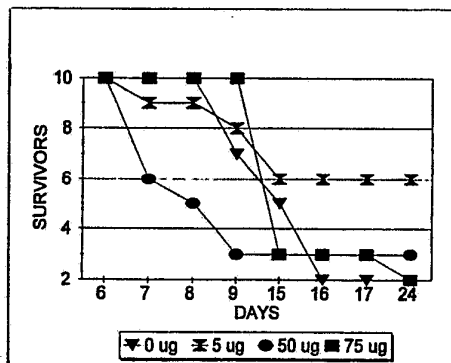
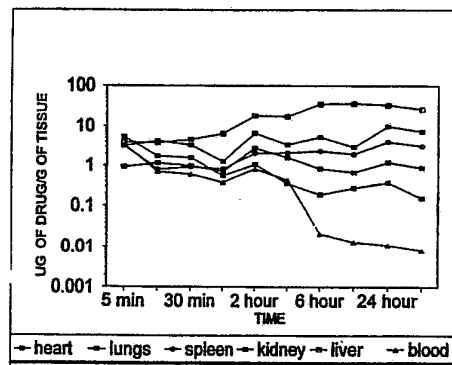
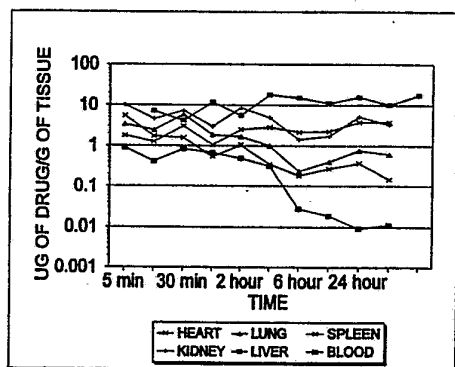




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 5 : A61K 37/02</p>	<p>A1</p>	<p>(11) International Publication Number: WO 93/21941 (43) International Publication Date: 11 November 1993 (11.11.93)</p>
<p>(21) International Application Number: PCT/CA93/00166 (22) International Filing Date: 21 April 1993 (21.04.93) (30) Priority data: 07/872,398 23 April 1992 (23.04.92) US (71) Applicant: KIRWOOD, Sheryl, Dana (legal representative of the deceased inventor) [CA/CA]; 423 Old Orchard Grove, North York, Ontario M5M 2G3 (CA). (71) Applicant (for all designated States except US): ALLELIX BIOPHARMACEUTICALS INC. [CA/CA]; 6850 Goreway Drive, Mississauga, Ontario L4V 1P1 (CA). (71)(72) Applicants and Inventors: TWIST, Michael [US/CA]; 55 Harbour Square, Apt. #1815, Toronto, Ontario M5J 2L1 (CA). BARNETT, Richard, W. [US/CA]; 45 Bristol Road East, Unit 22, Mississauga, Ontario L4T 3P5 (CA). SUMMER-SMITH, Martin [CA/CA]; 332 Whitehead Crescent, Bolton, Ontario L0P 1A0 (CA).</p>		<p>(72) Inventor: REID, Lorne, S. (deceased). (74) Agents: HIRONS, Robert, G. et al.; Ridout & Maybee, 101 Richmond Street West, Suite 2300, Toronto, Ontario M5H 2J7 (CA). (81) Designated States: AU, BB, BG, BR, CA, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i></p>

(54) Title: TREATMENT OF HERPESVIRUS INFECTION



(57) Abstract

Described herein are oligopeptides useful to inhibit replication of herpesviruses, especially herpes simplex viruses. In a preferred embodiment of the invention, the oligopeptide is a D-arginine nonamer having N- and C-terminal protecting groups.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

TREATMENT OF HERPESVIRUS INFECTIONField of the Invention

This invention relates to anti-viral compounds. More particularly, the invention relates to the use of peptide-based anti-viral agents for the treatment of herpesvirus infections.

Background to the Invention

The herpesviruses constitute a family of human pathogens related by a number of criteria, including life cycle, host range, polypeptide composition and genome structure. The herpesvirus family includes the herpesviridae that are divided into three sub-families; α -herpesvirinae which includes herpes simplex virus (HSV) type 1, which manifests as cold sores and type 2 which causes genital lesions, pseudorabies virus, equine abortion virus, and infectious bovine rhinotracheitis virus; β -herpesvirinae which includes human and murine cytomegalovirus (CMV); and gamma-herpesvirinae which includes the Epstein-Barr virus (EBV) which is responsible for infectious mononucleosis. Also included in the herpesvirus family is the Varicella Zoster virus (VZV) which is the causative agent of chicken pox, and the recently discovered human viruses HHV-6 and HHV-7.

