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Treatment of HIV-infection by interfering with host cell cyclophilin receptor activity

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ABSTRACT

An immunotherapeutic method for inhibiting HIV infection comprises administering to an individual a cyclophilin immunogen at a dose sufficient to induce an immune response to cyclophilin.

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ORIGINAL

COMPLETE SPECIFICATION

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Complete Specification for the invention entitled:

Treatment of HIV-infection by interfering with host cell cyclophilin receptor activity

The following statement is a full description of this invention, including the best method of performing it known to us:

TREATMENT OF HIV-INFECTION BY INTERFERING WITH HOST CELL CYCLOPHILIN RECEPTOR ACTIVITY

TECHNICAL FIELD OF THE INVENTION

The present invention provides pharmaceutical compositions for the treatment of HIV-infection targeting cyclophilin A and its corresponding human cellular binding partner or receptor as a target for intervention. The present invention provides anti-cyclophilin antibodies, derivatized cyclosporin A, and soluble forms of cyclophilin-binding partners and act presumably by interrupting the binding of cyclophilin A with its cellular binding partner(s) or receptor(s), as a treatment for HIV-infection. The present invention further provides genetic constructs designed to interfere with production and release of cyclophilin and its cognate cellular binding partner. The present invention further provides a screening assays for the identification of compounds which inhibit the interaction of cyclophilin and its cellular receptor.

BACKGROUND OF THE INVENTION

The human immunodeficiency virus (HIV) has been implicated as the primary cause of the slowly degenerative immune system disease termed acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983, *Science* 220:868-870; Gallo et al., 1984, *Science* 224:500-503). In humans, HIV replication occurs prominently in $CD4^+$ T lymphocyte populations, and HIV infection leads to depletion of this cell type and eventually to immune incompetence, opportunistic infections, neurological dysfunctions, neoplastic growth, and ultimately death.

HIV is targeted to $CD4^+$ cells because a CD4 cell surface protein (CD4) acts as the cellular receptor for the HIV-1 virus (Dalgleish et al., 1984, *Nature* 312:763-767; Klatzmann et al., 1984, *Nature* 312:767-768; Maddon et al., 1986, *Cell* 47:333-348). Viral entry into cells is dependent upon gp120 binding the cellular CD4 receptor molecules (McDougal et al., 1986, *Science* 231:382-385; Maddon et al., 1986, *Cell* 47:333-348), explaining HIV's tropism for $CD4^+$ cells, while gp41 anchors the envelope glycoprotein complex in the viral membrane. While these virus:cell interactions are necessary for infection, there is evidence that additional virus:cell interactions are also required.

HIV infection is pandemic and HIV-associated diseases represent a major world health problem. Attempts are also being made to develop drugs which can inhibit viral entry into the cell, the earliest stage of HIV infection. Here, the focus has thus far been on CD4, the cell surface receptor for HIV. Recombinant soluble CD4, for example, has been shown to inhibit infection of $CD4^+$ T cells by some HIV-1 strains (Smith et al., 1987, *Science* 238:1704-1707). Certain primary HIV-1 isolates, however, are relatively less sensitive to inhibition by recombinant CD4 (Daar et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:6574-6579). In addition, recombinant soluble CD4 clinical trials have produced inconclusive results (Schooley et al., 1990, *Ann. Int. Med.* 112:247-253; Kahn et al., 1990, *Ann. Int. Med.* 112:254-261; Yarchoan et

al., 1989, *Proc. Vth Int. Conf. on AIDS*, p. 564, MCP 137).

Thus, although a great deal of effort is being directed to the design and testing of anti-retroviral drugs, effective, non-toxic treatments are still needed.

Cyclophilin A is a 19 KD protein, which is expressed in a wide variety of cells. Cyclophilin A binds the immunosuppressive agent, cyclosporin A, and possesses peptidyl-prolyl cis-trans isomerase (PPIase), and protein folding or "chaperone" activities. Cyclophilin A is a member of the "immunophilin family", *i.e.*, a group of related cellular factors involved in regulating immunity. At least four types of mammalian cyclophilins have been cloned, cyclophilins A, B, C and hCyP3 (Friedman et al., 1993, *Proc. Natl. Acad. Sci. USA*, 90:6815-6819). A glycoprotein with a N-terminal signal sequence has been identified which binds cyclophilin C, but not cyclophilin A or B (Friedman et al., supra). The functional significance of this interaction has yet to be elucidated. A homologous protein was found in mouse macrophages, MAC-2 binding protein, and is postulated to be involved in the cascade of immunoregulatory events resulting from the absorption of the immunosuppressive drug cyclosporin A (Chicheportiche et al., 1994, *Proc. Natl. Acad. Sci. USA*, 269:5512-5577).

Very recently, a functional association of cyclophilin A with the Gag protein of HIV virions and specifically with the capsid antigen portion thereof, was noted (Thali et al., 1994, *Nature* 372:363-365). Cyclophilin was reported to be specifically incorporated into HIV-1 virions, and disruption of the Gag-cyclophilin interaction was reported to prevent both incorporation of cyclophilin A into virions and HIV-1 replication, indicating that the interaction of Gag with cyclophilin A is necessary for the formation of infectious HIV-1 virions (Franke et al., 1995, *Nature* 372:359-362).

Cyclophilin is recognized to be one of the host cell receptors for cyclosporin A -- a potent immunosuppressive drug which is widely used in prevention of graft rejection. Cyclosporin A is a member of a family of hydrophobic cyclic undecapeptides that exhibits potent immunosuppressive, antiparasitic, fungicidal and chronic anti-inflammatory properties. Cyclosporin A is thought to exert its immunosuppressive effects by inhibiting the early stages in T cell activation. Cyclosporin A has been found to block RNA transcription of the T cell growth factor, interleukin 2 (IL-2) and to inhibit expression of the IL2 receptor by precursor cytolytic T lymphocytes (Palacios, 1982, *J. Immunol.* 128:337). Cyclophilin A binds to cyclosporin A with a dissociation constant in the range of 10^{-8} mol/L, a value consistent with levels needed to elicit immunosuppression (Handschumacher et al., 1984, *Science* 226:544).

Cyclosporin A has been shown to interfere with Gag-cyclophilin interactions *in vitro*, block cyclophilin incorporation into virions, and inhibit the replication of HIV-1 in cell culture (Franke et al., *supra*; Thali et al., *supra*; Billich et al., 1995, J. Virol. 69:2451-2461). Cyclosporin A has not been shown to interact directly with the HIV-1 virus. It is hypothesized that cyclosporin A, and its analogs, interfere at two stages in the HIV life cycle by interacting with cyclophilin A -- during establishment of infection where cyclosporin A treatment inhibits

HIV infection prior to integration into the genome of the infected cell as measured by formation of circular HIV DNA and integration of proviral DNA into the host genome; and at a late stage of virus replication, when a reduction in shed virus particles but not viral antigen expression is observed (Billich et al., *supra*). Therefore, researchers have been focused on disrupting the interaction between the viral protein, Gag, and the cellular protein, cyclophilin A, in their attempts to develop an anti-HIV agent.

Despite the foregoing observations, the usefulness of cyclosporin A as a treatment for HIV-infection in patients is severely limited. First, cyclosporin A is a potent immunosuppressor, therefore its use in HIV-infected patients, who will become immunocompromised, is contraindicated. Moreover, the noted HIV-inhibitory effects of cyclosporin A and its analogs required drug concentrations that are 10 to 100-fold higher than those necessary to effectuate immunosuppression with cyclosporin A. The ability of cyclosporin to generally suppress the systemic immune system has been proposed as a treatment for HIV infection (see U.S. Patent No. 4,814,323). This method of treatment however has severe drawbacks, given the severely immunosuppressed state of HIV-infected patients.

SUMMARY OF THE INVENTION

The present invention provides a method for inhibiting HIV infection, comprising administering a cyclosporin derivative which is not internalized by cells, to a subject in an amount sufficient to inhibit HIV infection. Preferably, the cyclosporin derivative is PEG-cyclosporin. The present invention further provides an immunotherapeutic method for inhibiting HIV infection, comprising administering to an individual a cyclophilin immunogen at a dose sufficient to induce an immune response to cyclophilin. Preferably, the cyclophilin immunogen is administered with an adjuvant, and is glycated.

