6).

Current Treatment of Leishmaniais

There are three weaknesses in the current treatment for leishmaniasis:

1) The medications are not 100 percent effective. Treatment failures or relapses occur in about 10 to 25 percent of all forms of leishmaniasis (7). South American mucocutaneuos leishmaniasis has an initial cure rate of 50%, and visceral leishmaniasis in Sudan is notoriously resistant (8).

2) The antileishmanial agents require parenteral administration over a long period of time. The time range varies from 10 to 90 days, depending on an individual's response (47). This may require people in the Third World, where the disease is prevalent, to leave home for 1 to 3 months in order to receive treatment at a hospital. Intravenous administration severely limits access to the treatment, is expensive, burdens scarce hospital resources,

and increases the risk of infectious conditions such as AIDS, hepatitis B and septicemia.

3) The pharmacological agents are often toxic to humans, especially when initial treatment regimens fail, and secondary courses of drugs are needed (47).

All forms of leishmaniasis are initially treated with pentavalent antimony in the form of sodium stibogluconate (Pentostam) or of N-methyl glucanime antimonate (Glucantime). These drugs are injected intravenously at a dosage of 10-20 mg antimony/kg per day (Pentostam) or 28 mg antimony/kg per day (Glucantime) for 10-30 days. Treatment failures are classically treated with repeated administration of pentavalent antimony, or with pentamidine or amphotericin B. In general, the secondary treatment regimens are eventually effective (65).

The lack of progress in development of better antileishmanial agents is shown by the date of introduction of currently used drugs. The pentavalent antimonials Pentostam and Glucantime were introduced in 1947 and 1950, Pentamidine and Amphotericin B in 1940 and 1959 respectively (47).

Ideally, antileishmanial agents should be orally administrable, effective after a few administrations, relatively non-toxic to the patient, and inexpensive.

C. The Biological Role of Purines and the Chemotherapeutic Value of Purine Analogs.

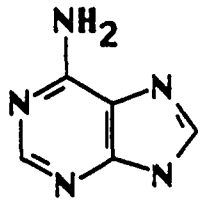
Purines are dicyclic nitrogenous bases. The structure of the parent compound is shown in Figure 3. The two major purine bases found in living organisms are adenine and guanine (66). Two other purines, hypoxanthine, xanthine, occur as intermediates in the metabolism of adenine and guanine (Fig. 3).

Purines occur in nature seldomly as free bases; they are found much more abundantly in the form of their nucleosides or nucleotides. A nucleoside is composed of a purine (or pyrimidine) base to which a ribose sugar is attached in B-linkage at the N9. A nucleotide is a nucleoside that has one or more phosphate groups on the hydroxal groups of the sugar (66).

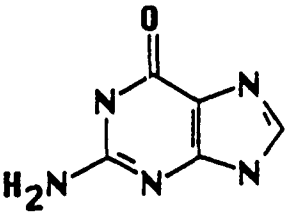
Nucleotides participate in a wide variety of biological processes. Perhaps the best known role of the purine nucleotides is, together with the pyrimidine nucleotides, to serve as the monomeric precursors of RNA and DNA. However, the purine ribonucleotides serve also in biological systems as the universal currency of energy, ATP; as metabolic regulators in the form of cyclic AMP (cAMP) and cyclic GMP; and as components of the widely used coenzymes FAD, NAD, and NADP and of an important methyl donor, S- adenosylmethionine (66).

A. Purine Bases

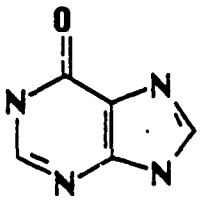
Adenine



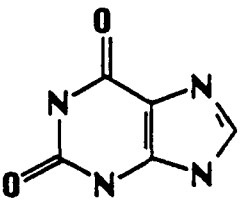
Guanine



Hypoxanthine

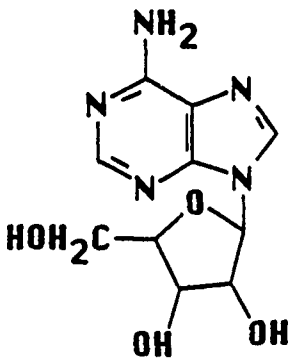


Xanthine



B. Purine Nucleosides

Adenosine



Guanosine

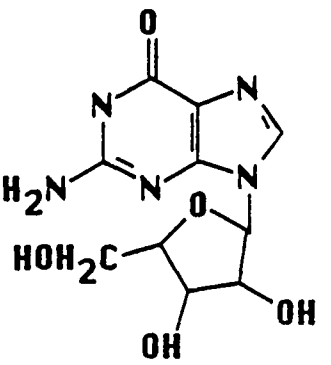


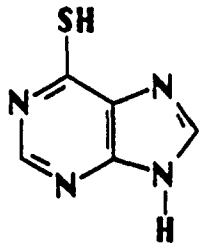
Figure 3. Chemical Structures of Purine Bases and Their Nucleotides.

Synthetic analogs of purines and its nucleosides or nucleotides are widely used in the medical sciences and clinical medicine. In the past, most of these uses have depended upon the role of nucleotides as components of nucleic acids for cellular growth and division (67). For a cell to divide, its nucleic acids must be replicated, requiring that the precursors of nucleic acids - the normal purine and pyrimidine ribonucleotides for RNA and the deoxyribonucleotides for DNA - be readily available. One of the most important components of the oncologist's pharmacopeia is the group of synthetic analogs of purine and pyrimidine bases and nucleosides (68).

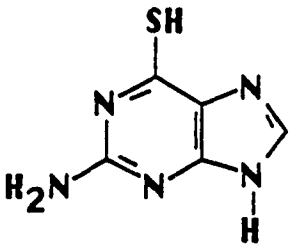
The pharmacological approach has been to use an analog in which either the heterocyclic ring structure or the sugar moiety has been altered in such a way as to induce toxic effects when the analog becomes incorporated into cellular constituents. Many of these effects result from inhibition by the drug of specific enzyme activities necessary for nucleic acid synthesis or from the incorporation of metabolites of the drug into the nucleic acids where they alter the required base pairing essential to accurate transmission of information (68).

The most commonly used analogs of the purine rings have substituents which do not occur naturally and which alter base pairing or the interaction of the nucleotides with specific enzymes (67). Some commonly used purine analogs

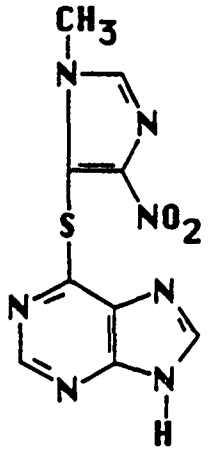
6-Mercaptopurine



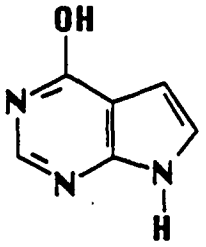
6-Thioguanine



Azathiopine



Allopurinol



Vidarabine

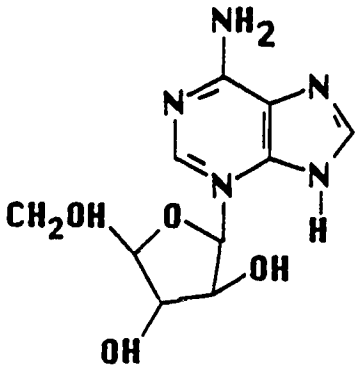


Figure 4. Chemical Structures of Some Purine Analogs Commonly Used as Chemotherapeutic Drugs.

include: 6-Mercaptopurine, which is used for the treatment of acute leukemias (Fig 4).

6-Thioguanine, which is also used in the treatment of acute leukemias.

Azathiopine is used in organ transplantation as a suppressor of events involved in immunologic rejection.

Allopurinol is an inhibitor of de novo purine biosynthesis and of xanthine oxidase. It is used for the treatment of hyperuricemia and gout.

Vidarabine is used in the treatment of Herpes simplex virus encephalitis (67).

D. Description of the Chemotherapeutic Agents Tested in this Study.

1. Purine Analogs.

The enzymes and pathways of purine metabolism are slightly different in the leishmanial protozoa to that in humans. Certain purine analogs, which have a slight structural alteration from the normal purine bases, are incorporated into normal purine metabolism by leishmanial cells, but not by human cells. As a result, these purine analogs are toxic to leishmanial organisms, but not to humans. This differential in toxicity between parasite and host provides the basis for effective chemotherapeutic agents.

In humans, purines are synthesized de novo from non-nucleotide precursors (amino acids, carbon dioxide, and ammonia) as well as from preformed bases through the salvage pathways. Salvage pathways permit the reutilization of purines or their derivatives from the degradation of nucleic acids or nucleotides.

In contrast, Leishmania spp. rely primarily on the salvage pathways for their source of nucleotides. They are thus dependent on nucleotide breakdown products of host tissue.

Allopurinol Riboside

Leishmanial promastigotes have been shown to have a purine nucleoside phosphotransferase which is either not present or poorly active in mammalian cells. This means that certain inosine analogs may be phosphorylated and incorporated into RNA by Leishmania spp. but not by mammalian cells; purine nucleotide synthesis in the parasite is modified, while that in the host is not, resulting in selective killing of the parasite. One such inosine analog that exploits this enzymatic difference between leishmanial and mammalian cells is allopurinol riboside.

Formycin A

Of these nucleotides, formycin A has been of particular interest due to its demonstrated chemotherapeutic and biological activity as an adenosine analog. Formycin A has shown antitumor, antibacterial, antifungal and antiviral

activity (14). This naturally occurring compound has two main metabolic routes: it can either be activated for incorporation in nucleic acids into its 5'-monophosphate form by the enzyme adenosine kinase (11) or it can act as a substrate for the enzyme adenosine deaminase (ADA)(12), and is thereby catabolized to the inosine analog formycin B (Figure 5B). Formycin B demonstrates a much lower level of chemotherapeutic activity.

Formycin A is catabolized in human tissue quite easily by adenosine deaminase, severely limiting its therapeutic potential (14). This has lead to research designed to develop a structural analog of formycin A which would not be a substrate for adenosine deaminase, but would still possess significant biological activity against leishmanial parasites. One approach has been to produce subtle changes in the pyrazolo[4,3-d]pyrimidine heterocyclic ring system. 9-deazainosine (Figure 5C) is one example of this.

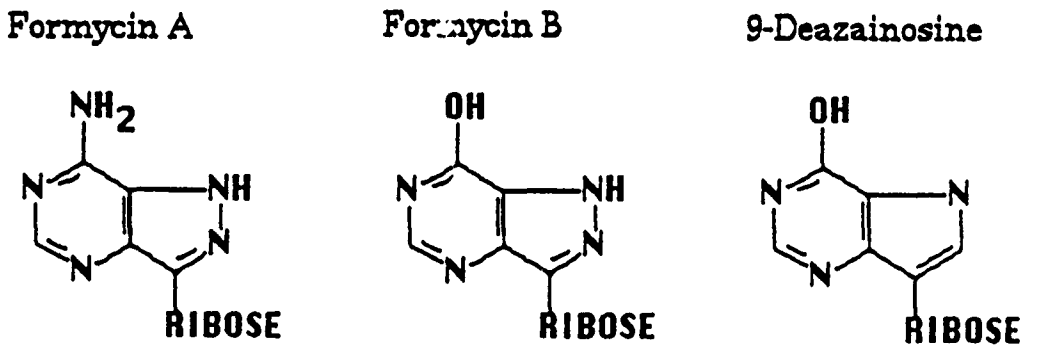
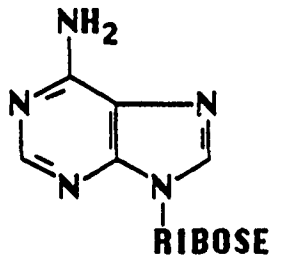


Figure 5. Chemical structure of formycin A, formycin B, and 9-deazainosine (30).

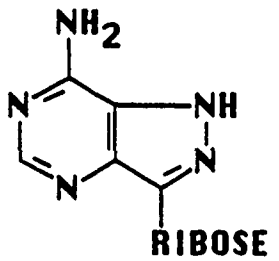
Normal Purines

Adenosine

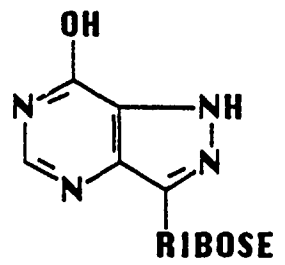


Purine Analogs

Formycin A



Formycin B



Sinefungin

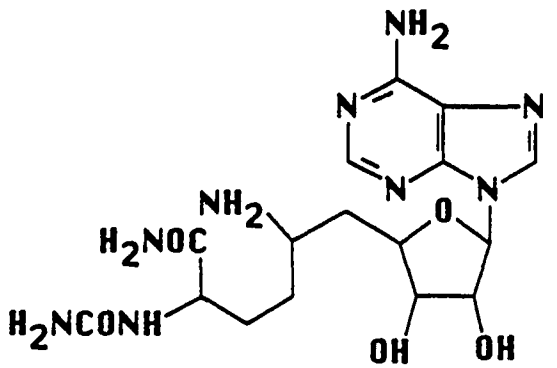
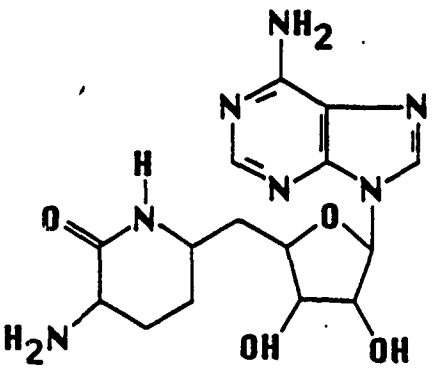


Figure 6A. Chemical Structures of the Chemotherapeutic Agents Tested in this Study: 1) Purine Analogs in Comparison to the Parent Purine Nucleoside, Adenosine.