The current types of herpes treatments are reviewed in "Antiviral Drugs: Pharmacokinetics, Adverse Effects and Therapeutic Use", M.C. Nahata, J. Pharm. Technol., 1987, 3:100. Among current anti-herpetic agents are acyclovir, and structurally related nucleoside analogues. The association of anti-herpetic activity with peptides has been noted, but is less common. In US 4,845,195, Colonno et al ascribe anti-herpetic activity to the pentapeptide Val-Val-Asn-Asp-Leu and various analogues (see also US 4,837,304).

Although some significant advances have been made in the treatment of herpesvirus infections, the need for effective, safe therapeutic agents for treating herpesvirus infections continues to exist.

It is a general object of the present invention to provide a method useful to treat herpesvirus infection in a mammal.

It is a more specific object of the present invention to provide a method useful to treat infection by a herpes simplex virus (HSV).

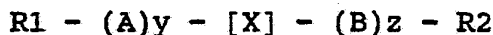
It is another object of the present invention to provide a pharmaceutical composition useful to treat herpesvirus infection.

It is another object of the present invention to provide a compound useful to inhibit herpesvirus replication.

SUMMARY OF THE INVENTION

It has now been found that oligopeptides that are substantially basic in nature i.e. have a substantially positive net charge, are particularly effective as inhibitors of herpesvirus replication. It has further been found that the anti-herpetic activity of such oligopeptides is not significantly reduced when amino acid components of such oligopeptides are in the more serum-stable D-amino acid form.

According to one aspect of the present invention, there is provided a compound capable of inhibiting herpesvirus replication, of the formula:



wherein

R1 is H or an N-terminal protecting group;

R2 is OH or a C-terminal protecting group;

X represents an oligopeptide consisting of 'n' amino acids and having a net positive charge selected from n, n-1 and n-2, wherein n is an integer from 6 to 12;

y is 0 or 1;

z is 0 or 1; and

A and B independently represent one or more amino acids residues which collectively are selected to retain the anti-herpetic nature of the compound, or a pharmaceutically acceptable salt thereof.

According to one embodiment of the invention, X in the above formula represents an oligopeptide comprising at least one D-amino acid, and more desirably consists essentially of D-amino acids. A preferred compound of the present invention consists of nine D-arginine residues having blocking groups at both the N- and C-termini.

According to another aspect of the invention, there is provided a pharmaceutical composition comprising a physiologically acceptable carrier and an effective amount of an anti-herpetic compound of the present invention. In embodiments of the present invention, the pharmaceutical composition is provided in a form suitable for topical or systemic administration.

According to another aspect of the present invention, there is provided a method for treating a patient infected with a herpesvirus, which comprises administering to the patient an effective amount of an anti-herpetic compound of the present invention. In

embodiments of the present invention, the anti-herpetic compound is administered to treat herpesvirus infection in humans or for veterinary purposes, particularly for the treatment of livestock.

The invention is now described in greater detail with reference to the accompanying drawings in which:

Brief Reference to the Drawings

Figures 1 and 2 illustrate tissue distribution of a representative compound following i.v. (Fig.1) and s.c. (Fig.2) administration thereof; and

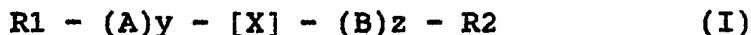
Figure 3 illustrates the anti-herpes effect of a representative compound, *in vivo*.

Detailed Description of the Invention and Its Preferred Embodiments

The present invention provides oligopeptide-based compounds having anti-herpetic activity, for use in the treatment of herpesvirus infections. The term "oligopeptide" is used interchangeably with the term "polypeptide," and refers to a compound having from 6 to about 100 or more amide-linked α -amino acid residues. As stated hereinabove, the term "herpesvirus" is intended to embrace the various members of the herpesvirus family, which unless otherwise indicated herein, refers collectively to species including all types of herpes simplex virus (HSV) such as HSV-1 and HSV-2, the Varicella Zoster viruses, and the Epstein-Barr viruses. The term "anti-herpetic" refers to the ability of a given compound to inhibit replication of at least one member of the herpesvirus family, as determined by a cell culture assay used conventionally in the art, such as the well

established plaque reduction assay. In the context of the plaque reduction assay for example, the anti-herpetic nature of a given compound is indicated by a reduction in plaque count following treatment of virally infected cells with the given compound, relative to a virally infected, untreated control.