The present invention further provides an immunotherapeutic method for inhibiting HIV infection, comprising administering to an individual an antibody that neutralizes cyclophilin, at a dose sufficient to inhibit HIV infection. Preferably, the antibody is a single-chain antibody, chimeric antibody, humanized antibody, or Fab fragment. The present invention further provides a method for inhibiting HIV infection comprising administering to a subject exogenous cyclophilin in an amount sufficient to inhibit HIV infection. The present invention further provides a pharmaceutical composition comprising a derivatized form of cyclosporin, wherein the cyclosporin is derivatized by reaction with polyethylene glycol to form a PEG-cyclosporin.

Lastly, the present invention provides a drug screening method to identify compounds or compositions which inhibit the interaction of cyclophilin and its cognate cellular binding partner or receptor *in vitro*, which drug screen method comprises: (a) adding a test compound, cyclophilin and a cyclophilin binding partner to a reaction mixture; and (b) detecting any

cyclophilin-cyclophilin binding partner complexes in the reaction mixture.

The invention is based, in part, on the applicants' discovery that an extracellularly supplied cyclophilin, or neutralizing antibody for cyclophilin A, as well as cyclosporin A derivatives modified to inhibit their internalization by host cells, each inhibit HIV replication. This discovery is described in examples which demonstrate that exogenous cyclophilin A, an extracellularly supplied neutralizing antibody for cyclophilin A, and an extracellularly supplied cyclosporin A derivative modified so as to be excluded from cell entry, each inhibit HIV replication. This result is surprising since cyclophilin A, a cellular protein having immunomodulatory activity, is believed to be incorporated into HIV virions by binding to HIV-Gag and is thought to be required for productive viral infection (*e.g.*, viral assembly and transmission) since, although certain cyclosporins have demonstrated efficacy against HIV infection in cellular host systems, these peptides have been thought to act within the infected cells. The Gag-cyclophilin A complex contained in the core of the virion is surrounded by a lipid envelope bearing HIV-envelope glycoproteins. As such, the Gag-cyclophilin complex is generally understood not to be exposed to exogenous cyclophilin, to the extracellular neutralizing antibody, or to cyclosporin A derivatized to inhibit internalization by the cells.

In particular, antibodies that neutralize cyclophilin can be used to inhibit HIV infection. In one embodiment of the present invention, neutralizing antibodies which have been generated outside the patient to be treated, for instance using cyclophilin as an antigen, can be administered in a passive immunotherapy approach. In a preferred embodiment, cyclophilin is used in conjunction with an adjuvant and is covalently modified, as by glycation, to improve antigenicity and the utility of the antibodies. In another embodiment of the present invention, cyclophilin can be formulated as an immunogen, for instance in conjunction with an adjuvant, for use as a "vaccine" to generate an active immune response in HIV-infected patients. In a preferred embodiment, cyclophilin is modified by advanced glycation to improve immunogenicity or to improve the characteristics of the elicited antibodies for the intended purposes.

Cyclosporins, which would ordinarily result in immunosuppression due to their intracellular interaction with cyclophilin A, can be derivatized to inhibit the entry of the drug into cells, for instance by the covalent attachment of bulky or charged substituents and thereby to render these derivatives non-immunosuppressive. In a preferred embodiment, a cyclosporin is modified by attachment of polyethylene glycol substituents. Such cyclosporin derivatives can be used to inhibit HIV-infection without inducing immunosuppression in HIV-infected patients.

The present invention further relates to the use of exogenous cyclophilin to interfere with HIV-infection, and to the use of cyclophilin derivatives that are unable to form complexes with HIV-Gag, yet are still able to bind the cyclophilin host receptor. Therefore, these cyclophilin derivatives are unable to be incorporated into virions, yet compete with or block the binding of

the HIV-virion-associated cyclophilin to host binding proteins.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows inhibition of HIV-1 infection in H9 cell cultures treated with exogenous cyclophilin at 40 μg/ml. Reverse transcriptase (RT) activity was assayed in culture supernatants 5 days post-infection, according to standard methods.

Figure 2 shows inhibition of HIV-1 infection in H9 cell cultures treated with exogenous cyclophilin (at 1 or 40 μg/ml) or with whole antiserum to glycated cyclophilin (α-AGE-CyP) or to non-modified cyclophilin (α-CyP). Reverse transcriptase (RT) activity was assayed in culture supernatants 3 days post-infection, according to standard methods.

Figure 3 shows a time course of inhibition of HIV-1 infection in human PBL cultures treated with exogenous cyclophilin at 40 μg/ml. p24 antigen levels in culture supernatants were assayed by a commercially available ELISA.

FIGURE 4. Inhibition of HIV-1 infection in human monocyte/macrophage cultures by cyclophilin, anti-cyclophilin antibodies, and cyclosporin A derivatized to inhibit cell entry. Inhibition was assessed as the percent inhibition of HIV pol retrotranscription measured in quantitative PCR assays by reference to appropriate controls.

DETAILED DESCRIPTION OF THE INVENTION

Cyclophilin (CyP) refers to the protein cyclophilin of which four types are known, CyPA, CyPB, CyPC and hCyP3, which is the receptor for cyclosporin A, and any derivative of cyclophilin thereof, or fragments or peptides having an amino acid sequence corresponding to cyclophilin.

Cyclosporin (CsA) refers to the immunosuppressive drug, cyclosporin, and any derivative of cyclosporin thereof, or fragments or peptides having an amino acid sequence corresponding to cyclosporin.

Cyclophilin binding partner as used herein means any cellular binding protein, cell surface binding protein, extracellular receptor, or intracellular binding protein, which has cyclophilin specific binding activity.

The present invention provides and pharmaceutical compositions for the treatment of HIV-infection; these agents are designed to interfere with the interaction between cyclophilin A and cellular binding proteins specific for cyclophilin A. In a first embodiment of the present invention, cyclophilin can be administered to inhibit HIV infection in patients. This cyclophilin may be a protein having a sequence substantially identical to human cyclophilin A, B or C, or to a cyclophilin of another species (*i.e.*, mouse, yeast) or the protein may be a mutant of any of the above native sequences. When administered in accordance with the present invention such treatment will have utility as an anti-HIV-infection treatment, by interfering with the interaction between virus-associated cyclophilin and host cell proteins.

In a second embodiment, neutralizing antibodies which have been generated against cyclophilin can be administered in a passive immunotherapy approach. The present invention also relates to the use of cyclophilin formulated as an immunogen. The use of cyclophilin as a "vaccine" will generate an active immune response useful in HIV-infected patients. In a preferred embodiment, cyclophilin for use as an immunogen is first modified by non-enzymatic glycosylation to improve antigenicity or the desired properties of the antigen-specific immune response.

Small molecules are cyclosporins, which would ordinarily result in immunosuppression due to their interaction with intracellular cyclophilin A, but which are derivatized so as to inhibit their entry into cells, for instance by the covalent attachment of bulky or charged substituents, such as polyethylene glycol. The extracellularly presented cyclosporin derivatives can be used to inhibit HIV-infection. This is thought to be attributable to blocking the interaction between cyclophilin A and its cellular binding proteins, which by their modification are without significant immunosuppressive activity.

The present invention additionally provides screening assays to identify compounds which inhibit HIV infection. Such compounds are selected for their activity in blocking the interaction between cyclophilin A and its cellular binding proteins. Such identified compounds would have utility in the treatment and prevention of HIV-infection.

The present invention is based, in part, on the applicants' surprising discovery that, when provided extracellularly, exogenous cyclophilin A, a neutralizing antibody for cyclophilin A, and cyclosporin A derivatives, which are modified to inhibit their entry into host cells, each inhibit HIV replication and infection in cell culture. An extracellularly supplied cyclosporin A, modified so as to be excluded from cell entry and therefore a non-immunosuppressive derivative, inhibits HIV-replication. These results are surprising since the only known HIV-infection related molecular interaction with cyclophilin, the Gag-cyclophilin A complex, is contained in the core of the HIV virion, which is surrounded by a lipid envelope bearing the HIV-envelope glycoproteins. As such, the Gag-cyclophilin complex should not be exposed to the extracellularly supplied cyclophilin A, neutralizing antibody or derivatized cyclosporin A. While not limited to any theory or explanation, the Applicants' hypothesis, that a host cell binding factor or receptor for cyclophilin exists, and is required in addition to the host CD4 receptor, for efficient infection of CD4+ host cells by HIV (*e.g.*, attachment, uncoating and translocation to the nucleus), was developed.