Cyclic Sinefungin



Cordycepin

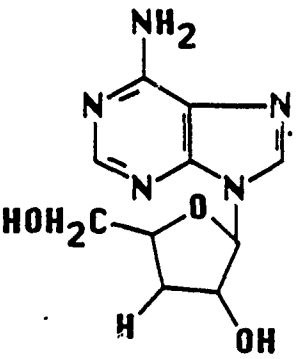
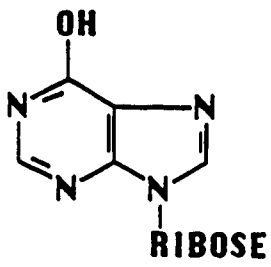


Figure 6A. Chemical Structures of the Chemotherapeutic Agents Tested in this Study: 1) Adenosine Analogs Continued.

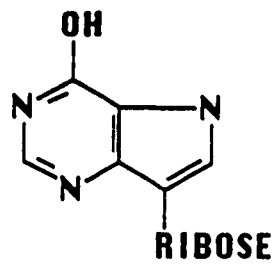
Normal Purines

Inosine

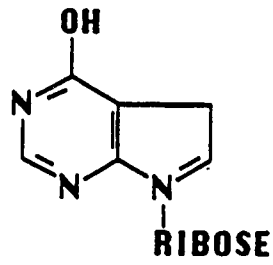


Purine Analogs

9-Deazainosine



7-Deazainosine



Allopurinol Ribose

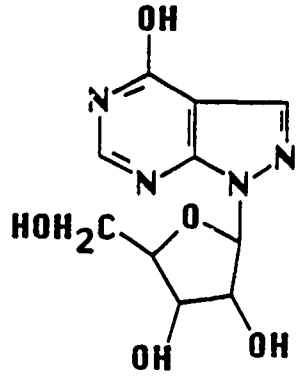


Figure 6A. Continued: 2) Purine Analogs in Comparison to the Parent Purine Nucleoside, Inosine.

Sinefungin

Sinefungin is a natural nucleoside isolated from cultures of Streptomyces incarnatus and S. griseolus. It is an adenosine analog, and also has structural similarity to the methyl donor S-adenosylmethionine. It is a powerful inhibitor of fungi (18,19,20), viruses (21), cancer and a variety of parasites, including Leishmania spp.(22,23,24). The mechanism of its toxicity on the latter is not clearly known. Recently it was reported that sinefungin inhibits protein methylases in Leishmania spp., probably due its structural similarity to S-adenosylmethionine (54). Other reports suggest that the major site of its inhibition of Leishmania spp. involves disturbance of DNA synthesis (55).

2. Other Compounds

DFMO (D,L-Difluoromethylornithine)

DFMO is an analog of the amino acid ornithine, and functions as an irreversible inhibitor of ornithine decarboxylase (56). The latter is the first enzyme, as well as the rate-controlling enzyme, in the biosynthetic pathway of polyamines. DFMO inhibition of ornithine decarboxylase results in a reduction of the intracellular concentrations of these polycations.

Polyamines are low-molecular-weight compounds found in all cells, where they are thought to participate in nucleic acid and protein synthesis and to stabilize certain macromolecules (52). The common polyamines are putrescine,

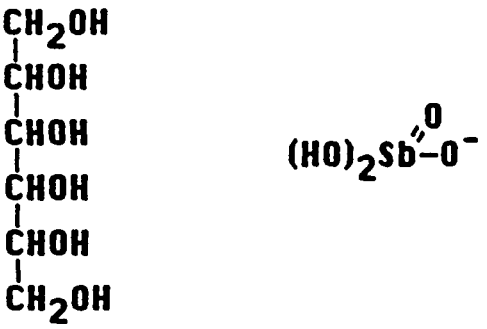
spermidine and spermine. The onset of growth in all prokaryotic and eukaryotic organisms thus far studied is associated with increased polyamine synthesis and the induction of ornithine decarboxylase (53). DFMO inhibition of ornithine decarboxylase has been found to limit growth of Human Cytomegalovirus (57), Trypanosomes (56), Pneumocystis carinii (52), and Leishmania spp.(48), with low toxicity to slow-growing host cells. DFMO in combination with purine analogs such as 9- deazainosine, allopurinol riboside or sinefungin have been found to be the most effective treatment for trypanosomes, the protozoal parasite similar to Leishmania spp. that causes African sleeping sickness (53).

Ketoconazole

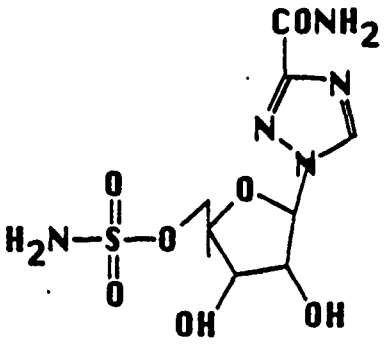
Ketoconazole is a synthetic imidazole analog. It is primarily used clinically as a broad spectrum antifungal agent (34). It exerts its antifungal activity through inhibition of lanosterol demethylation. This blocks the synthesis of ergosterol, the major sterol component of the fungal cell membrane (35). In mammalian cells, ketoconazole also inhibits lanosterol demethylation, with a subsequent decrease in the biosynthesis of cholesterol, the major sterol component of mammalian cell membranes (36). In addition, it interferes with cellular fatty acid and phopholipid biosynthesis.

Ketoconazole is also an effective antiviral agent, particularly against Herpes Simplex virus (37). Recently, it

Glucantime (Meglumine Antimoniate)



SRTC



Ketoconazole

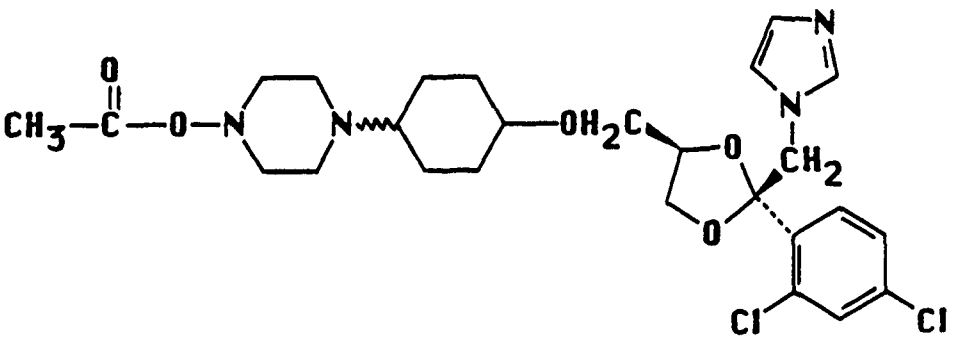


Figure 6B. Chemical Structures of the Chemotherapeutic Agents Tested in this Study: 2) Other Compounds.

has been shown to possess in vitro antileishmanial activity against Leishmania mexicana amastigotes in macrophages, by inhibiting the synthesis of ergosterol synthesis. Fifty percent inhibition of ergosterol biosynthesis in Leishmania was acheived at 0.21 μM ketoconazole. The identical effect on cholesterol synthesis in the uninfected macrophages was achieved at 1.5 μM (36).

SRTC

SRTC (5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide) is an experimental compound developed by Roland Roberts of Brigham Young University, Utah, for the treatment of protozoal infections, including leishmaniasis, malaria, and trypanosomiasis (African sleeping sickness). It is a pyrimidine analog. Not much more is known about it at present.

CHAPTER III

METHODOLOGY

A. Research Objectives

The research objectives of this study were:

1)Develop a valid and reliable assay to measure the toxicity of drugs on human-derived T4 cells in vitro.

2)Determine the toxicity of experimental antileishmanial agents on T4 cells in vitro, as measured by the inhibition of cell growth.

3)Determine the toxicity on T4 cells of drugs used in the treatment of the infectious complications of AIDS.

4)Test the protective effect against the toxicity of two promising antileishmanial agents, sinefungin and SRTC, on T4 cells by the addition of adenosine and other nutritional supplements to these cells.

5)Finally, the last objective of this study is to determine the mechanism by which sinefungin inhibits T4 cell growth, and how sinefungin is metabolized inside T4 cells.

B. Methods and Materials

Cell Culture

T4 cells of the CEM line were obtained from the Department of Virology at UMass Medical Center. They were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 1gm/liter Sodium bicarbonate, and 1% gentamycin. In experiments, cells were grown in polystyrene microwell

plates. The plates were incubated in a CO2 chamber within an incubator at 36°C. CO2 prevented the growth medium from becoming too alkaline for cell survival.

Assay Procedure

The starting cell stock was standardized at an absorbance of 0.500 at δ=660 nm, or a concentration of 300,000 cells/mm3, because different concentrations of cells grew at varying rates, and are thus inhibited to variable extents. The cell stock concentration was standardized by centrifuging the cells in a microcentrifuge for 2½ minutes and resuspending in fresh medium to an absorbance of 0.500.

Aliquots of the stirred cell stock (60 µl) were pipetted by a Rainin computerized multipipette into the plate wells, followed by 45 µl aliquots of double-concentrated RPMI medium. To the control wells, 45 µl of sterile, double-distilled water was added. To test wells, increasing concentrations of test drug were added. A row of blank wells containing sterile water and medium only, was used to check the sterility of the procedure.

Cells were counted at the beginning of an experiment (time 0 hours) and after 72 hours of incubation, when the cells were in the logarithmic phase of growth. A Coulter counter Zf model (Coulter Electronics, Hialeah, Florida) was used for counting. Percent inhibition was determined by:

$$\left[1 - \frac{\text{test cell count}}{\text{control cell count}}\right] \times 100$$

Data was entered on a AT&T microcomputer using a Lotus 1-2-3 spreadsheet, transferred to a Macintosh SE system, and

graphed on a Cricketgraph program. A curve of percent inhibition or cell number versus drug concentration was plotted.

Some purine analogs were supplied by Walter Reed Research Institute, Washington D.C., allopurinol riboside was donated by Burroughs- Wellcome, North Carolina, and others were obtained from Sigma Chemical Co., St Louis, MO.

Inhibition Reversal

The effects of a nutritional supplement on the inhibition of cellular growth caused by sinefungin and SRTC was tested to examine whether the the nutritional additive could reverse the cytotoxicity of the antileishmanial agent. Adenosine, methionine, S-adenosylmethionine and S- adenosylhomocysteine were tested on sinefungin, and adenosine was tested on SRTC. Cells were incubated with the reversal compound for 10 minutes before the antileishmanial agent was added, so that the nutritional supplement could enter the cell without having to compete with the drug. Solutions of the nutritional supplement replaced distilled water in the cell wells, so that the final volume of each cell well was kept constant at 150 µl.

Determination of the Mechanism of Sinefungin's Inhibition of T4 Cell Growth.

In order to determine the mechanism of action of sinefungin on T4 cells, we developed a method of quantifying the intracellular levels of sinefungin and its likely

metabolites. This method involves two steps: 1) Isolation and separation of the cytoplasmic nucleotide fraction, which contains the free intracellular sinefungin and its metabolites 2) Quantification of intracellular sinefungin levels over time using high pressure liquid chromatography (HPLC), as an indication of whether sinefungin is metabolized by T4 cells or not.

To do this, intracellular levels of sinefungin were compared in three sets of cells: a control containing no sinefungin, cells incubated with 1 mM sinefungin for 1 hour, and cells incubated with 1 mM sinefungin for 24 hours.

Isolation and Separation of the Cytoplasmic Nucleotide Fraction

Aliquots (35 ml) of cell stock were incubated with 1 mM sinefungin for 1 or 24 hours. They were then centrifuged at 10,000 RPM for 4 minutes, washed in 20 ml of 0.5 M ammonium hydroxide, recentrifuged at 10,000 RPM for 4 minutes, and the pellet was resuspended in 1 ml of CHES buffer at pH 9.0 . The cell solution was freeze-thawed four times, until all the cells lysed. The lyzed cell stock was incubated with 5'-nucleotidase for 37°C for 1.5 hours. This enzyme derived from snake venom removes a phosphate group from adenosine-5'-monophosphate, so that it does not elute on the HPLC chromatogram simultaneously with sinefungin. 100 µl of 0.1 M TCA was then added to the cell stock to precipitate the protein. The solution was then centrifuged in an Eppendorf

microcentrifuge at 14,000 RPM for two minutes. The supernatant was carefully removed by Pasteur pipette, and stored at -70°C.

Quantification of Intracellular Sinefungin Levels Over Time by HPLC.

The quantification of intracellular levels of sinefungin by HPLC analysis was performed on a Waters 6000A chromatograph, using a Waters 440 absorbance detector set at 254nm and a 25cm Whatman Hypersil ODS C18 column. The method used was obtained from Dr Lech Dudycz from the Dept of Pharmacology, University of Massachusetts Medical Center, Worcester, MA. Nucleotide samples were filtered by a 0.2 µM filter, and 100µl was injected automatically per run. A gradient solvent system was used to elute the nucleotides. Buffer A (50 mM KH_2PO_4, pH 3.0) was run for 5 minutes, followed by a linear gradient to 100 % Buffer B (50 mM KH_2PO_4: 50% acetonitrile) at 25 minutes, returning to 100% buffer A at 30 minutes. Flow rate was 1.75 ml/min. A Waters 720 data module was used to chart and calibrate the data.