In one of its aspects, the present invention provides a family of anti-herpetic oligopeptide-based compounds, of the formula:



wherein

R1 is H or an N-terminal protecting group;

R2 is OH or a C-terminal protecting group;

X represents an oligopeptide consisting of 'n' amino acids and having a net positive charge of 'n', 'n-1' or 'n-2', wherein n is an integer from 6 to 12;

y is 0 or 1;

z is 0 or 1; and

A and B each represent from 1 to 30 or more independently selected amino acids which collectively are selected to retain the anti-herpetic nature of the compound.

According to one embodiment, compounds of the invention belong to the family represented by the formula (Ia):



in which R1, R2 and X are as specified above. Preferred are compounds of formula (Ia) in which at least one of R1 and R2 is a protecting group.

A particularly preferred family of compounds is represented by the formula (Ib),



in which X is as specified above, Np represents an N-terminal protecting group, and Cp represents a C-terminal protecting group. The term "N-terminal protecting group" refers to a radical attached to the nitrogen atom which serves to protect the amino terminus of the oligopeptide from undesired biochemical attack. The term "C-terminal protecting group" refers to a radical attached to the C-terminus of the oligopeptide either via an oxygen or via the carbon of the terminal carboxyl group, which serves to protect the carboxyl terminus of the oligopeptide from undesired biochemical attack.

The compounds of the present invention incorporate a core oligopeptide designated by 'X' in each of the above formulae I, Ia and Ib. Oligopeptide X consists of from 6 to 12 amino acids, coupled for instance by amide linkage. Desirably, X consists of from 7 to 11 amino acids, and preferably consists of from 8 to 10 amino acids. The amino acid constituents of oligopeptide X are selected to confer on the oligopeptide a net positive charge selected from 'n', 'n-1' and 'n-2', where 'n' represents the number of amino acid incorporated within oligopeptide X. In other words, X is an oligopeptide consisting either entirely of positively charged amino acids (in the case where the net positive charge is 'n') or of substantially all positively charged amino acids (in the case where the net positive charge is 'n-1' and 'n-2'). The term "net positive charge" refers to the charge on the oligopeptide X as a whole, and is calculated simply by adding the number of positively charged amino acids resident in oligopeptide X and subtracting from that total the number of non-positively charged amino acids resident in

oligopeptide X. For instance, an oligopeptide X in which all but one amino acid is positively charged will have a "net" positive charge of 'n-1' in the case where the one amino acid has a neutral charge. The net charge on oligopeptide X will be 'n-2' in the case where the one amino acid has a negative charge. A charge of 'n-2' is also realized when two amino acids carrying a neutral charge are incorporated among otherwise positively charged amino acids. For the purposes of calculating net positive charge, the term "positively charged" refers to an amino acid having a side chain, possibly a β -carbon side chain but usually an α -carbon side chain, that is cationic in aqueous solution at neutral pH. The term "negatively charged" refers to an amino acid having a side chain that is anionic in aqueous solution at neutral pH. Amino acids having a neutral charge carry a side chain that exhibits either no charge (e.g. alanine) or both +/- charge (e.g. glutamine) in aqueous solution at neutral pH. Preferred compounds are those in which the net positive charge on oligopeptide X is n or n-1.

The terms "amino acid" and " α -amino acid residue" are used interchangeably herein with reference to naturally occurring and synthetic amino acids in either D- or L- form. Unless otherwise stated, the amino acid is the naturally occurring L-amino acid. Included, unless otherwise stated, are: (1) the amino acids having a neutral charge such as glycine; those amino acids having an aliphatic α -carbon side chain such as alanine, valine, norvaline, leucine, norleucine, isoleucine and proline; those having aromatic α -carbon side-chains such as phenylalanine, tyrosine and tryptophan; (2) the negatively charged amino acids, including those having acidic α -carbon side chains such as aspartic acid and glutamic acid; those having side chains which incorporate a hydroxyl group such as serine, homoserine, hydroxynorvaline, hydroxyproline and threonine; those

having sulfur-containing α -carbon side chains such as cysteine and methionine; and those having side chains incorporating an amide group such as glutamine and asparagine; and (3) the positively charged amino acids, including those having basic α -carbon side chains such as lysine, arginine, histidine, and ornithine (also herein referred to as "basic amino acids").