For example, compounds which may be used in accordance with the present invention encompass any compound which interferes with the interaction between cyclophilin and its cellular binding partner or receptor, including, but not limited to neutralizing antibodies against cyclophilin and the use of cyclophilin as an immunogen to generate an active immune response in HIV-infected patients. Other examples of compounds which may be used in accordance with the present invention include cyclophilin A and advantageously nonimmunosuppressive

derivatives of cyclosporin which inhibit the interaction between virus-associated cyclophilin A and its cellular binding partner(s) or receptor(s) and correspondingly inhibit HIV infection. Other examples of compounds which may be used in accordance with the present invention include, but are not limited to, peptides (such as, for example, peptides representing soluble portions of cyclophilin binding partner or receptor), phosphopeptides, small organic or inorganic molecules, or antibodies (including, for example, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ and FAb expression library fragments, and epitope-binding fragments thereof).

The present invention provides a use for neutralizing antibodies against cyclophilin, optionally raised against derivatized cyclophilin, in a passive immunotherapy approach and the use of cyclophilin, optionally a derivatized form of cyclophilin, as an immunogen to generate an active immune response in HIV-infected patients.

The present invention provides neutralizing antibodies which interact with cyclophilin, or with its cellular binding partner, and inhibit HIV infection. Such antibodies disrupt the interaction between cyclophilin and its host cellular binding partner or receptor thereby inhibiting HIV-infection. These neutralizing antibodies of the invention can be administered in a passive immunotherapy approach. In a preferred embodiment of the invention, the neutralizing antibodies are generated using glycated cyclophilin. Methods for glycating cyclophilin are described in WO 93/23081, which is incorporated herein in its entirety by reference.

Antibodies that are specific for cyclophilin A or its host cellular binding partner or receptor may be generated to inhibit HIV-infection, and these antibodies are thought to work in this regard by interfering with the interaction between virally-associated cyclophilin and its cellular binding partner or receptor. For passive immunotherapy approaches, such antibodies may be generated using standard techniques against the proteins themselves or against peptides corresponding to portions of the proteins. The antibodies include but are not limited to polyclonal, monoclonal, Fab fragments, single chain antibodies, chimeric antibodies, human antibodies, non-human antibodies or humanized antibodies.

Where fragments of the antibody are used, the smallest inhibitory fragment which binds to the target protein's binding domain is preferred. For example, peptides having an amino acid sequence corresponding to the domain of the variable region of the antibody that binds to the target protein may be used. Such peptides can be synthesized chemically or produced via recombinant DNA technology. Alternatively, single chain neutralizing antibodies which bind to intracellular target epitopes may also be administered. Such single chain antibodies may be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population by utilizing, for example, techniques such as those described in Marasco et al. (*Proc. Natl. Acad. Sci. USA* 90:7889-7893, 1993).

For the production of antibodies, various host animals may be immunized by injection

with the protein, including but not limited to rabbits, mice, and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to modification of the antigen of interest by the process of advanced glycation or by advanced glycation products, or by administration of the antigen with Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, key hole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum.*

Many methods may be used to introduce the passive vaccine formulations described above, these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed, or into the tissue where the pathogen resides within the body.

In immunization procedures, the amount of immunogen to be used and the immunization schedule will be determined by a physician skilled in the art and will be administered by reference to the immune response and antibody titers of the subject.

The present invention further provides a use of cyclophilin, formulated as an immunogen, and used as a "vaccine" to generate an active immune response in HIV-infected patients that are not severely immunocompromised or in populations at risk for HIV exposure and infection. In a preferred embodiment, active immunization comprises reacting the immunogen, cyclophilin, with glucose or another reducing sugar under conditions which lead to the formulation of irreversible covalent adducts, known as Advanced Glycation Endproducts (AGEs). Methods for glycating cyclophilin are described in WO 93/23081 which is incorporated herein in its entirety by reference.

An active immune response generated against cyclophilin A, or a cellular binding partner or receptor therefore, which interferes with the interaction between cyclophilin and its cellular binding partner, may be generated to inhibit HIV-infection. For active immunization, a vaccine comprising an immunogen consisting of cyclophilin or its cellular binding partner or receptor, or fragments or peptides having an amino acid sequence corresponding to the domains required for cyclophilin binding to its cellular binding partner or receptor, may be utilized to generate an immune response in the HIV patient or a person at risk for HIV exposure. These immunogens optionally may be modified by advanced glycation or advanced glycation products and may be formulated with an adjuvant to increase the immunological response, such as potentially useful human adjuvants, *i.e.*, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum.*

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the vaccine

formulation via the natural route of infection of the pathogen for which the vaccine is designed.

In immunization procedures, the amount of immunogen to be used and the immunization schedule will be determined by a physician.

The present invention further provides cyclosporin derivatives that are modified so as to inhibit their entry into or internalization by host cells, such that the derivatives of cyclosporin are correspondingly not interactive with intracellular cyclophilin A. Such cyclosporin derivatives of the present invention are believed to act to inhibit HIV viral entry when presented extracellularly. Such cyclosporin derivatives inhibit infection by HIV, yet should not result in immunosuppression of the HIV-infected patient. Therefore, the invention encompasses derivatives of cyclosporin which prevent or minimize internalization of the molecule by the cell, yet do not prevent interaction with the cyclophilin, thereby inhibiting HIV-infection. Cyclosporin may be derivatized by the addition of a bulky substituent to the protein. Examples of such substituents, include but are not limited to, charged substituents (*e.g.*, spermine or spermidine), polynucleotides with and without modified backbones, carbohydrates (*e.g.*, polyacrylic acid, polysodium acrylate, polycesium acrylate, polymethacrylic acid), amphiphilic block copolymers (*e.g.*, polystyrene poly) (sodium acrylate), and amphiphilic homopolymers.

In a preferred embodiment of the invention, cyclosporin is derivatized by reaction with polyethylene glycol, resulting in a "pegylated" cyclosporin. Pegylated cyclosporin may be prepared by standard chemical methods. The preferred method of preparing the pegylated cyclosporin of the invention, involves reacting methoxypolyethylene glycol-succinimidyl succinate with 8-amino-cyclosporin A and 4-dimethylamino pyridine in methylene chloride with stirring for two days at room temperature in the dark. To block any unreacted sites, ethanolamine was then added and the mixture incubated at room temperature with stirring for another 24 hours. The derivatized cyclosporin A was purified from the reaction mixture by normal phase HPLC.

The present invention further provides the use of exogenous cyclophilin to inhibit the interaction between cyclophilin and its cellular binding partner or receptor. Examples of exogenous forms of cyclophilin include but are not limited to human cyclophilin A, other human cyclophilins, the cyclophilins of other species (*i.e.*, mouse, yeast) derivatized cyclophilin, recombinant cyclophilin, cyclophilin fusion proteins or peptide fragments expressing the domain of cyclophilin which binds to its host cell receptor. Both forms of cyclophilin, which do and do not form complexes with the Gag protein and are not incorporated by the HIV virion yet bind to the host cell cyclophilin binding partner or receptor and block binding of the Gag-cyclophilin complex are encompassed.

A further embodiment of the invention relates to the use of exogenous cyclophilin binding protein(s) to inhibit the interaction between cyclophilin and its cellular binding partner. Examples of exogenous forms of cyclophilin binding partners or receptors include but are not

limited to derivatized cyclophilin binding partners, recombinant cyclophilin binding partners, fusion proteins or peptide fragments expressing the domain of the cyclophilin binding partner or receptor which binds to cyclophilin.