C. Operational Definitions

ED50 (Effective dose 50): The dose of a compound that causes a 50% decrease in the absolute number of pathogenic parasites relative to untreated controls.

ID50 (Inhibition dose 50): The dose of a compound that causes 50% inhibition in the growth rate or absolute numbers of host cells relative to untreated controls.

Therapeutic index: The toxicity of an chemotherapeutic agent to the host relative to its toxicity to the pathogenic parasite. In this study, it is defined specifically as

$$\left(\frac{\text{ID50 in T4 cells}}{\text{ED50 in } \textit{Leishmania} \text{ spp.}} \right)$$

Percent Inhibition: The inhibition of cellular growth caused by an agent as compared to undisturbed cellular growth of controls. It is measured by direct cell counts with a Coulter Cell Counter, and expressed as:

$$\left[1 - \frac{\text{test cell count}}{\text{control cell count}}\right] \times 100$$

Human CEM T4 (CD4) Cells: This is the human T4-lymphoblastoid cell line, CCRF-CEM. They were originally derived from leukemic human T-helper/inducer (T4) lymphocytes, and are grown by tissue culture methods. The term T4 or CD4 is the name given to the membrane-surface proteins by which these cells can be biochemically identified.

Purine Analogs: These are compounds that have a chemical structure very similar to that of the purines adenine, guanine, inosine, xanthine and hypoxanthine which are utilized for the formation of DNA and RNA. The analogs of purines can inhibit normal purine nucleotide pathways, thereby killing the cells whose metabolism they inhibit. The most common mechanism of purine analog toxicity is via their phosphorylation to adenosine- or guanosine-triphosphate analogs which inhibits DNA and RNA synthesis. The other common mechanism is via their inhibition of intracellular

enzymes involved in purine base or nucleotide metabolic pathways.

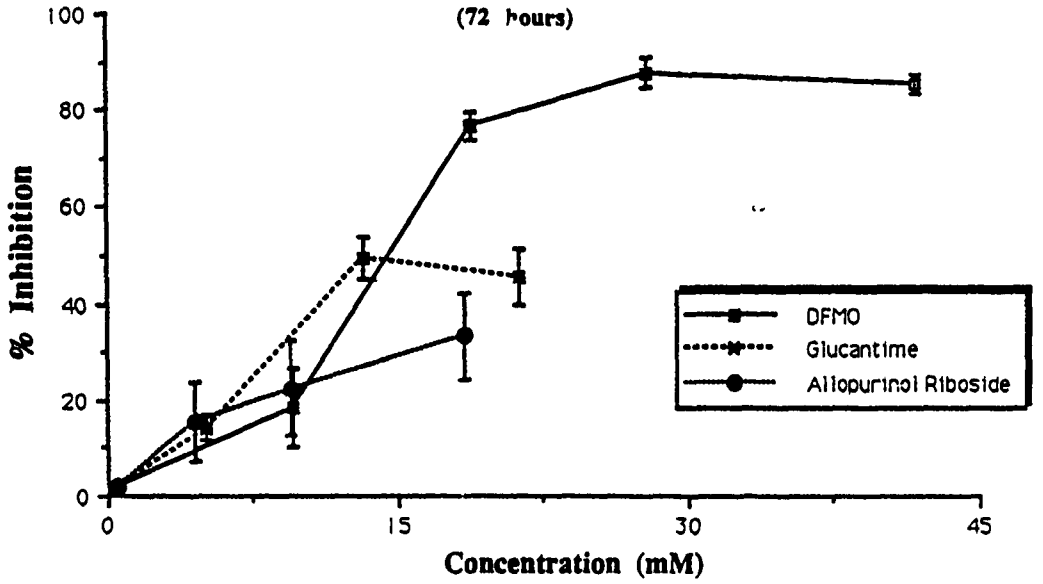
Nutritional Supplements for Inhibition Reversal: These are natural compounds found in food or food supplements that are involved in the metabolic pathways known or suspected to be inhibited by the purine analogs being tested. They include the regular purine bases and nucleosides of which the test compound is an analog. For example , the effects of allopurinol riboside can be reversed by adenine and related compounds (28), and Formycin A, an adenosine analog, can be reversed by adenosine (29) in certain organisms. If the purine analog is hypothesized to inhibit an enzyme, the enzymatic product usually reverses the toxicity of the compound.

RESULTS

B. Inhibition of T4 Cell Growth By Antileishmanial Agents

The effect of drug dose on T4 cell growth is shown in Figures 7,8,9, and 10 (Individual graphs for each drug are shown in Appendix A). Toxicity to T4 cell growth is represented as percent inhibition of cells grown with a drug relative to untreated controls. Compounds that are relatively non-toxic to T4 cells will require a high concentration to inhibit T4 cell growth, and will thus have a high ID50 (Inhibition dosage causing 50 percent less growth in drug-treated cells relative to controls). Thus a high ID50 is indicative of low toxicity to T4 cells, and vice versa.

The plots in Figures 7-10 are arranged in decreasing order of ID50s i.e. in increasing order of toxicity. One-way analysis of variance indicates a statistically significant relationship between drug dose and growth inhibition at $p<0.001$ with $\alpha=0.05$ for all the compounds tested, except for allopurinol riboside which is statistically significant at $p<0.05$. The ID50s of the compounds are summarized in Table 1. The drugs are arranged in increasing order of toxicity from top to bottom. Each curve in Figures 7-10, and each ID50 value in Table 1, is based on the results of 3 to 8 experiments, and in each experiment 4 individual test wells were used to give a mean and its standard error (represented by error bars).



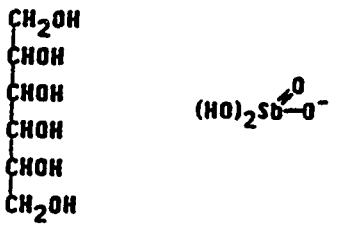
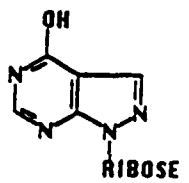
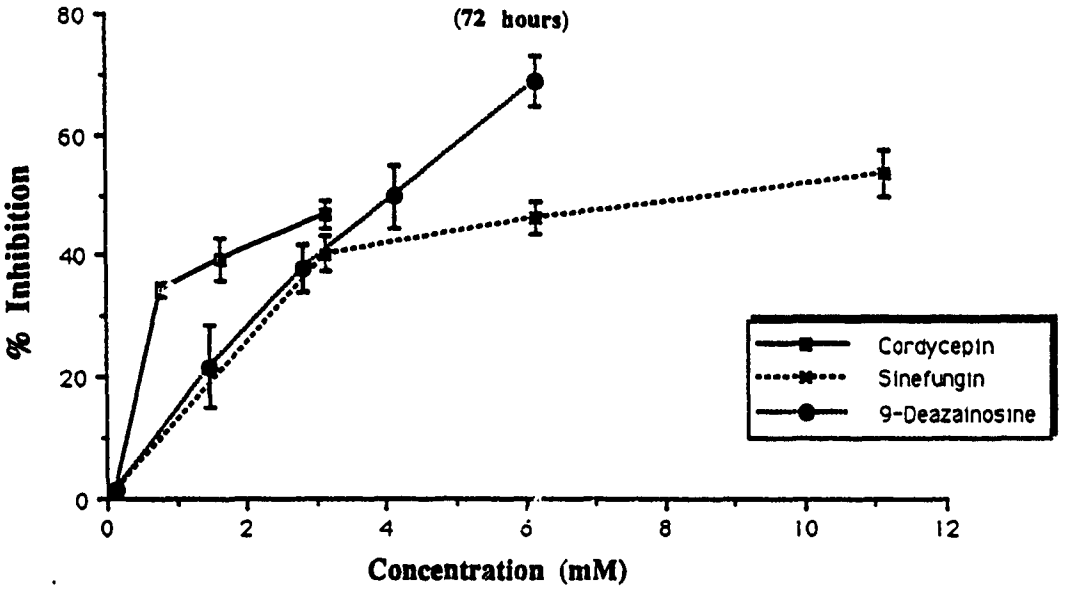
Drug	1 way ANOVA p-Value	Chemical Structure
DFMO	0.001	
Glucantime	0.0001	
Allopurinol Riboside	0.0274	

Figure 7. Inhibition of T4 Cell Growth by the Drugs DFMO, Glucantime and Allopurinol Riboside. The 1-Way Analysis of Variance and Chemical Structure for Each Drug Are Shown Below the Graph.



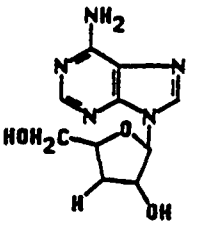
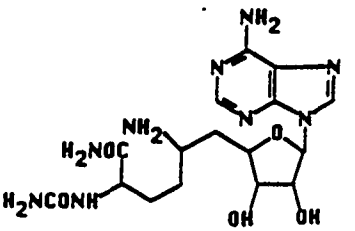
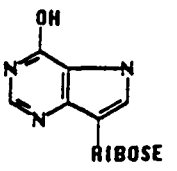
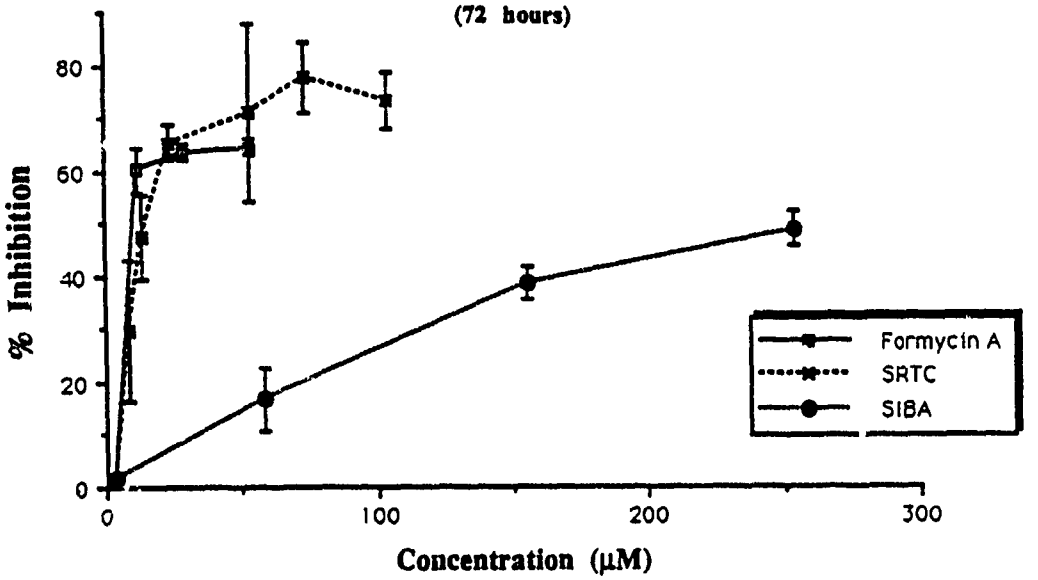
Drug	1 way ANOVA p-Value	Chemical Structure
Cordycepin	0.0001	
Sinefungin	0.0001	
9-Deazainosine	0.0001	

Figure 8. Inhibition of T4 Cell Growth by the Drugs Cordycepin, Sinefungin and 9-Deazainosine. The 1-Way Analysis of Variance p Value and Chemical Structure for Each Drug Are Shown Below the Graph.



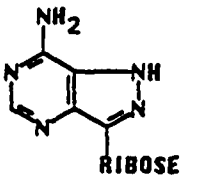
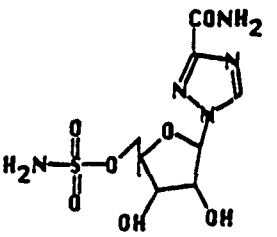
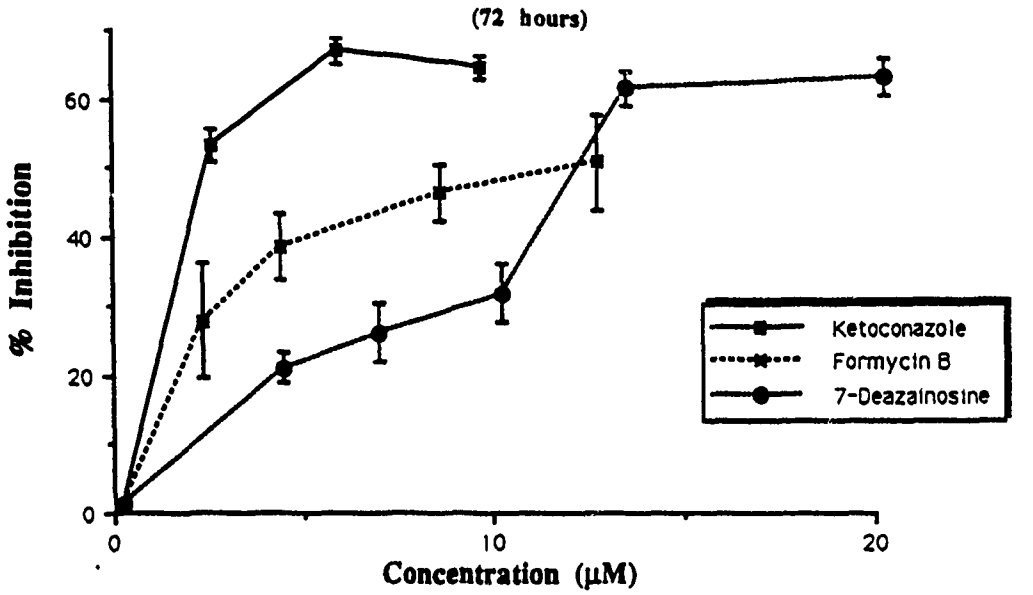
Drug	1 way ANOVA p-Value	Chemical Structure
Formycin A	0.0001	
SRTC (5'-o-sulfamoyl-1-ß-D-ribofuranosyl triazole-3-carboximide)	0.0001	
SIBA	0.0004	

Figure 9. Inhibition of T4 Cell Growth by the Drugs Formycin A, SRTC, and SIBA. The 1-Way Analysis of Variance p Value and Chemical Structure for Each Drug Are Shown Below the Graph.