According to a preferred aspect of the present invention, X comprises at least one amino acid in the D-isomer form. The oligopeptide X may, for example, comprise alternating L- and D-amino acids. Most preferably the oligopeptide consists essentially of D-amino acids.

In specific embodiments of the present invention, oligopeptide X in the above formulae I, Ia and Ib has a sequence selected from among the group consisting of;

i) an oligopeptide consisting of from 6 to 11 basic amino acids and one amino acid other than a basic amino acid, wherein each basic amino acid is independently selected from among the group consisting of arginine, lysine, histidine and ornithine, and said one amino acid is selected from among the group consisting of glutamine, serine, histidine, asparagine and homoglutamine. Especially suitable oligopeptides are those in which each basic amino acid is independently selected from arginine and lysine, and the non-basic amino acid is glutamine; and

ii) an oligopeptide consisting essentially of from 7 to 12 basic amino acids, wherein each basic amino acid residue is independently selected from among the group consisting of lysine and arginine.

According to specific embodiments of the present invention, X represents an oligopeptide selected from among the group consisting of:

i) an oligopeptide comprising amino acids arranged in the sequence

Arg-Y2-Y3-Arg-Arg-Y4-Arg-Arg-Arg wherein each of Y2, Y3 and Y4 is a basic amino acid, and at least one of Y2, Y3 and Y4 is arginine;

ii) an oligopeptide comprising from 6 to 11 arginines and one glutamine; and

iii) an oligopeptide homopolymer consisting of from 7 to 12 arginines.

According to preferred embodiments of the present invention, X in the above formula I, Ia and Ib represents an oligopeptide, preferably consisting essentially of D-amino acids, having an amino acid sequence selected from:

Arg-Lys-Lys-Arg-Arg-Lys-Arg-Arg-Arg;
Arg-Lys-Lys-Arg-Arg-Ser-Arg-Arg-Arg;
Arg-Lys-Lys-Arg-Arg-His-Arg-Arg-Arg;
Arg-Lys-Lys-Arg-Arg-Asn-Arg-Arg-Arg;
Arg-Lys-Lys-Arg-Arg-homoGln-Arg-Arg-Arg;
Arg-Lys-Lys-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Lys-Arg-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Lys-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Lys-Arg-Arg-Lys-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Lys-Arg-Arg-Arg;
Arg-Gln-Arg-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Gln-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Arg-Gln-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg;

Arg-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gln-Arg;
Arg-Arg-Arg-Gln-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg;
Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg; and
Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg.

Particularly preferred compounds of the present invention are those of formula I, Ia and Ib in which X represents either homopolymeric D-arginine, having 8, 9 or 10 amide-linked D-arginine residues, or an oligopeptide comprising one at least one D-Gln residue and 7, 8 or 9 D-Arg residues. The presently most preferred compounds are those wherein X in the above formulae I, Ia and Ib represents an oligopeptide consisting essentially of D-amino acids and having a sequence selected from:

D-[Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg];
D-[Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg]; and
D-[Arg-Arg-Arg-Gln-Arg-Arg-Arg-Arg].

As noted hereinabove, the compounds of the present invention are desirably those of formula (Ib)



wherein X represents an oligopeptide as defined above, Np represents an N-terminal protecting group, and Cp represents a carboxyl terminal protecting group. Any chemical group which serves to protect peptide ends from undesired chemical attack can be used. Carboxyl terminal protecting groups and N-terminal protecting groups employed conventionally in the art of peptide synthesis are most desirably incorporated in the compounds of the present invention. Useful N-terminal protecting groups

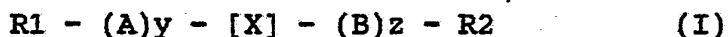
include, for example, lower alkanoyl groups of the formula R-C(O)- wherein R is a linear or branched lower alkyl chain comprising from 1-5 carbon atoms. A preferred N-terminal protecting group is acetyl, CH₃C(O)-. Also useful as N-terminal protecting groups are amino acid analogues lacking the amino function.