In a further embodiment of the present invention, a therapeutic modality for HIV-infection embodying the genetic engineering of cells to produce or release cyclophilin constitutively or in response to suitable inducers and the supply of said cells to an HIV patient by engraftment of exogenously transfected cells or treatment of the patient by gene therapy to transfect endogenous cell populations is envisioned. This therapeutic modality is likewise suitable to implement HIV treatment by expression and release of cyclophilin mutants, molecular decoys or other mimics of cyclophilin that inhibit HIV infection, for instance by interfering with the interactions between virus associated cyclophilin and such cellular binding proteins, therefore, as are required for productive infection. Therapeutic modalities capitalizing on the genetic engineering of cells to express and release soluble forms of cyclophilin-binding proteins or the cyclophilin-binding domains thereof will likewise find utility in the practice of the present invention and are contemplated hereunder.

Cyclophilin may be derivatized by a variety of methods. In a preferred embodiment, cyclophilin is pegylated by reacting cyclophilin with methoxypolyethylene glycol-succinimidyl succinate and 4-dimethylamino pyridine in methylene chloride with stirring for two days at room temperature in the dark. To block any unreacted sites, ethanolamine is added and the mixture incubated at room temperature with stirring for another 24 hours. The pegylated cyclophilin is purified from the reaction mixture by normal phase HPLC.

The recombinant forms of cyclophilin can be produced by synthetic techniques or via recombinant DNA technology. Recombinant DNA can be used to construct expression vectors containing differentially expressed cyclophilin protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. Alternatively, RNA capable of encoding differentially expressed cyclophilin protein sequences can be chemically synthesized using, for example, synthesizers.

A variety of host-expression vector systems can be utilized to express the cyclophilin coding sequences. Such host-expression systems represent vehicles by which the coding sequences of interest can be produced and subsequently purified, but also represent cells which can, when transformed or transfected with the appropriate nucleotide coding sequences, produce and release cyclophilin protein *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g., E. coli, B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing cyclophilin protein coding sequences; yeast (*e.g., Saccharomyces, Pichia*) transformed with recombinant yeast expression vectors containing cyclophilin protein coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g., baculovirus*) containing cyclophilin

protein coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing cyclophilin protein coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified can be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278, in which the cyclophilin protein coding sequence can be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; and pIN vectors. pGEX vectors can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene protein can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. Successful insertion of cyclophilin coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus. These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed.

In mammalian host cells, a number of expression systems, including for instance viral-based expression systems, can be utilized and either the protein expressed on the cells or the cells expressing the protein may be utilized. In cases where an adenovirus is used as an expression vector, the cyclophilin coding sequence of interest can be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene can then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing cyclophilin protein in infected hosts.

The following assays are designed to identify compounds or compositions that bind to cyclophilin A, its cellular binding partner or receptor and interfere with the interaction between cyclophilin A and its cellular receptor. Those compounds identified as inhibitors of the interaction between cyclophilin A and its cellular binding partner or receptor would have utility as anti-HIV agents. The principle of the assays to identify compounds which inhibit the interaction of cyclophilin as the target gene or protein of interest and its cellular binding partner or receptor involves preparing a reaction mixture of the test compound and cyclophilin

and its host receptor or a cellular preparation comprising, in part, said cyclophilin-binding activity and for a time sufficient to allow the components to interact and bind, thus forming a complex which can be removed and/or detected. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of cyclophilin A and its cellular binding partner or receptor. Control reaction mixtures are incubated without the test compound or with a control agent. The formation of any complexes between the target gene protein and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the target gene protein and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal target gene protein may also be compared to complex formation within reaction mixtures containing the test compound and a mutant target gene protein.

The assay for compounds that interfere with the interaction of the target gene products and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the target gene product or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target gene products and the binding partners, *e.g.*, by competition, can be identified by conducting the reaction in the presence of the test substance; *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the target gene protein and interactive cellular or extracellular binding partner. Alternatively, test compounds that disrupt preformed complexes, *e.g.*, compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the target gene protein or the interactive cellular or extracellular binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtitre plates are utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the target gene product or binding partner, and optionally drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted

components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

Alternatively, a homogeneous assay can be used. In this approach, a preformed complex of the target gene protein and the interactive cellular or extracellular binding partner is prepared in which either the target gene product or its binding partners is labeled, but the signal generated by the label is quenched due to complex formation (U.S. Patent 4,109,496). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt target gene protein/cellular or extracellular binding partner interaction can be identified.

The target gene product can be prepared for immobilization using recombinant DNA techniques routinely used in the art. For example, the target gene coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular binding partner can be purified and used to raise a monoclonal antibody. This antibody can be labeled with the radioactive isotope ^{125}I. In a heterogeneous assay, the GST-target gene fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed components. The interaction between the target gene protein and the interactive cellular or extracellular binding partner can be detected by measuring the amount of radioactivity that remains associated with

the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

Alternatively, the GST-target gene fusion protein and the interactive cellular or extracellular binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the target gene product/binding partner interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads. Such cyclophilin fusion protein constructs are similarly useful to identify and isolate cellular cyclophilin binding partners and mutants thereof.

The compounds of the present invention, including but not limited to, anti-cyclophilin antibodies, recombinant cyclophilin, derivatized cyclosporin and derivatized cyclophilin A, have utility as pharmacological compositions for the treatment and prevention of HIV-infection. Those compounds identified in the screening assays of the invention which inhibit the interaction between cyclophilin A and its cellular binding partner or receptor, also have utility in pharmacological compositions for the treatment and prevention of HIV-infection.

A compound of the invention can be administered to a human patient by itself or in pharmaceutical compositions where it is mixed with suitable carriers or excipients at doses to treat or ameliorate various conditions involving HIV-infection. A therapeutically effective dose further refers to that amount of the compound sufficient to inhibit HIV infection. Therapeutically effective doses may be administered alone or as adjunctive therapy in combination with other treatments for HIV infection or associated diseases. Techniques for the formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, latest addition.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, and optionally in a depot or sustained release formulation.

Furthermore, one may administer the agent of the present invention in a targeted drug delivery system, for example in a liposome coated with an anti-CD4 antibody. The liposomes will be targeted to and taken up selectively by cells expressing CD4.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers, such

as Hank's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers.

Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form in ampoules or in multi-dose containers, with an added preservative. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Many of the compounds of the invention identified as inhibitors of the interaction between cyclophilin A and its host receptor may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc.; or bases. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. Examples of pharmaceutically acceptable salts, carriers or excipients are well known to those skilled in the art and can be found, for example, in

Remington's Pharmaceutical Sciences, 18th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA, 1990. Such salts include, but are not limited to, sodium, potassium, lithium, calcium, magnesium, iron, zinc, hydrochloride, hydrobromide, hydroiodide, acetate, citrate, tartrate, malate sales, and the like.

Example 1

This example provides *in vitro* experiments and assays demonstrate the effectiveness of recombinant cyclophilin to inhibit HIV infection of human peripheral blood lymphocytes (PBLs) or cultures of the H9 T cell line. Monitoring the progression of the infection will identify the effective dose range of soluble recombinant cyclophilin to interfere with infection.

Mouse cyclophilin (cyclophilin A) was cloned from RAW 264.7 cells. Macrophages plated at a cell density of 1 x 10^6 cells/ml in RPMI/10% FBS were stimulated with LPS at 1 μg/ml. Six hours after the addition of LPS the medium was removed and total RNA isolated with RNAzol (Biotecx; Houston, TX) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed, and resulting cDNA amplified by PCR using a set of cyclophilin-specific primers (5'-CCA-TGG-TCA-ACC-CCA-CC-3' and 5'-ACG-CTC-TCC-TGA-GCT-ACA-GA-3') which span the cyclophilin coding region. A single DNA amplification product of the expected size was obtained, cloned into the plasmid pT7Blue and transformed into Nova Blue competent *E. coli* using the pT7Blue T-Vector Kit (Novagen; Madison, WI).