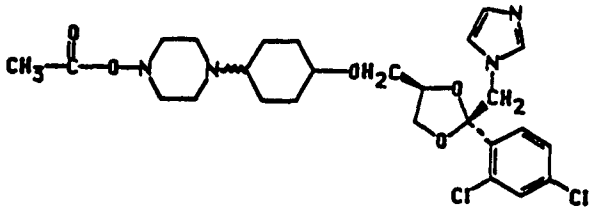
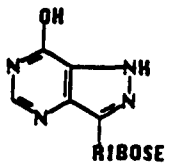
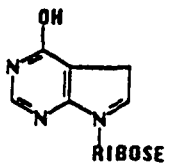
Drug	1 way ANOVA p-Value	Chemical Structure
Ketoconazole	0.0001	
Formycin B	0.0001	
7-Deazainosine	0.0001	

Figure 10. Inhibition of T4 Cell Growth by the Drugs Ketoconazole, Formycin B, and 7-Deazainosine. The 1-Way Analysis of Variance p Value and Chemical Structure for Each Drug Are Shown Below the Graph.

Table 1. The toxicity of antileishmanial agents on T4 cells. This is measured by the dose that causes fifty percent inhibiton of T4 cell growth relative to untreated control T4 cell growth.

Compound	ID50 (μM)
Allopurinol Riboside	>12,300
Glucantime *	>12,000
Sinefungin	11,000
DFMO $	10,400
9-deazainosine	4,000
Cyclic sinefungin	>3,000
Cordycepin	3,000
SIBA °	250
Formycin B	13
SRTC #	13
7-deazainosine	12
Formycin A	<8
Garlic ∞	11 μg protein/ml (0.001 of a clove/ml)
Ketoconazole	2.3

* = Meglumine antimoniate
$ = DL-α-difluoromethylornithine
° = 5-deoxy-5(isobutylthio)-3-adenosine
= 5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide
∞ = Allium sativum. This is made from a crude extract of raw garlic which is diluted in double-distilled water and sterile-filtered.

Of the 14 compounds tested, allopurinol riboside and Glucantime have the lowest growth inhibition effect. They are both currently used in the treatment of leishmaniasis. After these two, sinefungin and DFMO have the least T4 cell cytotoxicity. These are drugs in experimental trials for the treatment of viral and protozoal infections. The two compounds with the greatest toxicity are garlic and ketoconazole. Ketoconazole is the treatment of choice for eosophageal or systemic candidiasis in AIDS patients.

These toxicities of drugs on T4 cells are particularly valuable when compared to the dose of the same drugs required to effectively kill or inhibit parasitic organisms in humans or other hosts. The selective toxicity of an agent to the infectious parasite in comparison to the host can be measured by the therapeutic index i.e. toxicity in the host relative to the toxicity in the parasite. In order to assess the therapeutic value of the compounds tested here, I have compared the T4 cell toxicities of this study to leishmanial toxicities reported in the literature. Table 2 shows the results of this comparison, as measured by the therapeutic index, i.e. $\left(\frac{\text{ID50 in T4 cells}}{\text{ED50 in } \underline{\text{Leishmania}} \text{ spp.}}\right)$

In this study, we have tried to focus on Leishmania mexicana, a species causing muco-cutaneous leishmaniasis. However, where data is lacking for this species, I have used data from other species.

Because of the wealth of information in Table 2, I have tried to simplify the digestion of its contents by leaving out the superscript numbers which indicate the reference source for each data point. In Appendix B, the reference sources of Table 2 are included.

The therapeutic indices calculated from the toxicity to the promastigote form of Leishmania spp. indicate sinefungin to be an extremely promising antileishmanial agent. 9-deazainosine also has a high therapeutic index for both the amastigote and promastigote form, and thus holds promise as

an effective treatment for leishmaniasis. Allopurinol riboside, which is currently in clinical trial for visceral leishmaniasis in Kenya and India (44), has high therapeutic indices for both the promastigote and amastigote form, but significantly less than sinefungin and 9-deazainosine. Formycin B and cordycepin have moderately high therapeutic indices, but both these compounds have been found to have high systemic toxicities in rats and hamsters (44). Sinefungin, 9-deazainosine and allopurinol riboside have been found to have low systemic toxicities in rats and hamsters (24,41,43).

The exceptionally high therapeutic index of sinefungin could result from its breakdown to relatively non-toxic catabolic products by the enzyme adenosine deaminase. This enzyme, which is present in the fetal calf serum supplemented to the growth medium, is responsible for the first step in the catabolism of adenosine to inosine, and ultimately to the final breakdown product of purines, uric acid. Since sinefungin is an adenosine analog, it could be catabolized by adenosine deaminase, which would result in a spuriously elevated therapeutic index for sinefungin. In order to test this hypothesis, we compared the inhibition of T4 cell growth by sinefungin at 5 levels of fetal calf serum, ranging from 5% to 20% of the growth medium (v:v). A 3mM dose of sinefungin was used at all levels of fetal calf serum. The results are shown in Figure 11. No trend or significant

Table 2. The Therapeutic Value of Compounds in the Treatment of Leishmania spp.

Compound	The toxicity of antileishmanial agents on: Leishmania (ED_{50}) 1 Promastigote (uM)	2 In Macrophages (uM)	3 In Hamsters (mg/kg/day)	Mammalian cells (ID_{50}) 4 T4 cells (uM)	5 Other cells (uM)	Therapeutic Index 6 promastig. Col 4÷1	amastig. Col 4÷2
Sinefungin	0.005 (L.M)	-	4.0 (L.D)	11,000	-	2,200,000	-
9-deazainosine	40.0 (L.M)	1.0 (L.T)	19.0 (L.D)	4,000	>1,000	100	4,000
Allopurinol Riboside	200.0 (L.M) 7.0 (L.D)	76-190(L.T) 86-213(L.D)	485.0 (L.D)	>>12,300	>2,000	>62 >1,757	>92 >82
Formycin B	0.1 (L.M)	0.04 (L.T)	1.5 (L.D)	13	200	13	325
Formycin A	1.0 (L.D)	0.04 (L.T)	<<13.0 (L.D)	<8	-	8	200
7-deazainosine	<2.0 (L.D)	0.2 (L.T)	-	12	20	6	60
SRTC	11.6 (L.M)	-	-	13	-	1.1	-
Cyclic sinefungin	-	-	-	>3,000	-	-	-
Cordycepin	25.0 (L.M)	-	-	3,000	-	120	-
SIBA	20.0 (L.M)	-	-	250	-	12.5	-
Allium sativum (Garlic)	>28ug protein/ml (L.M)	-	-	11 ug protein/ml (0.001 of a clove/ml)	-	<0.4	-
DFMO	30.0 (L.D)	>1,098 (L.D)	-	10,400	-	347	9.5
Ketoconazole	-	1.0 (L.M)	-	2.3	10% at 10uM	-	2.3
Meglumine antimoniate (Glucantime)	>130,000(L.B)	17.5 (L.D)	16.0 (L.D) 660.0 (L.B)	>12,000	-	0.09	686

(L.M.)= Leishmania mexicana 0227 (L.B)= Leishmania braziliensis (L.D)= Leishmania donovani (L.T)= Leishmania tropica

Colum 1 = The concentration of drug that inhibits the growth of the promastigote form of Leishmania spp. to half that of untreated controls.

Colum 2 = The concentration of drug that eliminates 50% of leishmanial amastigotes from within human macrophages in vitro relative to untreated controls.

Colum 3 = The concentration of a drug that suppresses leishmanial amastigote numbers in hamster livers to half that of untreated controls after four days of treatment.

Colum 4 = The concentration of a drug that inhibits the growth of CD4 cells by 50 % relative to untreated controls.

Col 6 = $\frac{\text{50\% Inhibition level on T4 cells}}{\text{50\% Inhibition dose on promastigote Leishmania}}$ 4÷2= $\frac{\text{50\% Inhibition level on T4 cells}}{\text{50\% Inhib. dose on amastigote Leishmania in macrophages}}$

difference in the inhibition of T4 cell growth by sinefungin was observed. It thus appears that the latter is not influenced by the levels of adenosine deaminase in the growth medium at a concentration greater than 5%. The growth of control cells was limited by a fetal calf serum concentration of less than 5%, so that the effect of this level of fetal calf serum on sinefungin inhibition of T4 cells could not be tested. However, since the fetal calf serum used was heat-inactivated to destroy the adenosine deaminase enzyme, it is highly unlikely that sufficient enzyme to completely catabolize sinefungin is present in less than 5% fetal calf serum. It thus appears that the presence of adenosine deaminase in the serum added to the growth medium is not causing artificially low toxicity of sinefungin to T4 cells, and consequently a spuriously elevated therapeutic index.

Garlic has the lowest therapeutic index in Table 2. Its value for this indicator of less than unity illustrates that it is more toxic to T4 cells than Leishmania mexicana. This is shown graphically in Figure 11b. In fact, garlic is extremely toxic to T4 cells - its ID50 is the equivalent of the extract of one thousandth of a raw clove per milliliter of cell stock.

B. The Protective Effect of Nutritional Supplementation

The protective effect of adenosine against the cytotoxicity of SRTC (5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide) on the T4 cells was determined at

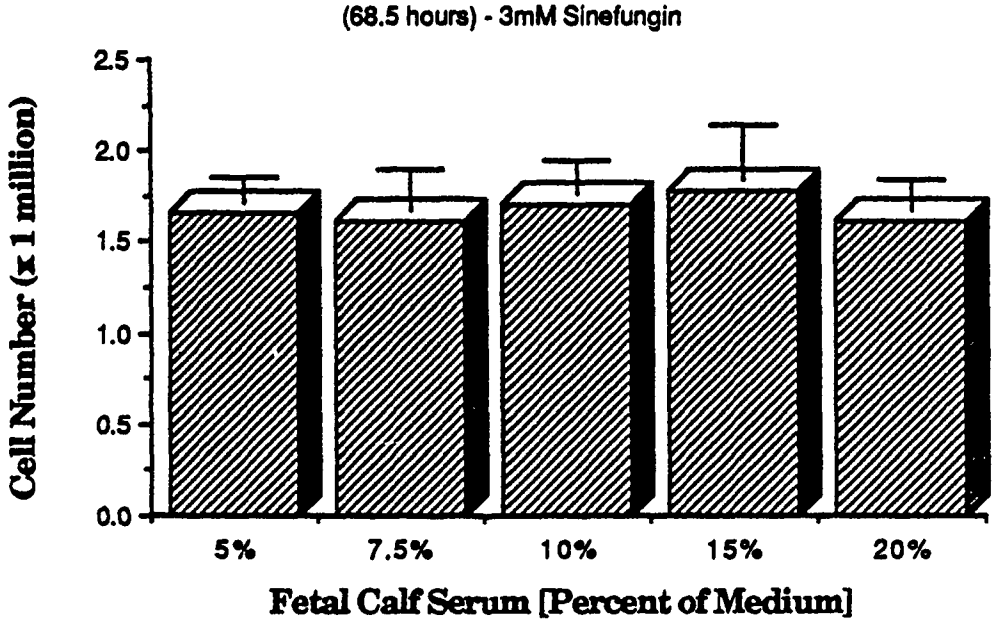


Figure 11. The Effect of Different Fetal Calf Serum Concentrations on the Inhibition of T4 Cell Growth by Sinefungin.