Preferred C-terminal protecting groups are, similarly, those used conventionally in the art of peptide synthesis. Such C-terminal protection may be achieved by incorporating the blocking group via the carbon atom of the carboxylic function, for example to form a ketone or an amide, or via the oxygen atom thereof to form an ester. Thus, useful carboxyl terminal protecting groups include, for example, ester-forming alkyl groups, particularly lower alkyl groups such as e.g., methyl, ethyl and propyl, as well as amide-forming amino functions such as primary amine (-NH₂), as well as monoalkylamino and dialkylamino groups such as methylamino, ethylamino, dimethylamino, diethylamino, methylethylamino and the like. C-terminal protection can also be achieved by incorporating as the C-terminal amino acid a decarboxylated amino acid analogue, such as agmatine. Of course, N- and C-protecting groups of even greater structural complexity may alternatively be incorporated, if desired.

Especially preferred compounds of the invention, which conform to formula (Ib), are acetyl-[(D-Arg)₉]-NH₂; acetyl-(D-Arg)₃-(D-Gln)-(D-Arg)₅-NH₂; and acetyl-[D-(Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)]-NH₂.

It will be appreciated that the oligopeptide may be conjugated, either through its C-terminus or its N-terminus to other amino acids without necessarily sacrificing the anti-herpetic activity exhibited by the oligopeptide, as determined by the assays herein

described. The present invention thus further embraces anti-herpetic polypeptide compounds which incorporate the oligopeptides described herein and conform to the general formula (I), i.e.



wherein at least one of y and z is 1, A and B independently represent one or more amide-linked, amino acids, and $R1$, $R2$ and X are as specified above. Desirably, $R1$ represents an N-terminal protecting group, Np , and $R2$ represents a carboxyl terminal protecting group, Cp , wherein Np and Cp are as defined above.

Specifically contemplated compounds of formula I are anti-herpetic compounds in which the oligopeptide X is flanked at the C-terminus and/or at the N-terminus by another unit of oligopeptide X . The repeating units of oligopeptide X may be linked directly by amide bond, or through a peptide linker of from 1 to about 10 amino acids in length.

The compounds of the present invention can be readily prepared by standard, well-established solid-phase peptide synthesis methods (SPPS), general descriptions of which appear, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd Edition, 1984, Pierce Chemical Company, Rockford, Illinois; and in M. Bodanszky and A. Bodanszky, The Practice of Peptide Synthesis, 1984, Springer-Verlag, New York; Applied Biosystems 430A Users Manual, 1987, ABI Inc., Foster City, California.

In general, a suitably protected amino acid is attached through its carboxyl group ($-COOH$) to a derivatized, insoluble polymeric support, e.g. cross-linked polystyrene or polyamide resin. "Suitably protected" refers to the presence of protecting groups on the alpha-

amino group (α -NH₂) and side-chain functional group (if present) of the amino acid. Synthesis proceeds in a stepwise, cyclical fashion by successively removing the α -NH₂ protecting group, then coupling an activated amino acid to the newly freed α -NH₂. Activation of the -COOH group of the incoming amino acid can be effected directly via a carbodiimide, e.g. dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC), via formation of the symmetric acid anhydride, or preferably by formation of an "active ester", e.g. hydroxybenzotriazole (HOBT), pentafluorophenyl, para-nitrophenyl or N-hydroxysuccinimide esters. Suitable side-chain protecting groups generally are stable to all of the reagents, solvents and reaction conditions used during synthesis, yet removable under conditions which will not affect the integrity of the final peptide product.

The two preferred methods of solid phase peptide synthesis are the BOC and Fmoc methods, so called for their use of the tert-butyloxycarbonyl and 9-fluorenylmethyloxycarbonyl groups, respectively, to protect the α -NH₂ of the amino acid residues.

In the more established BOC method, the acid-lability of the BOC group is exploited and trifluoroacetic acid (TFA) treatment is used to effect its removal. The preferred amino acid side-chain protecting groups (for examples see Table 1 below) are relatively stable in weak acid, e.g. TFA. Most can be cleaved by very strong acids such as hydrofluoric (HF) or trifluoromethanesulfonic acid (TFMSA). A few side-chain protecting groups, e.g. His(Dnp) & Met(O), may require a separate deprotection step, e.g. thiophenol or ammonolysis, mercaptopyridine or mercaptoethanol treatment, respectively. After synthesis, the peptide is typically cleaved from the resin and simultaneously deprotected by HF treatment at low temperature, e.g. 0°C.