The recombinant pT7Blue clone containing murine cyclophilin cDNA was digested with the restriction enzymes *Nde*I and *Bam*HI, and the cyclophilin insert was isolated and ligated in the *Nde*I/*Bam*HI-digested pET14b vector (Novagen) allowing for its expression as a histidine fusion protein. The cyclophilin insert was placed behind the T7 promoter which was under the control of the *lac* repressor (allowing for induction by the addition of IPTG to the medium). The vector also contained a gene for ampicillin resistance (allowing for selection in carbenicillin-containing medium). The cyclophilin-containing vector was used to transform *E. coli* DH5α cells. These cells were streaked out, and cells grown up from a single recombinant colony were isolated and stored in 20% glycerol at -70 °C for later use. Murine cyclophilin-containing pET14b plasmid DNA was then prepared and used to transform *E. coli* BL21(DE3) expression strain (Novagen). One hundred milliliter cultures (seeded in 500 ml erlenmeyer flasks) were grown at 37°C with vigorous shaking until the absorbance at 600 nm was between 0.6 and 1.0 OD units. At that time, IPTG was added to the cultures (1 mM final) and the incubation continued at 37 °C for an additional 3 hours. Bacteria were harvested by centrifugation and the cell pellets frozen at -70 °C.

For protein purification, a bacterial pellet (corresponding to 100 mls of culture) was thawed and resuspended in 4.0 ml Binding Buffer in preparation for subsequent purification using His-Bind Resin (Novagen). The bacteria were lysed by adding an equal volume of

washed glass beads (106 μm; Sigma) and vortexing the mixture vigorously for 10 minutes. Glass beads were removed by centrifugation at 1000 x g for 10 minutes, and the bacterial extract clarified by centrifugation at 38000 x g for 30 min. The cleared bacterial lysate was sterile-filtered through a 0.22 micron syringe filter, and immediately loaded onto a column containing 2.5 ml His-Bind Resin (Novagen). The column was washed extensively according to the manufacturers directions.

After washing, the recombinant material was eluted with 1 M imidazole. The eluate, which contained the cyclophilin fusion protein as assessed by SDS-PAGE, was subjected to proteolytic cleavage with thrombin to remove the His-Tag leader sequence from the amino terminus of the expressed peptide. For the cleavage reaction, thrombin (0.5 Units per mg recombinant protein) and the recombinantly expressed peptide were incubated in Thrombin Cleavage Buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl and 2.5 mM $CaCl_2$) for 2 hours at 20 °C. The mixture was then concentrated using a Centriprep-10 concentration device (Amicon Corp., Danvers, MA) at 4 °C. To purify recombinant murine cyclophilin away from both thrombin and the cleaved His-Tag leader sequence, the concentrated mixture was subjected to high performance gel filtration chromatography over a Superose 12 column (Pharmacia, Piscataway, NJ) equilibrated in PBS, pH 7.4. Cyclophilin eluted as a sharp peak with an apparent molecular mass of 19 kDa. Fractions containing cyclophilin (16 & 17) were pooled, concentrated using a Centriprep-10 concentration device (Amicon Corp.), and dialyzed against 0.01 M sodium phosphate buffer, pH 7.4. Protein concentration was measured by Bradford assay with bovine gamma globulin as standard (Bio-Rad Laboratories, Richmond, CA). The material was greater than 98% pure as judged from silver-stained SDS polyacrylamide gels

The H9 T cell line was cultured in RPMI medium supplemented with 10% fetal calf serum and 1% pen/strep. Cells were seeded at a density of $0.5x10^6$ cells/ml and, every 2nd day, one-half of the cell suspension was removed and replaced with fresh medium, to keep cell density below $1x10^6$ cells/ml. Cells were infected with HIV-1_{RF} strain at a multiplicity index of 10 ng p24 per $1x10^6$ cells. After a 2 hr adsorption at 37 °C, 5% CO_2, non-bound virus was washed out, and incubation was continued in fresh medium with or without rCyP at the indicated doses. Samples were removed for RT and p24 analysis (according to standard methods) every 2nd day, and half of the cell suspension substituted with fresh medium at that time.

Cultures of purified normal human peripheral blood lymphocytes (PBLs) were activated/stimulated with PHA/IL-2 for 48 hours prior to infection. Cultures were infected with HIV in the presence or absence of exogenous recombinant cyclophilin (rCyP) and re-fed every three days with rCyP containing medium at the indicated doses. Monitoring the progression of the infection identified the effective dose range of soluble cyclophilin to interfere with HIV infection.

The results of these studies are illustrated in Figures 1, 2 and 3. Figure 1 illustrates that on day 5 after infection, as assayed by RT activity in the culture supernatants, experimental HIV infection was inhibited in H9 T cell cultures treated with recombinant murine cyclophilin at 40 μg/ml. Figure 2 shows in part that the presence of cyclophilin at 40 μg/ml, but not at 1 μg/ml, inhibited HIV infection in H9 cell cultures, as measured by RT activity post-infection. In additional experiments, these inhibitory effects seen with a cultured T cell line were confirmed and extended to normal human peripheral blood lymphocyte cultures, reinforcing the utility of this therapeutic modality against HIV infection in humans. Figure 3 shows a time course of the inhibitory effect of exogenously supplied recombinant cyclophilin at a dose of 40 μg/ml in PBL cultures, as measured by the appearance of p24 antigen in culture supernatants as assayed by a p24 ELISA kit.

Example 2

This example provides *in vitro* experiments and assays demonstrating the effectiveness of antibodies raised against cyclophilin (anti-CyP) or against an experimentally glycated preparation of CyP (anti-AGE-CyP). The anti-AGE-CyP antibodies recognize soluble CyP in its native configuration as indicated by immunoprecipitation studies; the anti-CyP antiserum did not.

An aliquot of purified recombinant murine cyclophilin (500 μg/ml final) was reacted with glucose (1 M final) in 0.5 M sodium phosphate buffer (pH 7.4) for three months at 37 °C in a non-humidified air incubator. Incubation of proteins with glucose under these conditions has been shown to lead to the formation of irreversible covalent adducts, known as Advanced Glycation Endproducts (or AGEs), via the extended system of Maillard reactions that lead to the spontaneous modification of proteins by reducing sugars. Upon completion, the reaction mixture was dialyzed to remove non-covalently attached low-molecular weight reactants, aliquoted into eppendorf tubes (0.125 μg per tube), and stored frozen at -20 °C until used for immunization of rabbits.

Polyclonal rabbit anti-cyclophilin antisera were raised. Female New Zealand White rabbits were immunized with cyclophilin which had been emulsified in 1.0 ml saline plus 1.0 ml complete Freund's adjuvant. The immunogen (20 μg human cyclophilin) was administered by subcutaneous injection at four sites on the dorsum of the rabbit. Animals were administered booster injections monthly with the same antigen emulsified in 1.0 ml saline and 1.0 ml incomplete Freund's adjuvant. Blood was withdrawn 7 days and 14 days following each booster injection, and the serum fraction was recovered and stored frozen at -20 °C. Pre-immune and immune sera were tested for reactivity by Western blotting and immunoprecipitation against both recombinant human cyclophilin and recombinant murine cyclophilin. Pre-immune sera were negative in both assays. On western blots the immune sera

was positive against both antigens. Immunoprecipitation experiments were negative.

The recombinant murine cyclophilin used for immunization and boosting was produced in *E. coli* by the procedure described in example 1. At each time point, rabbits were immunized and boosted with 50 μg murine cyclophilin. Pre-immune and immune sera were tested for reactivity by Western blotting and immunoprecipitation against both recombinant human cyclophilin and recombinant murine cyclophilin. Pre-immune sera were negative in both assays. On western blots the immune sera was positive against both antigens. Immunoprecipitation experiments were negative.

At each time point, rabbits were immunized and boosted with 125 μg AGE-modified murine cyclophilin. Pre-immune and immune sera were tested for reactivity by Western blotting and immunoprecipitation against both recombinant human cyclophilin and recombinant murine cyclophilin. Pre-immune sera were negative in both assays. On western blots and in immunoprecipitation experiments the immune sera was positive against both antigens indicating that antibody raised against AGE-modified murine cyclophilin recognized soluble cyclophilin, as well as, immobilized cyclophilin.