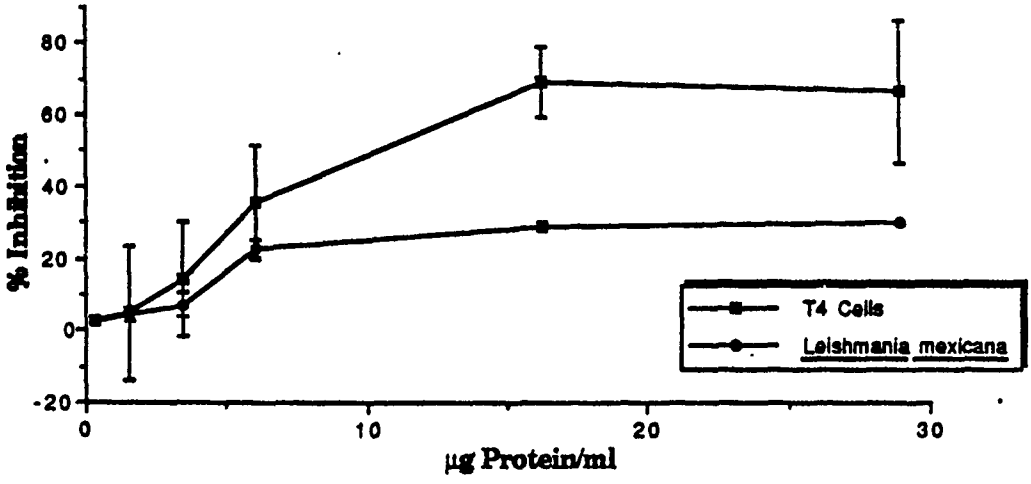
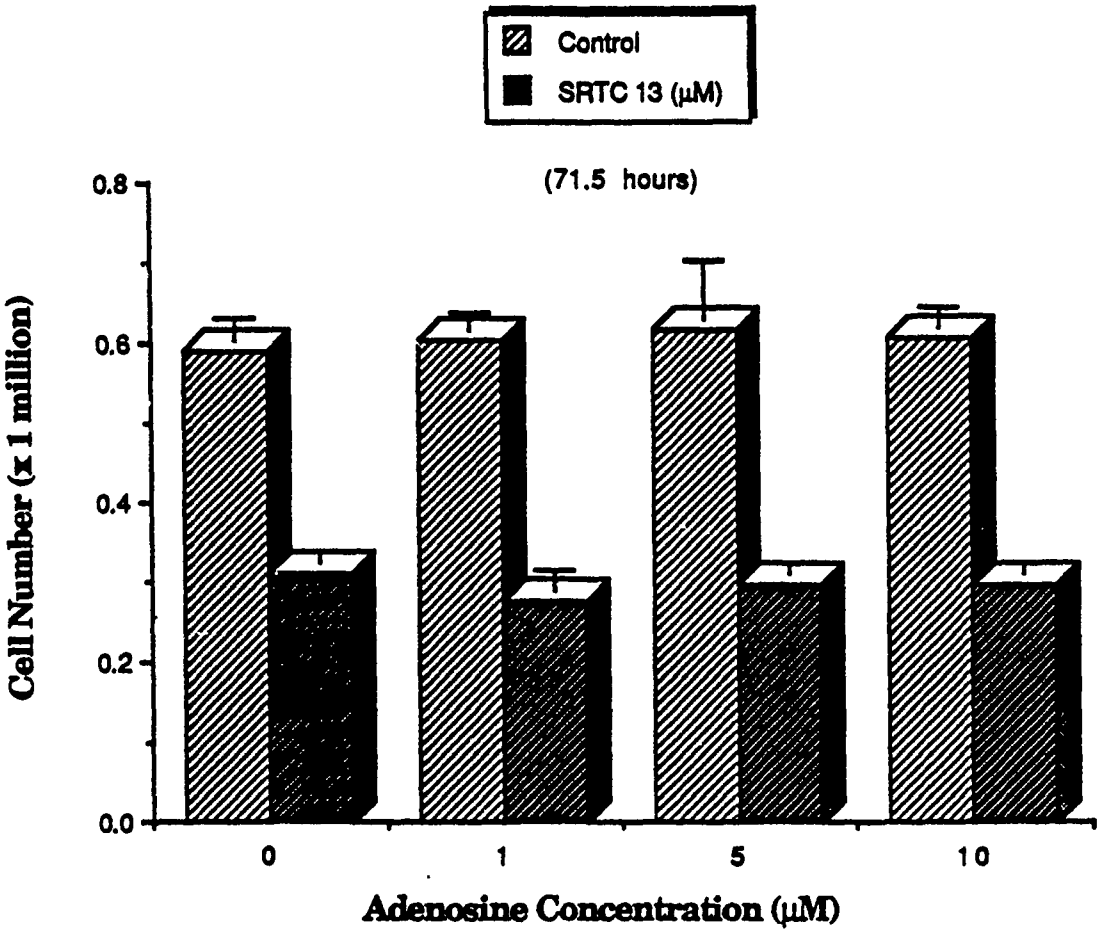


Figure 11b. A Comparison of the Growth Inhibition of Garlic on T4 Cells and Leishmania mexicana #227.

concentrations ranging from 1 μM to 10 μM adenosine supplement. The results, shown in Figure 12, illustrate that adenosine had no effect on SRTC cytotoxicity. For sinefungin, we tested the protective effect of adenosine, methionine, S-adenosylmethionine, and simultaneous adenosine and methionine supplementation to the T4 cells. Figure 13 illustrates that adenosine had no protective effect against the growth inhibition of sinefungin on T4 cells, Figure 14 illustrates methionine had no such protective effect as well. Figure 15 indicates that S-adenosylmethionine did not significantly reverse the T4 cell inhibition caused by sinefungin. However, S-adenosylmethionine inhibited the growth of control cells, which were not treated with sinefungin. Figure 16 indicates that simultaneous addition of adenosine and methionine did have a protective effect against the toxicity of sinefungin on T4 cells. The concentrations which provided the optimal protective effect were 50 μM of adenosine and 100 μM of methionine.

3) The T4-Lymphocyte-Specific Toxicity of Medications Used to Treat the Infectious Complications of AIDS.

Two of the compounds we tested, DFMO and ketoconazole, are currently used in treating the infectious complications of the acquired immunodeficiency syndrome (50,52). Ketoconozole is the treatment of choice for esophagitis



*5'-o-sulfamoyl-1-ß-D-ribofuranosyl triazole-3-carboxamide

Figure 12. The Effect of Adenosine Supplementation on the Inhibition of T4 Cell Growth By SRTC (5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide).

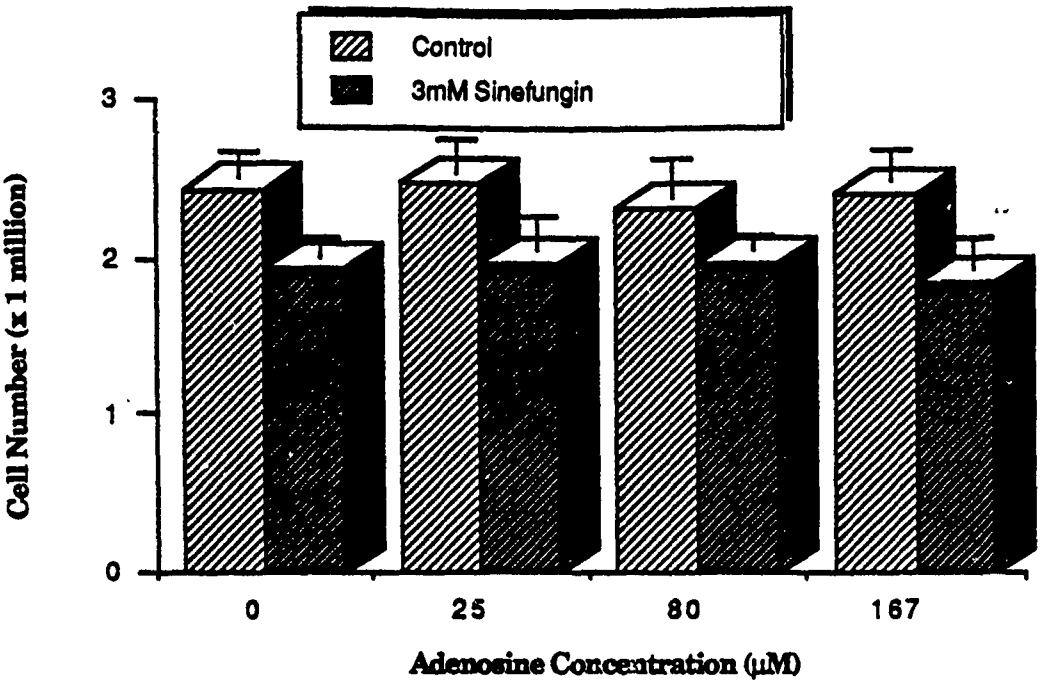


Figure 13. The Effect of Adenosine Supplementation on the Inhibition of T4 Cell Growth By Sinefungin.

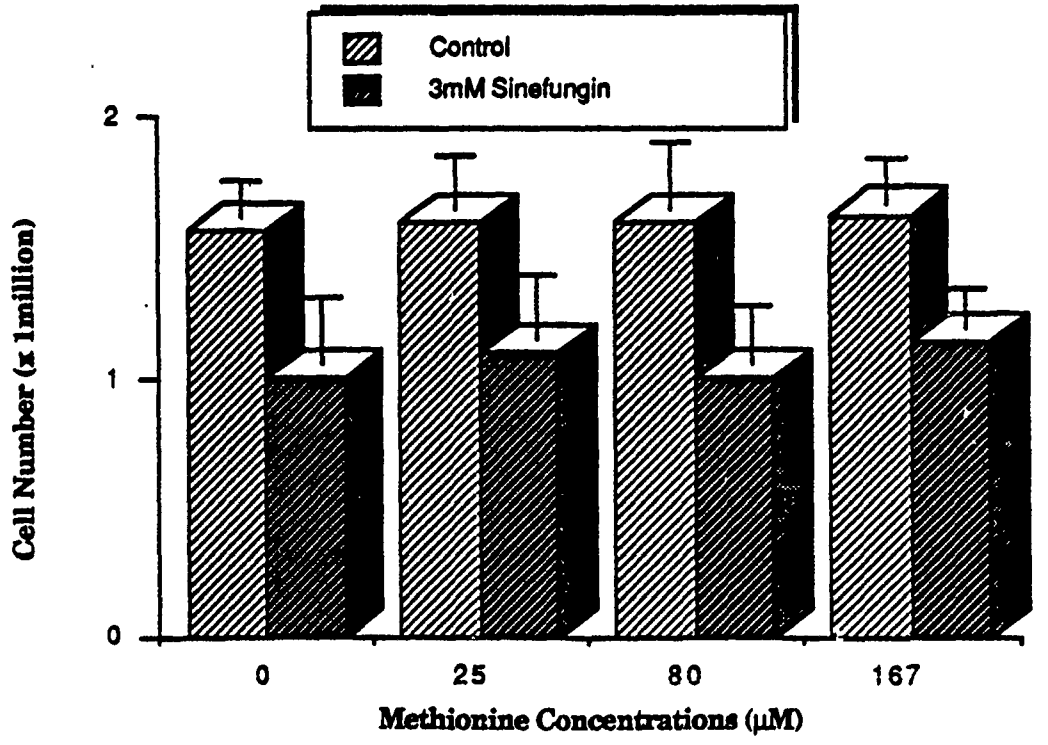


Figure 14. The Effect of Methionine Supplementation on the Inhibition of T4 Cell Growth By Sinefungin.

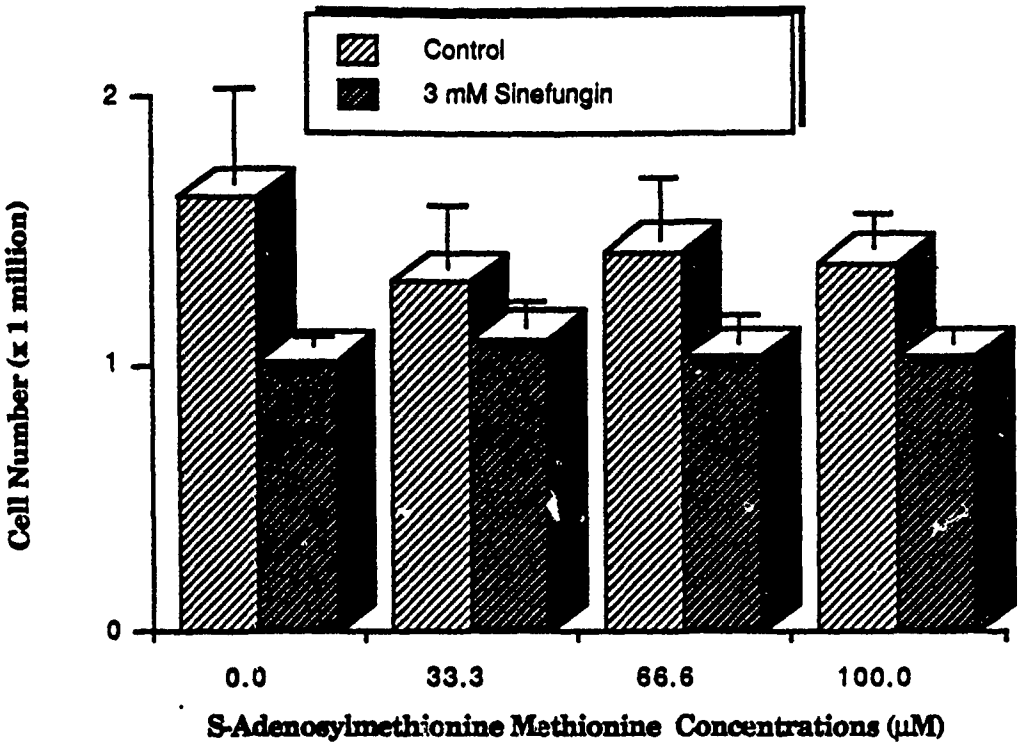


Figure 15. The Effect of S-Adenosylmethionine Supplementation on the Inhibition of T4 Cell Growth By Sinefungin.