TABLE 1 - Examples of Side-Chain Protecting Groups Used in the BOC Method

<u>Residue</u>	<u>Side-Chain Moiety</u>	<u>Protecting Group</u>
Arginine	guanidino	p-toluenesulfonyl (Tos); methoxybenzenesulfonyl (Mts); nitro.
Aspartic Acid, Glutamic Acid	carboxyl	ortho-benzyl (OBzl)
Cysteine	sulfhydryl/thiol	p-methylbenzyl (CH ₃ Bzl)
Histidine	imidazole N-H	2,4-dinitrophenyl (Dnp); (Tos)
Lysine	amino	2-chlorobenzoyloxycarbonyl (Cl-Z)
Methionine	sulfide/thioether	sulfoxide (O); none
Serine, Threonine	hydroxy	benzyl (Bzl)
Tryptophan	indole N-H	formyl (CHO)
Tyrosine	hydroxy	2-bromobenzoyloxycarbonyl (Br-Z)

In the more recently developed FMOC method the base labile FMOC group is removed using a mild organic base, e.g. piperidine, thereby allowing the use of side-chain protecting groups which are labile to milder acid treatment, e.g. TFA (for examples see Table 2). An acid labile ether resin such as HMP-resin (para-hydroxymethylphenoxymethyl polystyrene) is used as the solid support, permitting simultaneous cleavage/deprotection in TFA.

Table 2 - Examples of Side-Chain Protecting Groups Used in the FMOC Method

<u>Residue</u>	<u>Side Chain Moiety</u>	<u>Protecting Group</u>
Arginine	guanidino	4-methoxy-2,3,6-trimethyl-benzenesulfonyl (Mtr); pentamethylchroman-6-sulfonyl
Aspartic Acid, Glutamic Acid	carboxyl	t-butyl ester (OtBu)
Cysteine	sulfhydryl/thiol	trityl (Trt); acetamidomethyl (Acm)
Histidine	imidazole N-H	Trt
Lysine	amino	t-butyloxycarbonyl (BOC)
Serine, Threonine, Tyrosine	hydroxyl	t-butyl (tBu)

Suitably protected and/or preactivated D- and/or L-amino acids, derivatized and/or preloaded resins, and all ancillary reagents and solvents required for either BOC or FMOC peptide synthesis are commercially available from several suppliers. In addition, automated peptide synthesizers with optimized, pre-programmed BOC and/or FMOC synthesis cycles are available from numerous commercial sources.

Incorporation of N- and/or C- protecting groups can also be achieved using protocols conventional to solid phase peptide synthesis methods. For incorporation of C-terminal protecting groups, for example, synthesis of the desired peptide is typically performed using, as solid phase, a supporting resin that has been chemically modified so that cleavage from the resin results in a peptide having the desired C-terminal protecting group. To provide peptides in which the C-terminus bears a primary amino protecting group, for instance, synthesis is performed using a p-methylbenzhydrylamine (MBHA) resin so that, when peptide synthesis is completed, treatment with hydrofluoric acid releases the desired C-terminally amidated peptide. Similarly, incorporation of an N-methylamine protecting group at the C-terminus is achieved using N-methylaminoethyl-derivatized DVB resin, which upon HF treatment releases peptide bearing an N-methylamidated C-terminus. Protection of the C-terminus by esterification can also be achieved using conventional procedures. This entails use of resin/blocking group combination that permits release of side-chain protected peptide from the resin, to allow for subsequent reaction with the desired alcohol, to form the ester function. FMOC protecting groups, in combination with DVB resin derivatized with methoxyalkoxybenzyl alcohol or equivalent linker, can be used for this purpose, with cleavage from the support being effected by TFA in dichloromethane. Esterification of the suitably activated carboxyl function e.g. with DCC, can

then proceed by addition of the desired alcohol, followed by deprotection and isolation of the esterified peptide product.

Incorporation of N-terminal protecting groups can be achieved while the synthesized peptide is still attached to the resin, for instance by treatment with suitable anhydride and nitrile. To incorporate an acetyl protecting group at the N-terminus, for instance, the resin-coupled peptide can be treated with 20% acetic anhydride in acetonitrile. The N-protected peptide product can then be cleaved from the resin, deprotected and subsequently isolated.