To prepare a cyclophilin affinity resin, recombinant murine cyclophilin was coupled to Sepharose beads according to a protocol recommended by the manufacturer. In brief, three grams of freeze-dried CNBr-activated Sepharose 4B (Pharmacia; Piscataway, NJ) was rehydrated in 1 mM HCl (yielding 10.5 ml swollen gel), and washed for 15 minutes with 1 mM HCl (200 ml/g freeze dried powder) on a sintered glass funnel. Following the removal of fluid on the sintered glass funnel, 5.0 ml gel was transferred to a 50 cc tube and was mixed with 10 ml of 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.0) containing 25 μg of recombinant murine cyclophilin. The mixture was rocked at room temperature for 2 hours. At that time, 0.5 ml of 1 M ethanolamine-HCl (pH 9) was added to quench excess reactive groups, and rocking was continued for another 2 hours. The binding of cyclophilin to the gel matrix was essentially complete, as indicated by the absence of detectable protein in the supernatant. The gel was then transferred to a sintered glass funnel and washed with three alternate cycles of 0.1 M sodium acetate (pH 4.0) containing 0.5 M NaCl and 0.1 M Tris-HCl (pH 8.0) containing 0.5 M NaCl (40 ml each wash). The final volume of swollen gel was about 5.0 ml.

Murine cyclophilin affinity resin (5.0 ml packed volume) was transferred to a 10 ml glass walled chromatography column. After allowing the resin to pack, the column was washed successively with 10 bed-volumes of 10 mM Tris (pH 7.5), 10 bed-volumes of 100 mM glycine (pH 2.5), 10 bed-volumes 10 mM Tris (pH 8.8), 10 bed-volumes 100 mM triethylamine (pH 11.5, prepared fresh), and lastly, 10 bed-volumes 10 mM Tris (pH 7.5). Five milliliters polyclonal rabbit anti-murine AGE-modified cyclophilin which had been dialyzed overnight against 10 mM Tris (pH 7.5) was loaded onto the column. The column was washed with 10 bed-volumes 20 mM Tris (pH 7.5), followed by 10 bed-volumes 10 mM Tris/0.5 M NaCl (pH 7.5), and then cyclophilin specific antibodies were eluted by washing the column

with 100 mM glycine-HCl (pH 2.5) and collecting 1.0 ml fractions.

H9 T cell line was cultured in RPMI medium supplemented with 10% fetal calf serum and 1% pen/strep. Cells were seeded at a density of $0.5x10^6$ cells/ml and, every 2nd day, one-half of the cell suspension was removed and replaced with fresh medium, to keep cell density below $1x10^6$ cells/ml. Cells were infected with HIV-1_{RF} strain at a multiplicity index of 10 ng p24 per $1x10^6$ cells. After a 2 hr adsorption at 37 °C, 5% CO_2, non-bound virus was washed out, and incubation was continued in fresh medium. Samples were removed for RT and p24 analysis every 2nd day, and half of the cell suspension substituted with fresh medium at that time.

Monocyte cultures were prepared from the whole blood of HIV-negative donors. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-Hypaque gradient, resuspended at $6-8x10^6$ cells/ml in DMEM supplemented with 10% heat-inactivated normal human serum (NHS) and plated in a PRIMARIA flask. After a 2 h, 37 °C incubation, adherent cells were washed 3 times with DMEM, and re-fed with DMEM + 10% NHS + 1 ng/ml M-CSF (Sigma). After a 24 h incubation, cells were washed with Ca^{++}- and Mg^{++}-free PBS and then incubated in PBS+10 mM EDTA for 3-5 min on ice. Cells were detached with a rubber policeman, washed, counted, and resuspended in DMEM+10% NHS+1 ng/ml M-CSF. At that point, cells were >98% monocytes by the criteria of cell morphology and nonspecific esterase staining. Monocytes were plated at a density 10^6 cells/ml in PRIMARIA plates and allowed to differentiate *in vitro* for 6 days with half the medium changed every 2 days. On day 7 after plating, cells were exposed to a monocytotropic strain HIV-1_{ADA} (multiplicity of infection 100 ng of p24 per 10^6 cells) for 2 hr, washed, and cultured in DMEM+10%NHS.

Recombinant mouse cyclophilin at various concentrations (ranging from 1 to 200 μg/ml), and anti-cyclophilin antibodies (1:40 dilution) were added to cells together with the virus, and were likewise present throughout all subsequent incubations.

At various intervals after infection, cells were washed and resuspended in 1xPCR buffer with Proteinase K (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2.5 mM $MgCl_2$; 0.1 mg/ml gelatin; 0.45% NP40; 0.45% Tween 20; 250 mg/ml Proteinase K) at $6x10^6$ cells/ml. After a 3 hr digestion at 60 °C, Proteinase K was inactivated by heating to 95 °C for 10 min., and lysates were used for PCR analysis with primers specific for the HIV-1 *pol* gene (PCR I and PCR J), HIV *pol* PCR primers: sense, 5'-GAAGCTCTATTAGATACAGG-3'; anti-sense, 5'-TCCTGGCTTTAATTTTACTGG-3'; probe, 5'-GGAATTGGAGGTTTATCAAAGT-3'; HIV-1 2-LTR circle DNA, or cellular α-tubulin gene as a control (AT1 and AT2). PCR reactions in 50 μl were prepared as follows: 25 ml cell lysate; 1 μl of 10 mM dNTP mixture; 1 μl of each primer; 12.5 μl of 2xPCR buffer without Proteinase K; 0.25 μl of Taq polymerase (5 U/ml or 1.25 U per reaction). 35 cycles of PCR were performed, each composed of 30 sec, 95 °C denaturation; 30 sec, 60 °C annealing; 45 sec, 72 °C extension. Cycles were preceded by a 6 min, 95 °C denaturation, and followed by a 7 min, 72 °C final extension. PCR products were

visualized by hybridization to a ^{32}P-labeled probe after a Southern transfer, and results were quantitated on a direct imager system (Packard).

In H9 cell cultures (Figure 2), antisera raised against recombinant cyclophilin or glycated cyclophilin inhibited HIV infection, as measured by RT activity 3 days post-infection.. These data were confirmed and extended using non-transformed cells in cultures of human monocytes/macrophages.

The effects in monocyte cultures were assayed by PCR 48 hr after infection. A dose-dependent inhibition of HIV-1 cDNA synthesis by cyclophilin was observed (Figure 4), with 50% inhibition (IC_{50}) at 50 μg/ml. Inhibition seen for HIV-1 cDNA forms produced late *in vivo* reverse transcription process (after the second strong stop) indicating that viral replication stopped prior to completion of nuclear translocation was affected. Similar results were observed with anti-cyclophilin antibodies (Figure 4), which had been affinity-purified from anti-AGE-CyP antisera.

Example 3

This example provides *in vitro* experiments and assays demonstrate the effectiveness of pegylated cyclosporin as an inhibitor of HIV-infection. Human monocyte/macrophage cell cultures are challenged with an inoculum of HIV in the presence and absence of a "pegylated" cyclosporin analogue. This cyclosporin analogue has been modified by a reaction with polyethylene glycol so as to prevent cell entry. This pegylated CsA is not immunosuppressive in a standard T cell proliferation assay. Monitoring the progression of the infection will identify the effective dose range of pegylated cyclosporin to interfere with HIV infection.

Replication of HIV virions, which have been shown to contain cyclophilin, are inhibited by the immunosuppressive drug, cyclosporin A (CsA). To determine whether CsA acts extracellularly or intracellularly in this regard, a CsA analogue which cannot penetrate the cell membrane has been synthesized as follows: 8-amino-cyclosporin A (0.5 mgs) was reacted with methoxypolyethylene glycol-succinimidyl succinate (10 mgs) and 4-dimethylamino pyridine (0.2 mgs) in methylene chloride with stirring for two days at room temperature in the dark. To block any unreacted sites, ethanolamine (5 μl) was then added and the mixture incubated at room temperature with stirring for another 24 hours. The reaction mixture was transferred to -70 °C until CsA-PEG purification by normal phase HPLC was performed.

The H9 T cell line was cultured in RPMI medium supplemented with 10% fetal calf serum and 1% pen/strep. Cells were seeded at a density of $0.5x10^6$ cells/ml and, every 2nd day, one-half of the cell suspension was removed and replaced with fresh medium, to keep cell density below $1x10^6$ cells/ml. Cells were infected with HIV-1_{RF} strain at a multiplicity index of 10 ng p24 per $1x10^6$ cells. After a 2 hr adsorption at 37 °C, 5% CO_2, non-bound virus was washed out, and incubation was continued in fresh medium. Samples were removed for RT and p24 analysis every 2nd day, and half of the cell suspension substituted with fresh medium

at that time.