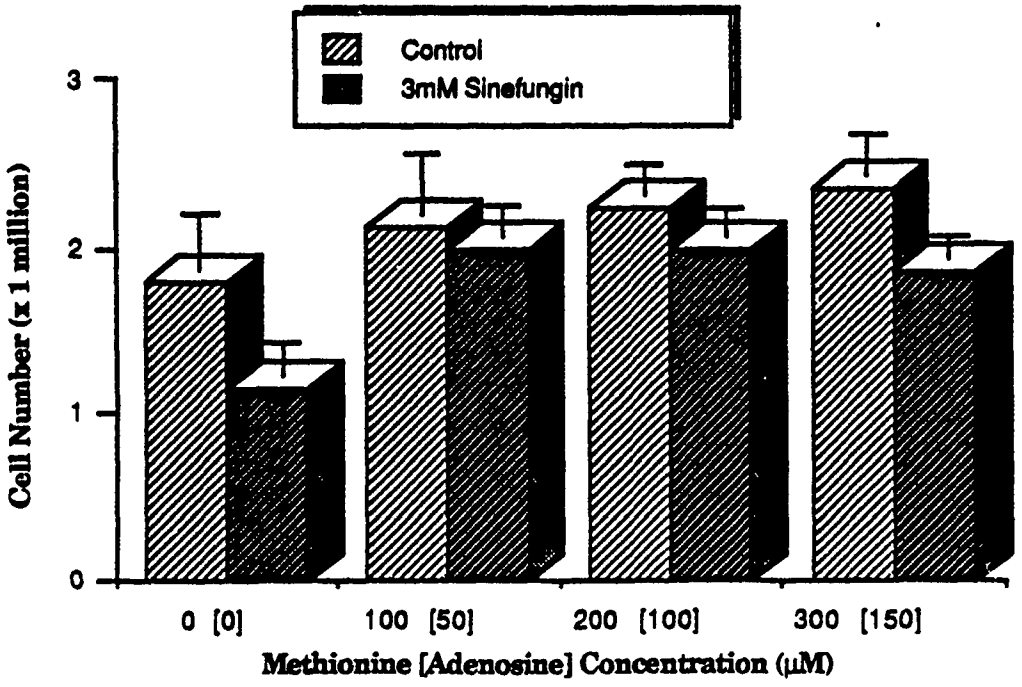
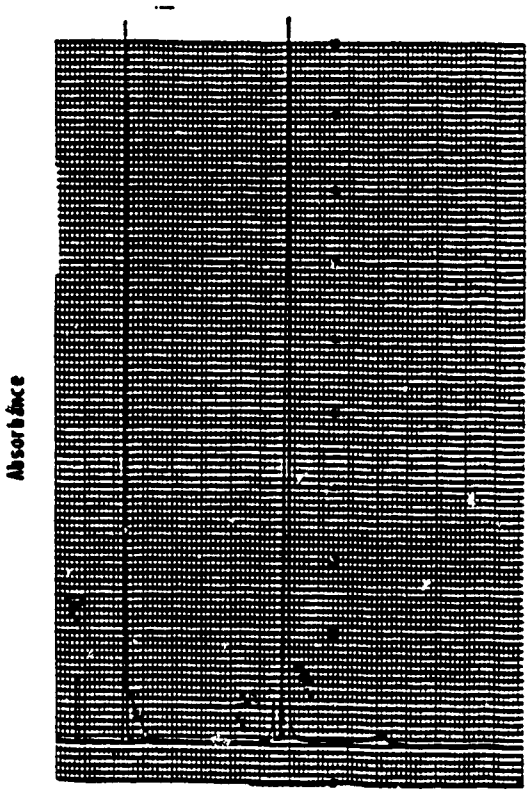


Figure 16. The Effect of Simultaneous Adenosine and Methionine Supplementation on the Inhibition of T4 Cell Growth By Sinefungin.

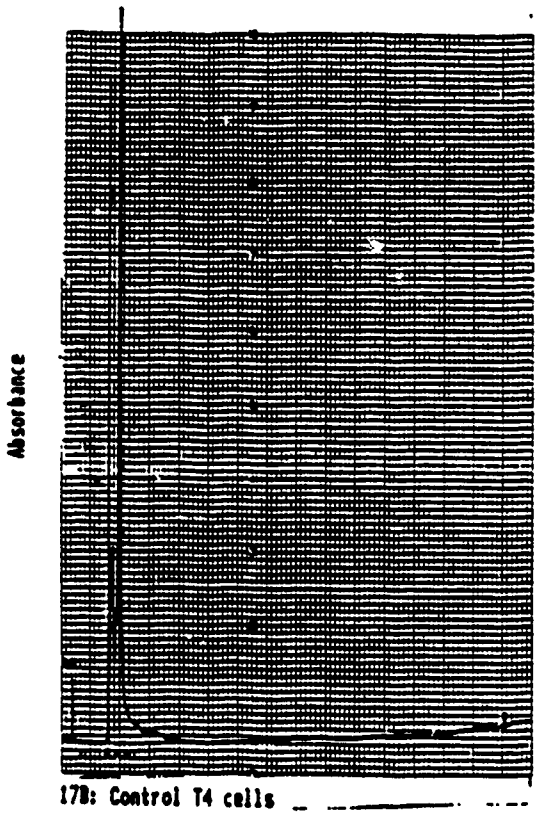
resulting from Candida spp. infections, an opportunistic infection that occurs on average in 31% of AIDS cases (50). It is also the second-line agent for systemic Cryptococcus neoformans and Coccidioides immitis infections in patients with AIDS (50). DFMO has been used to effectively treat Pneumocystis carinii pneumonia in AIDS patients (60), but is still considered an experimental medication (52). Pneumocystis carinii pneumonia (PCP) is the most common life-threatening process in AIDS patients, and a mortality rate of 60% per episode of PCP has been quoted when the conventional therapies are used (61). In order to assess their therapeutic value, we calculated the therapeutic index for these two compounds. This therapeutic index is of crucial importance in the management of AIDS, because the toxicity of medications on T4 cells would further compromise a weak immune system precisely in the area already weakened by the HIV. Table 3 shows the results.

The therapeutic index of ketoconazole for all of the pathogens it is used to treat is low. It is close to 1 for all the organisms, suggesting there is little therapeutic value in its use. This is because ketoconazole exhibits high toxicity to T4 cells.

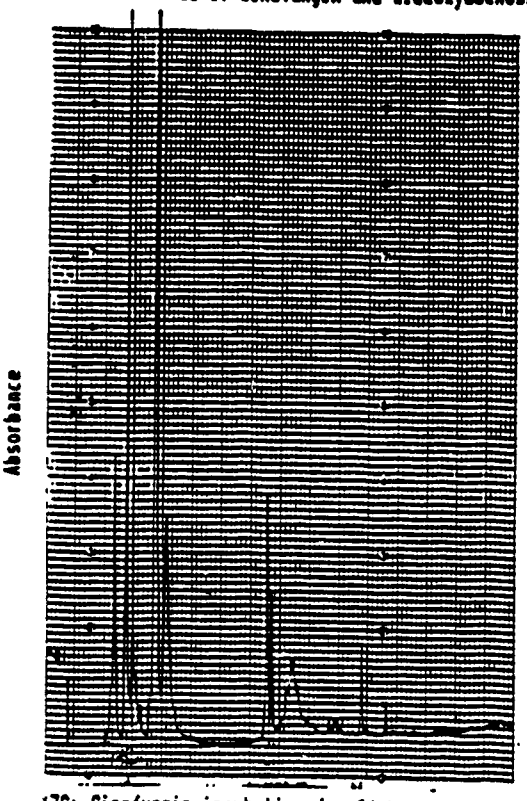
The analysis of intracellular levels of sinefungin by HPLC are shown in Figure 17. Figure 17A is a chromatogram of standards of sinefungin and dideoxyadenosine. Dideoxyadenosine is used as an internal standard, since there are very low concentrations of this nucleoside present in a cell. Sinefungin has an elution time of 4 minutes. The chromatogram of control cells, i.e. with no sinefungin added, is shown in figure 17B. The intracellular concentration of



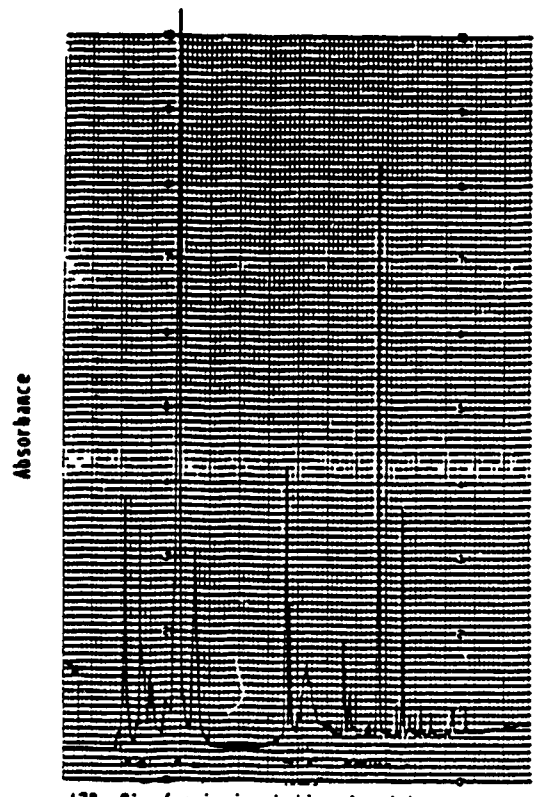
17A: Standards of sinefungin and dideoxyadenosine



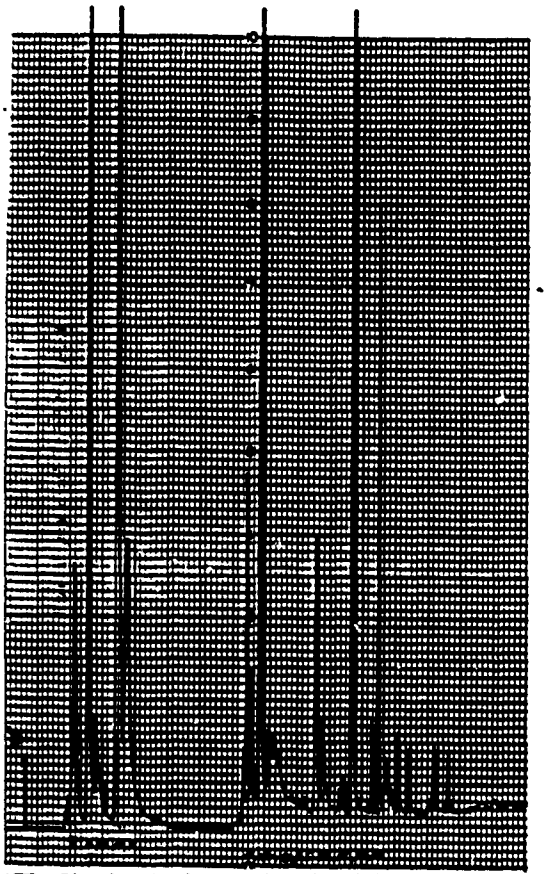
17B: Control T4 cells



17C: Sinefungin incubation for 24 hours



17D: Sinefungin incubation for 1 hour



17E: Sinefungin incubation for 1 hour
+ Standards of sinefungin and dideoxyadenosine

Figure 17: HPLC analysis of intracellular sinefungin levels in T4 cells incubated with 1mM sinefungin for 1 hour and 24 hours relative to untreated control T4 cells.

17A: Standards of sinefungin (0.5 mg/ml) and dideoxyadenosine (0.75 mg/ml).

17B: Nucleotide-containing extract of the intracellular contents of control T4 cells, i.e. incubated without any sinefungin.

17C: Nucleotide-containing extract of the intracellular contents of T4 cells incubated with sinefungin for 24 hours.

17D: Intracellular extract of T4 cells incubated with sinefungin for 1 hour.

17E: Combination of standard mix and intracellular extract of T4 cells incubated with sinefungin for 1 hour.

sinefungin in cells incubated with sinefungin for 1 hour and 24 hours is shown in figures 17D and 17C respectively. In order to determine which peak in chromatogram 17D represents sinefungin, we added sinefungin and dideoxyadenosine standards to a sample of cells incubated with sinefungin for 1 hour, giving chromatogram 17E.

The intracellular concentration of sinefungin is higher at 24 hours than at 1 hour. At both time intervals, the concentration of sinefungin in the nucleotide extract solution is less than that of the standard mix, i.e. <22.5 µg/ml. In samples incubated with sinefungin for both 1 and 24 hours, several peaks are seen which are not present in the control. They have an elution time in the range of 14 to 28 minutes. What these peaks are exactly is not known. Time limited the further investigation as the identity of them. There are significantly more peaks in the 1 hour sample (Fig. 17C) than in the 24 hour sample (Fig. 17D).

DISCUSSION

In this study we have examined both current and experimental medications for leishmaniasis and AIDS to explore their toxicity to human T4 cells in vitro. These cells perform an essential role in mounting a normal immune response. They activate B cells to produce antibodies in response to antigen, stimulate cytotoxic T cells to attack foreign antigen, cause suppressor T cells to proliferate and thereby shut down an immune reaction when it is no longer needed, and stimulate macrophages to phagocytose and present foreign antigen. Consequently, a drug that will decrease the T4 cell number of a patient will seriously impair the entire range of their immune response.

The T4-cell-specific toxicity data of medications tested in this study were compared with literature reports of the toxicity of the same medications on the relevant infectious organisms. The ratio of toxicity on T4 cells relative to pathogen for each chemotherapeutic agent was calculated, and this therapeutic index was used as a measure of the chemotherapeutic value of the particular compound.

A. Therapeutic Value of the Antileishmanial Agents Tested

The compounds vary widely in their toxicity to T4 cells (Table 1), even though structurally they may have only slight differences. For example, a comparison of the chemical structures of 7-deazainosine and 9-deazainosine indicates

that 7-deazainosine has a nitrogen missing from the 7th position, while 9-deazainosine has a nitrogen in the 9th position, of the heterocyclic ring of a regular purine nucleotide (Figure 18). This slight change results in 330 times greater toxicity of 7-deazainosine on T4 cells than 9-deazainosine.