Monocyte cultures were prepared from the whole blood of HIV-negative donors. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-Hypaque gradient, resuspended at 6-8x10^6 cells/ml in DMEM supplemented with 10% heat-inactivated normal human serum (NHS), and plated in a PRIMARIA flask. After a 2 h, 37 °C incubation, adherent cells were washed 3 times with DMEM, and re-fed with DMEM+10%NHS+1 ng/ml M-CSF (Sigma). After a 24 h incubation, cells were washed with Ca^{++}- and Mg^{++}-free PBS and then incubated in PBS+10 mM EDTA for 3-5 min on ice. Cells were detached with a rubber policeman, washed, counted, and resuspended in DMEM+10% NHS+1 ng/ml M-CSF. At that point, cells were >98% monocytes by the criteria of cell morphology and nonspecific esterase staining. Monocytes were plated at a density 10^6 cells/ml in PRIMARIA plates and allowed to differentiate *in vitro* for 6 days with half the medium changed every 2 days. On day 7 after plating, cells were exposed to a monocytotropic strain HIV-1_{ADA} (multiplicity of infection 100 ng of p24 per 10^6 cells) for 2 hr, washed, and cultured in DMEM+10%NHS.

As shown in Figure 4, cyclosporin derivatized to inhibit cell entry was effective at a dose of 0.1 μg/ml to inhibit HIV infection of monocyte/macrophage cultures as judged by PCR analysis of HIV *pol* gene retrotranscription. Although pegylated CsA was non-immunosuppressive, it showed approximately the same antiviral effect as CsA itself. In a parallel study with H9 cells, pegylated CsA was also approximately equipotent with CsA.

Example 4

This example provides cross-linking studies to determine the identity of cyclophilin-binding partners on H9 cell membranes. Disuccinimidyl suberate (DSS), a homobifunctional N-hydroxy-succinimide ester, was used as a cross-linking reagent. H9 cells are harvested, washed in ice-cold PBS, 2.5 x 10^7 cells transferred to a clean tube, and cells pelleted by centrifugation. This pellet was resuspended in 0.5 ml cross-linking buffer, and ^{125}I-cyclophilin added. After a 60 minute incubation on ice, cells were pelleted at 500 x g, and supernatant discarded. The pellet was resuspended to 2.5 x 10^6 cells/ml in cross-linking buffer, and DSS added to a final concentration of 20 ug/ml. This reaction mixture was rocked at 4 °C for 20 minutes, at which time 1 volume of TE buffer, pH 7.4 was added to stop the reaction. Cells were washed 3 times in ice-cold TE buffer (pH 7.4), and membranes extracted by homogenization in an NP40 lysis buffer. NP40 extracts were analyzed by SDS-PAGE electrophoresis and autoradiography to identify proteins crosslinked to iodinated cyclophilin. Additional analyses, in which cells were fractionated into membrane, cytosolic, and/or nuclear fractions further characterized the subcellular localization of cyclophilin-binding partners.

Recombinant murine cyclophilin was iodinated using the Bolton-Hunter method, or the commercially available Iodogen method. Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxy-5-([^{125}I] iodophenyl-propionate) was be purchased from NEN.

Example 5

This example provides an immunization protocol to generate monoclonal anti-AGE-modified cyclophilin begins with immunization of mice. Four BALB/C mice were immunized by i.p. injection with 25 μg AGE-modified murine cyclophilin in 1:1 mixture of saline and adjuvant (Ribi). Twenty-one days post-immunization, mice were boosted with 25 μg AGE-modified murine cyclophilin in RIBI adjuvant, and mice were boosted with this amount of antigen in adjuvant every 21 days. After the second boost, mice were tail-bled (approximately 50 μl) and the serum from each mouse analyzed for immunoreactivity against cyclophilin in a direct ELISA in which the plate was coated with recombinant murine cyclophilin. When a mouse screened positive for anti-cyclophilin IgG, the mouse was given a final boost of antigen and then euthanized for spleen removal and hybridoma production. Resulting hybridomas were screened for production of cyclophilin-specific monoclonal antibodies.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An immunotherapeutic method for inhibiting HIV infection, comprising administering to an individual a cyclophilin immunogen at a dose sufficient to induce an immune response to cyclophilin.

2. The method of claim 1 wherein the cyclophilin immunogen is administered with an adjuvant.

3. The method of claim 2 wherein the cyclophilin immunogen is glycated.

4. An immunotherapeutic method for inhibiting HIV infection, comprising administering to an individual an antibody that neutralizes cyclophilin, at a dose sufficient to inhibit HIV infection.

5. The method of claim 4 in which the antibody is a single-chain antibody, chimeric antibody, humanized antibody, or Fab fragment.

6. A method for inhibiting HIV infection, comprising administering to a subject exogenous cyclophilin in an amount sufficient to inhibit HIV infection.

7. A drug screening method to identify compounds or compositions that inhibit the interaction of cyclophilin and its cognate cellular binding partner or receptor *in vitro*, which comprises:
   (a) adding a test compound, cyclophilin and a cyclophilin binding partner to a reaction mixture;
   (b) detecting any cyclophilin-cyclophilin binding partner complexes in the reaction mixture.

8. A method according to claim 1, claim 6 or claim 7, substantially as herein described.

Dated this 27th day of June 2000

The Picower Institute of Medical Research
By its Patent Attorneys
Davies Collison Cave

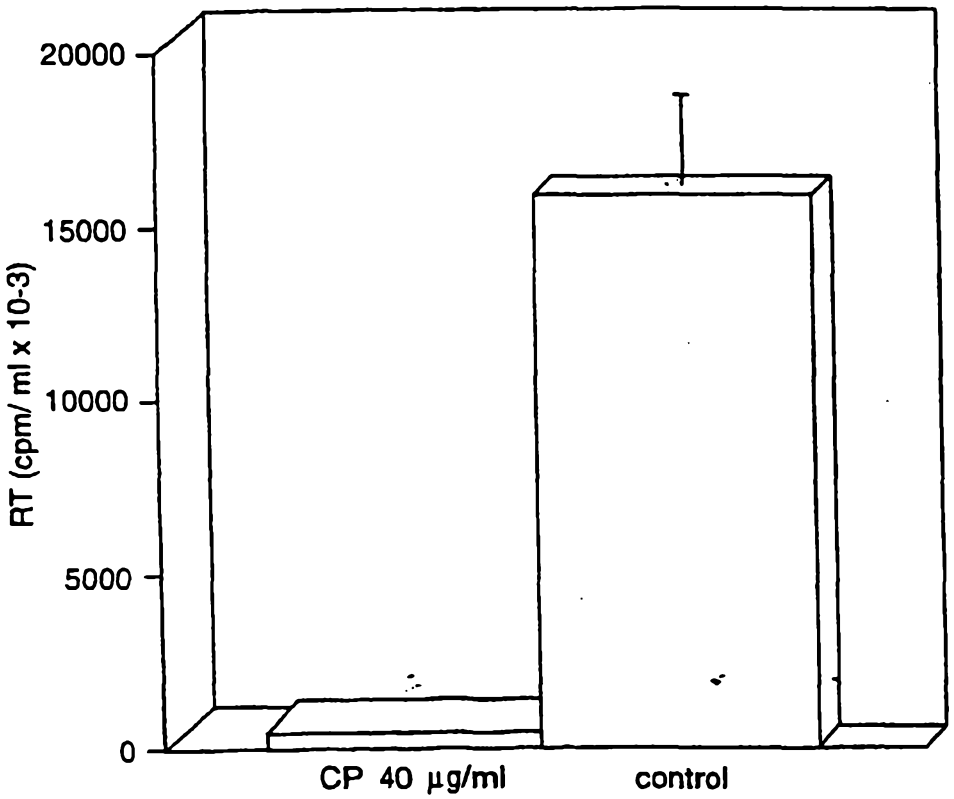

Fig. 1

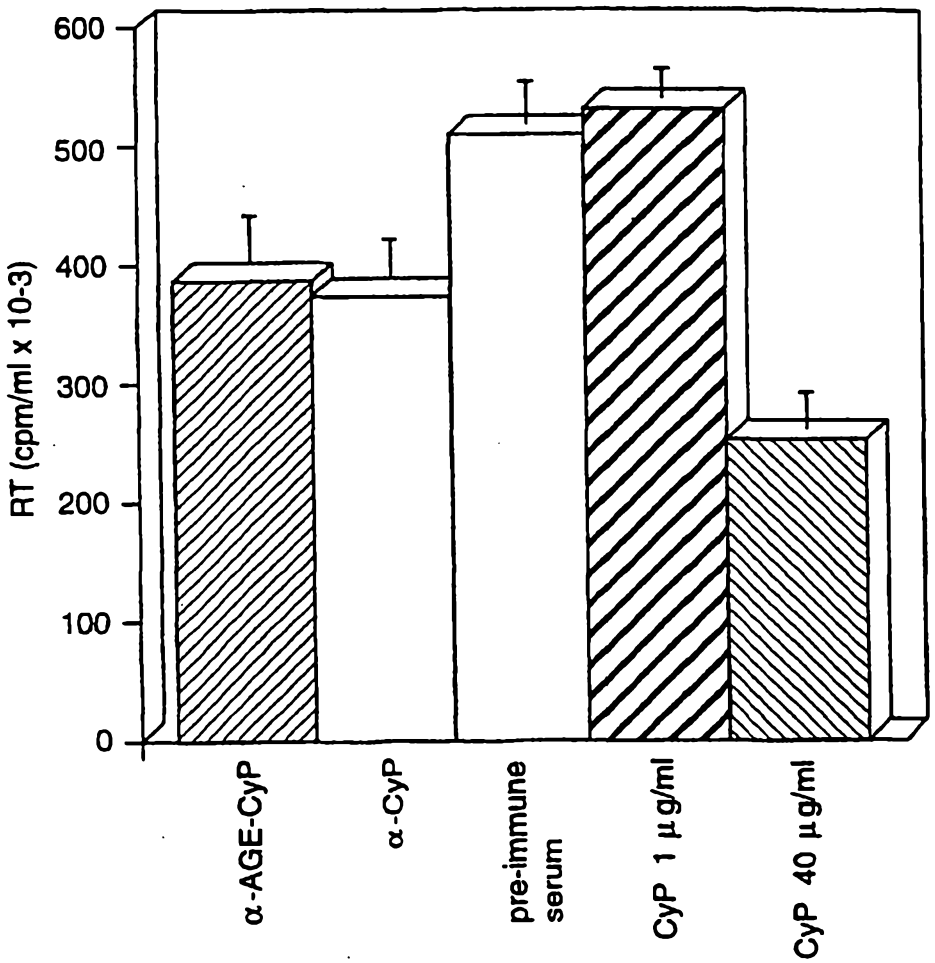
600
500
400
300
200
100
0
RT (cpm/ml x 10-3)
α-AGE-CyP
α-CyP
pre-immune serum
CyP 1 µg/ml
CyP 40 µg/ml

Fig. 2

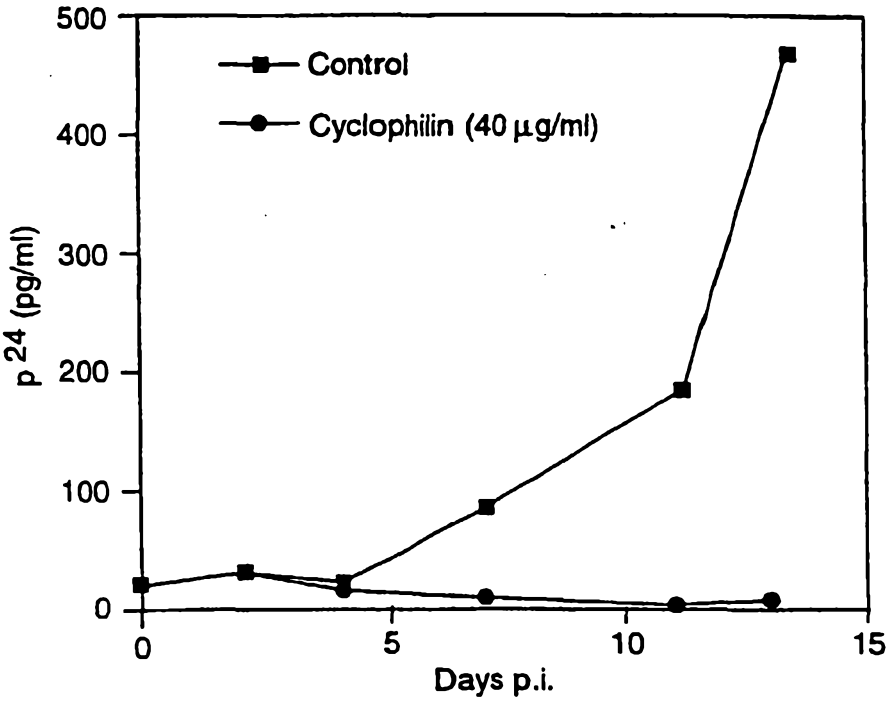
500
400
300
200
100
0
p24 (pg/ml)
Control
Cyclophilin (40 µg/ml)
0
5
10
15
Days p.i.

Fig. 3

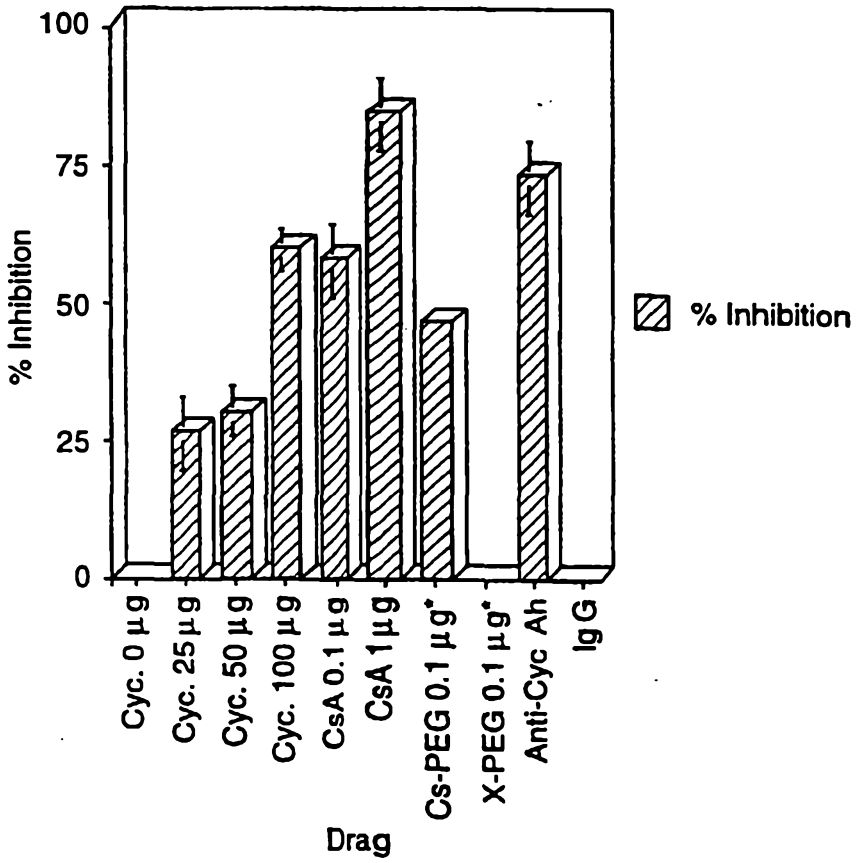
100
75
50
25
0
% Inhibition
% Inhibition
Cyc. 0 µg
Cyc. 25 µg
Cyc. 50 µg
Cyc. 100 µg
CsA 0.1 µg
CsA 1 µg
Cs-PEG 0.1 µg*
X-PEG 0.1 µg*
Anti-Cyc Ah
IgG
Drag

Fig. 4