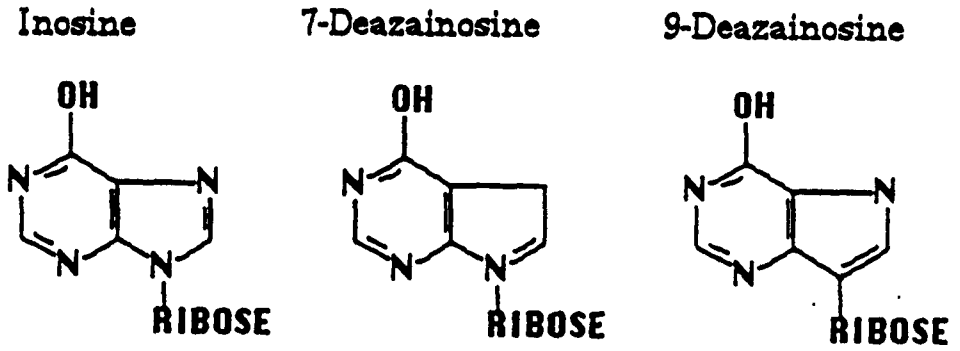


Figure 18. A Comparison of the Structures of Inosine with the Nucleoside Analogs, 7-Deazainosine and 9-Deazainosine.

The toxicity to leishmanial cells, in contrast, is much the same for the two compounds (Table 2, page 47). The biochemical basis for this difference in toxicity is attributed to whether cells phosphorylate the nucleoside to their mono-,di-, or tri-phosphate forms. The latter act as analogs of adenosine tri-phosphate, and thereby inhibit RNA synthesis. In leishmanial cells, both 7-deazainosine and 9-deazainosine are phosphorylated to tri-phosphate analogs of adenosine. In mammalian cells, only the 7-deazainosine is phosphorylated (41). Consequently, 9-deazainosine is a valuable chemotherapeutic agent while 7-deazainosine is not.

This is reflected in their therapeutic indexes: for L. tropica amastigotes, 9-deazainosine has an index of 4,000, while 7-deazainosine has an index value of 60.

Although the therapeutic index provides a valuable measure of the effectiveness of a drug, there are several limitations with such an indicator. Firstly, because of the ethical limitation on experimental testing of agents on humans, we do not have data on the ED50s of these agents on leishmanial infections in humans. We thus need to extrapolate from effective doses against leishmaniasis in animal and cell culture models to the equivalent effective antileishmanial doses in humans.

Secondly, in animals the ED50s against Leishmania are measured as daily intake in milligram per kilogram of body weight per day. There are few analyses of the resulting in vivo plasma or tissue concentrations of drugs that result from a given quantity of daily intake. Thus it is difficult to calculate the therapeutic index of compounds in T4 cells relative to the in vivo ED50. Consequently, the ED50s in cell culture systems were used to calculate the therapeutic index.

Thirdly, Leishmania spp. exist in two forms during their lifecycle: as promastigotes in the gut of the sandfly, and as amastigotes in the macrophages of humans and other hosts. The ED50 in the latter is a more valid measure to use in a therapeutic index, because it is the pathogenic form in humans. However, it is difficult and expensive to culture

Leishmania spp. within another cell line. Since the promastigote form is relatively simple and inexpensive to culture, and the toxicities are generally comparable , ED50s in promastigotes are often used as a measure of the antileishmanial potency of a compound. However, ED50s in promastigote and amastigote forms are not always comparable, as for example in the case of Glucantime which is very toxic to the amastigote form, but relatively non-toxic in the promastigote culture system. Where data is available, I have calculated therapeutic indexes based on ED50s in both promastigote and amastigote forms.

Fourthly, the different species of Leishmania vary significantly in their biochemical metabolism and thus their sensitivity to pharmacological agents. For example, in Table 2 (page 47), the ED50 of Glucantime on Leishmania donovani, a species causing visceral leishmaniasis, is 16 mg/kg/day, while on Leishmania braziliensis, a species causing cutaneous leishmaniasis, it is 660 mg/kg/day. There are at least two reasons for this: i) because the two species have different biochemical metabolisms, their sensitivities to drugs are different. ii) because of the pharmokinetics of drugs, they are generally not distributed evenly throughout all the tissues of the body. Thus the concentration of Glucantime reaching the body surface or skin, where L. braziliensis infections are located, is much lower than that reaching the spleen and liver, where L. donovani infections are located.

Glucantime is one of the two pentavalent antimonial drugs that are currently the treatment of choice for this disease, even though it has severely toxic side-effects. This drug has a therapeutic index of 686 in L. donovani amastigotes, a favorable ratio in terms of selective toxicity. Allopurinol riboside is currently in clinical trial for the treatment of leishmaniasis. It has a therapeutic index of >82 in L. donovani and >92 in L. mexicana. Although this is a fairly high therapeutic index, it is less than that of Glucantime, and considerably less than other experimental compounds we tested. Consequently, from Table 2, Allopurinol riboside would not appear to be a particularly valuable antileishmanial agent. However, it has a very high therapeutic index for L. donovani promastigotes (>1,757), which may be responsible for the selection of this compound for clinical trials.

Sinefungin shows an extremely high therapeutic index (2,200,000) in the promastigote form of L. mexicana strain #227, which is notoriously resistant to other forms of pharmacological treatment. This high therapeutic index is due to sinefungin's high toxicity, or low ED50, to L. mexicana. The ED50 for L. donovani parasites in hamsters was low too, but not nearly as low as the ED50 in promastigotes. No literature reports about the ED50 of sinefungin on leishmanial amastigotes could be found. This data would

provide valuable insight into the antileishmanial value of this compound.

With an in vivo hamster test system, sinefungin was shown to be 5 times more effective against L. donovani and 30 times more effective against L. braziliensis than Glucantime (24). In addition, no toxic effects of sinefungin on mice at therapeutic doses was observed (28), and it was not found to be mutagenic on the Ames test (24). The results of Table 2 suggest that sinefungin may be a very effective treatment for L. mexicana. The determination of the ED50 of sinefungin on leishmanial amastigotes, especially on the L. mexicana species, would provide valuable insight into the antileishmanial value of this compound.

Formycin A and formycin B have reasonably high therapeutic indexes on L. tropica amastigotes, but these compounds have been found to have strong systemic effects on rats and hamsters, and so are not being considered further as antileishmanial agents (28). 7-deazainosine, SRTC, and SIBA have low ID50s on T4 cells (i.e. relatively high toxicity) and consequently do not show promise as chemotherapeutic agents.

Ketoconazole, which is used to treat yeast and viral infections effectively (37, 51), has recently been recommended as a potential antileishmanial agent (36). However, in our study we found it to have high toxicity to T4 cells (ID50= 2.3 μM), the highest toxicity of the compounds

listed in Table 2 (page 47). Thus ketoconazole does not appear to be a promising antileishmanial agent. It would be valuable to determine the T4-lymphocyte- specific toxicity of ketoconazole in human patients, to determine whether the latter is consistant with our findings in vitro.

DFMO is another agent that has recently received attention as an antileishmanial agent (48), because it has been found to be an effective inhibitor of other protozoal parasites, including the trypanosomes T. b. rhodiense and T.b. gambiense (52), the infectious agents associated with African sleeping sickness, T. b. brucei, the malarial parasites Plasmodium berghei and P. falciparum (48), as well as Giardia lamblia and Pneumocystis carinii (52). Bacchi et al. report an effective synergistic effect of DFMO, a polyamine synthesis inhibitor, and 9-deazainosine, an RNA synthesis inhibitor, in the in vivo treatment of African sleeping sickness in mice (53).

In our study we found DFMO to have low toxicity to T4 cells (ID50 = 10,400 μM). This results in a high therapeutic index (347) of DFMO for L. donovani promastigotes. However, one study in 1983 reports a very low toxicity of DFMO to L. donovani amastigotes in mouse macrophages of >1,100 μM (49). No confirmatory data for this finding were found, but if it is valid, it suggests DFMO is not a valuable treatment for leishmaniasis.

Recently, great interest has been shown in garlic due to its demonstrated effective antimicrobial and antiviral properties (31,32 ,45,46). For this reason, we decided to test it on Leishmania mexicana #227 promastigotes and T4 cells in our laboratory. Our findings of a therapeutic index of less than 1 calls into question an aspect of using garlic as a cure which is not often considered: it may have strong immunosuppressive properties.

There is, however, an alternative explanation. We used a sterile-filtered, crude extract of raw garlic in culture with T4 cells. It is quite likely that the molecules that are toxic to T4 cells in vitro would be broken down during digestion in a human or animal. In contrast, the active molecule responsible for the antimicrobial effect of garlic, which has been identified as diallyl thiosulfinate (32), does not appear to be broken down in the digestive process. However, the in vitro findings of this study strongly recommend a simple investigation of whether eating garlic depresses T4 lymphocyte count in humans, before any further research on the antimicrobial or antiviral properties of garlic are done.

B. The Protective Effect of Nutritional Supplementation Against the Toxicity of SRTC and Sinefungin on T4 Cells.

The observation that both adenosine (Fig. 13) and S-adenosylmethionine (Fig. 14) do not reverse the T4 cell growth inhibition caused by sinefungin indicates that this

effect does not result from sinefungin acting as either an adenosine or S-adenosylmethionine analog. In L. mexicana, however, S-adenosylmethionine has been found to completely reverse the growth inhibition effect of sinefungin (24).

The finding that supplementation of both adenosine and methionine together does reverse inhibition by sinefungin (Fig. 16), while S-adenosylmethionine does not, appears contradictory. Presumably the effect of adenosine and methionine supplementation would be to provide substrates for S-adenosylmethionine synthesis, thereby increasing intracellular S-adenosylmethionine levels, which would cause the observed reversal in T4 cell growth inhibition by sinefungin. Yet S-adenosylmethionine itself does not cause the equivalent reversal. This may occur because S-adenosylmethionine does not enter the cell or the appropriate intracellular compartment to reverse the effect of sinefungin, while adenosine and methionine are able to enter.

The ability of S-adenosylmethionine to reverse growth inhibition caused by sinefungin in L. mexicana, but not in T4 cells, suggests a reason for the extremely high therapeutic index of sinefungin: in Leishmania spp., sinefungin is probably acting as a S-adenosylmethionine analog, disturbing the vital methylation functions of this molecule, while in T4 cells this does not appear to be happening. One mechanism by which sinefungin may disturb S-adenosylmethionine metabolism in leishmanial cells is by acting as an irreversible

ENZYMES STUDIES

We continued our study of DNA polymerases and S-adenosylmethionine synthatases of L. mexicana for the purpose of looking for differences between parasite and host which could possibly be exploited for drug development.

For the first time, we were able to devise an isolation procedure which yielded a very active DNA polymerase B like and were able to determine its molecular weight (38,000), and purified it over 23,342 fold.

We isolated two different S-adenosylmethionine synthatases from L. mexicana and are presently characterizing them.

TABLE 3

Purification of DNA Polymerase α-Like

SAMPLE	VOLUME	PROTEIN	TOTAL UNITS	UNITS /mg	PURIFIC.
CRUDE	20mL	1.09g	114	0.104	1
15K SUP.	18mL	878.4mg	131.4	0.15	1.44
DEAE-23	35mL	673.4mg	163.9	0.24	2.31
P11	3mL	75.3mg	162.9	2.16	20.8
S300	11.4mL	390ug	334.4	857.4	8244.2

TABLE 4

Purification of DNA Polymerase β-Like

SAMPLE	VOLUME	PROTEIN	TOTAL UNITS	UNITS /mg	PURIFIC.
CRUDE	20mL	1.09g	43.6	0.04	1
15K SUP.	18mL	878.4mg	60.2	0.07	1.75
DEAE-23	35mL	673.4mg	214.2	0.31	7.75
P11	3mL	75.3mg	143.1	1.9	47.5
S300	34.3mL	1.26mg	1176.5	933.7	23342

CONCLUSION

Sinefungin and 9-deazainosine appear to be very promising antileishmanial agents. The former appears to be particulary effective against the leishmanial species L. mexicana. The effect of sinefungin on leishmanial amastigotes in macrophages needs to be tested. If the therapeutic index for amastigotes is as high as it is for promastigotes, sinefungin warrants clinical trial for the treatment of leishmaniasis.

Raw garlic extract is very toxic to T4 cells in vitro, signifcantly more than its toxicity to L. mexicana. A clinical study to determine whether raw garlic ingestion supresses T4 lymphocyte numbers in humans would be valuable in light of the current enthusiasm over its reported health effects.

DFMO appears to be a potentially valuable treatment for Human Cytomegalovirus and Pneumcystis carinii pneumonia infections in AIDS cases, either alone or in synergism with other agents. Ketoconazole is toxic to T4 cells in vitro. A clinical trial to determine whether ketoconazole ingestion in humans depresses their blood T4 cell number would be a valuable confirmatory study. These findings suggest that ketoconazole should be used with caution for treating AIDS patients, since this drug may further impair their T4 cell function.

MILITARY SIGNIFICANCE

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo synthesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogues inhibiting key enzymes in purine pathway should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

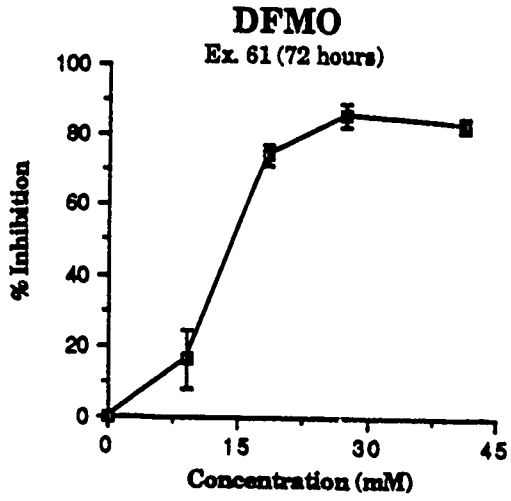
To date there is no safe, effective, and quality-controlled antiparasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

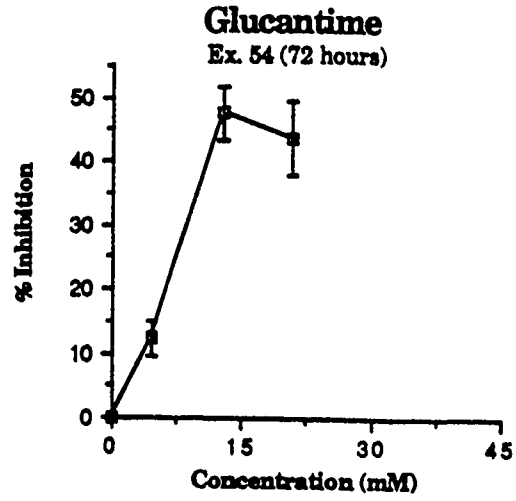
The elucidation of the biochemical mode of action of promising compounds and the identification of unique enzyme systems will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

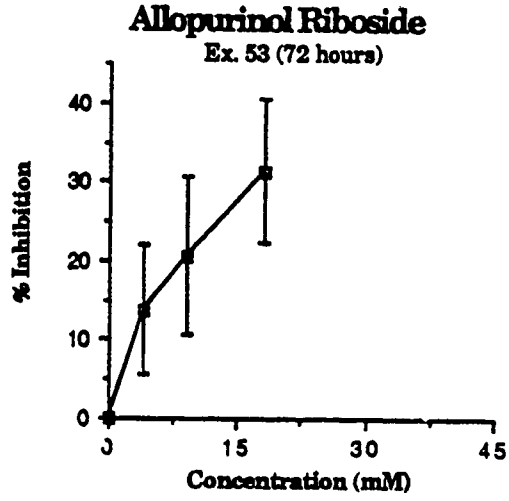
Targeting of already promising compounds may increase the efficacy of these compounds for the various disease states of leishmaniasis and be more cost effective than the development of more than one drug.

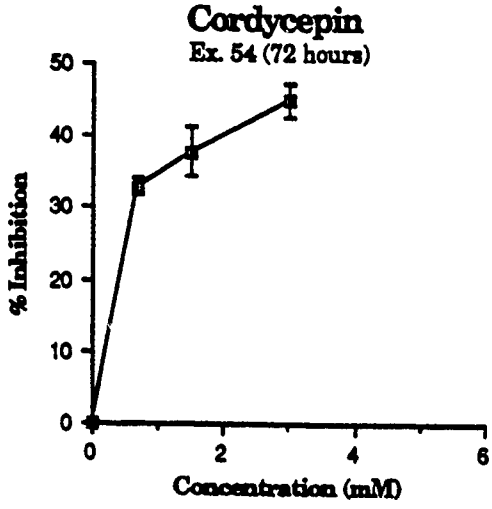
Targeting will also allow the reduction in toxicity of certain compounds, and also be more cost effective since less drug should be required.

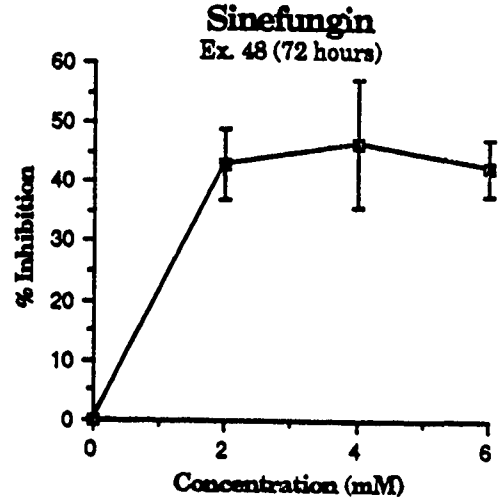
APPENDIX A. Individual Graphs of T4 Cell Growth Inhibition by the Drugs Tested in This Study.

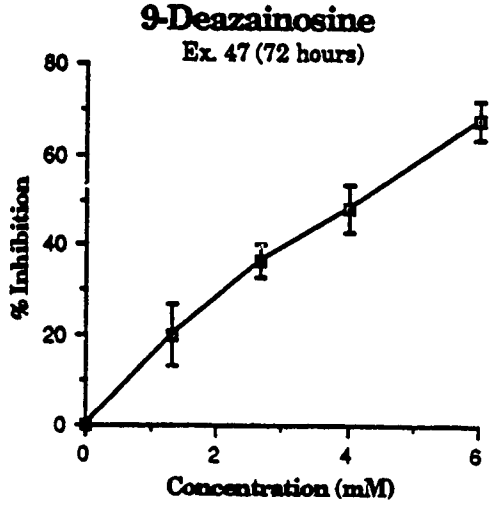


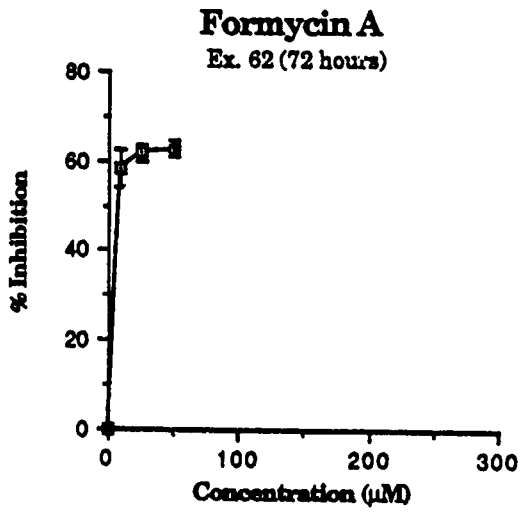


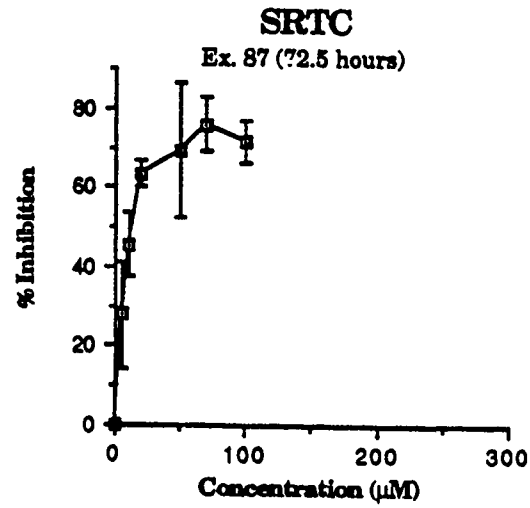


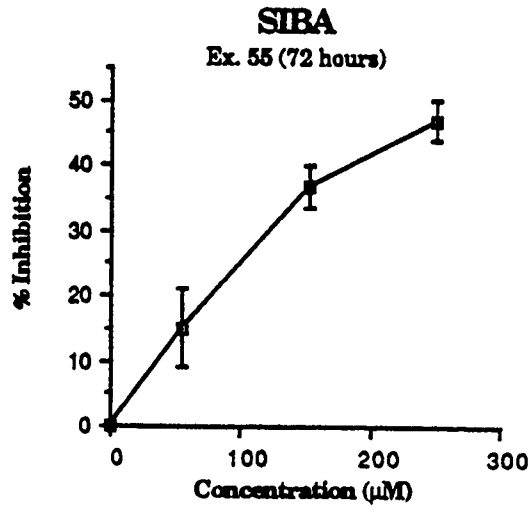

Cordycepin
Ex. 54 (72 hours)
% Inhibition
50
40
30
20
10
0
0
2
4
6
Concentration (mM)

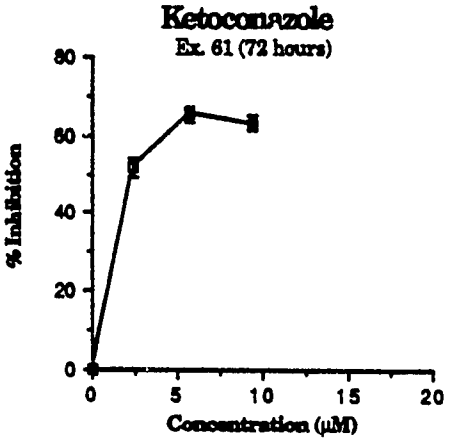

Sinefungin
Ex. 48 (72 hours)
% Inhibition
60
50
40
30
20
10
0
0
2
4
6
Concentration (mM)

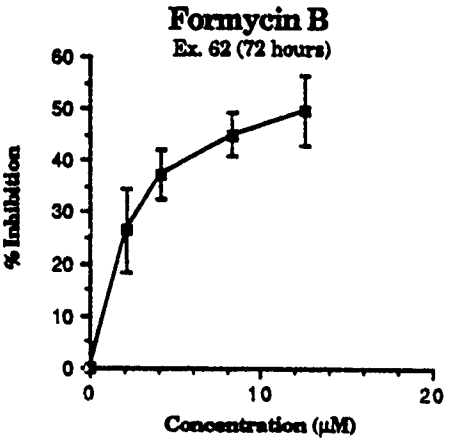

9-Deazainosine
Ex. 47 (72 hours)
% Inhibition
80
60
40
20
0
0
2
4
6
Concentration (mM)

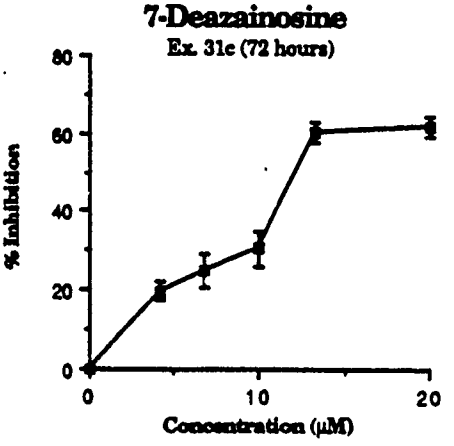

Formycin A
Ex. 62 (72 hours)
% Inhibition
80
60
40
20
0
0
100
200
300
Concentration (μM)


SRTC
Ex. 87 (72.5 hours)
% Inhibition
80
60
40
20
0
0
100
200
300
Concentration (μM)


SIBA
Ex. 55 (72 hours)
% Inhibition
50
40
30
20
10
0
0
100
200
300
Concentration (μM)


Ketoconazole
Ex. 61 (72 hours)
% Inhibition
Concentration (μM)
0
20
40
60
80
0
5
10
15
20


Formycin B
Ex. 62 (72 hours)
% Inhibition
Concentration (μM)
0
10
20
30
40
50
60
0
10
20


7-Deazainosine
Ex. 31c (72 hours)
% Inhibition
Concentration (μM)
0
20
40
60
80
0
10
20

APPENDIX B. Therapeutic Value of Compounds in the Treatment of Leishmania spp. - Reference Sources Included.

	The toxicity of antileishmanial agents on: Leishmania (ED_{50})			Mammalian cells (ID_{50})		Therapeutic Index	
	1	2	3	4	5	6	
Compound	Promastigote	In Macrophages	In Hamsters	T4 cells	Other cells	promast.	amastig.
	(uM)	(uM)	(mg/kg/day)	(uM)	(uM)	Col 4÷1	Col 4÷2
Sinefungin	0.005 (L.M)[24]	-	4 (L.D)[24]	11,000	-	2,200,000	-
9-deazainosine	40.0 (L.M)[38]	1.0(L.T)[38]	19(L.D)[48]	4,000	>1,000 [40]	100	4,000
Allopurinol Riboside	200.0 (L.M)[38] 7.0 (L.D)[40]	76-190(L.T)[38] 86-213(L.D)[44]	485(L.D)[44]	>>12,300	>2,000 [40]	>62 >1,757	>92 >82
Foraycin B	0.1 (L.M)[38]	0.04(L.T)[38]	1.5(L.D)[44]	13	200 [40]	13	325
Foraycin A	1.0 (L.D)[40]	0.04(L.T)[38]	<<13(L.D)[44]	<8	-	8	200
7-deazainosine	<2.0 (L.D)[40]	0.2(L.T)[38]	-	12	20 [40]	6	60
SRTC	11.6 (L.M)[38]	-	-	13	-	1.1	-
Cyclic sinefungin	-	-	-	>3,000	-	-	-
Cordycepin	25.0 (L.M)[38]	-	-	3,000	-	120	-
SIBA	20.0 (L.M)[38]	-	-	250	-	12.5	-
Allium sativum (Garlic)	>28pg protein/ml	(L.M)[38] -	-	11 pg protein/ml (0.001 of a clove/ml)	-	<0.4	-
DFMO	30.0 (L.D)[40]	>1,098(L.D)[49]	-	10,400	-	347	9.5
Ketoconazole	-	1.0 [39]	-	2.3	10% at 10uM[39]	-	2.3
Meglumine antimoniate (Glucantime)	>130,000(L.B)[47]	17.5(L.D)[47]	16 (L.D)[24] 660 (L.B)[24]	>12,000	-	0.09	686

(L.M.)= Leishmania mexicana 4227 (L.B)= Leishmania braziliensis (L.D)= Leishmania donovani (L.T)= Leishmania tropica

Column 1 = The concentration of drug that inhibits the growth of the promastigote form of Leishmania spp. to half that of untreated controls.

Column 2 = The concentration of drug that eliminates 50% of leishmanial amastigotes from within human macrophages in vitro relative to untreated controls.

Column 3 = The concentration of a drug that suppresses leishmanial amastigote numbers in hamster livers to half that of untreated controls after four days of treatment.

Column 4 = The concentration of a drug that inhibits the growth of CD4 cells by 50 % relative to untreated controls.

Col 6 = $\frac{\text{50\% Inhibition level on T4 cells}}{\text{50\% Inhibition dose on promastigote Leishmania}}$ 4÷2= $\frac{\text{50\% Inhibition level on T4 cells}}{\text{50\% Inhib. dose on amastigote Leishmania in macrophages}}$

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