Once the desired peptide sequence has been synthesized, cleaved from the resin and fully deprotected, the peptide is then purified to ensure the recovery of a single oligopeptide having the selected amino acid sequence. Purification can be achieved using any of the standard approaches, which include reversed-phase high-pressure liquid chromatography (RP-HPLC) on alkylated silica columns, e.g. C₄-, C₈-, or C₁₈- silica. Such column fractionation is generally accomplished by running linear gradients, e.g. 0-50%, of increasing % organic solvent, e.g. acetonitrile, in aqueous buffer, usually containing a small amount of TFA, e.g. 0.1%. Alternatively, ion-exchange HPLC can be employed to separate peptide species on the basis of their charge characteristics. Column fractions are collected, and those containing peptide of the desired/required purity are pooled together. The peptide is typically then treated to exchange the cleavage acid (e.g. TFA) with a pharmaceutically acceptable acid, such as acetic, hydrochloric, phosphoric, maleic, tartaric, succinic and the like, to provide a water soluble salt of the peptide.

Following purification, it is desirable to analyze the oligopeptide further to ensure its chemical authenticity and purity. This is most conveniently achieved through amino acid composition analysis. To analyze amino acid composition, a sample of purified oligopeptide is completely hydrolysed in aqueous acid, e.g. HCl, and the resulting mixture of amino acids separated, identified and quantitated via HPLC, e.g. Waters Pico-Tag system, or automated analyzer, e.g. Beckman 6300 Amino Acid Analyzer. A more definitive measure of authenticity is full sequence analysis of the peptide. Several protein sequenators which sequentially degrade the peptide and identify the linear order of its amino acids are used for this purpose, and are available from several commercial sources. High-resolution mass spectrometry methods can also be applied, to generate exact molecular weight information.

For use in treating humans, the oligopeptide compounds of the invention are desirably of "pharmaceutical grade" purity, a term used herein with reference to an oligopeptide preparation that migrates as a single peak on HPLC, exhibits uniform and authentic amino acid composition and sequence upon analysis thereof, and otherwise meets standards set by the various national bodies which regulate quality of pharmaceutical products. It will be appreciated that strict standards of purity may not be required for use of the present compounds and compositions in the veterinary field.

The present invention provides, in another of its aspects, anti-herpetic compositions that comprise a physiologically tolerable carrier and an effective amount of an anti-herpetic compound of the invention. In this context, the term "effective amount" means an amount of the compound sufficient to cause a reduction in replication of the viral target. Such reduction is most properly revealed by assaying virus titer in serum samples derived from the

patient, before and after treatment.

For the treatment of humans infected with herpesvirus, compounds exhibiting pharmaceutical grade purity are combined with pharmaceutically acceptable carriers to generate compositions suitable for administration. Any of the carriers conventionally used in the pharmaceutical industry particularly for peptide-based drugs may be employed, such as diluents, excipients and the like. Reference may be made to "Remington's Pharmaceutical Sciences", 17th Ed., Mack Publishing Company, Easton, Penn., 1985 for guidance on drug formulations generally. According to one embodiment of the invention, the compounds are formulated for administration by injection, either subcutaneously or intravenously, and are accordingly provided as aqueous solutions in sterile and pyrogen-free form and optionally buffered or made isotonic. Thus, the compounds may be administered in distilled water or, more desirably, in saline or 5% dextrose solution. The compounds herein designated as preferred compounds are substantially water-soluble. Water solubility of these and other compounds of the invention may be enhanced, if desired, by incorporating a solubility enhancer, such as cetyltrimethylammonium bromide or chloride. Lyoprotectants, such as mannitol, sucrose or lactose and buffer systems, such as acetate, citrate and phosphate may also be included in the formulation, as may bulking agents such as serum albumin.

Alternatively, the compounds of the present invention may be formulated for administration by routes other than injection. Compositions for topical application, such as creams, lotions or ointments can be used, as may aerosol inhalable formulations. Oral dosage forms, such as tablets, capsules and the like, formulated in accordance with standard pharmaceutical practise, may also be employed. Cream, lotion and ointment formulations will be

useful particularly for application to virally-induced skin lesions. Appropriate triglyceride bases and gels can be used to prepare creams and ointments, and surfactants and antimicrobial agents may be incorporated, as is conventional.

The present invention provides, in another of its aspects, a method for treating a mammal, including a human, infected with a herpesvirus, which comprises the step of administering to the mammal a pharmaceutical composition comprising an effective amount of an anti-herpetic compound of the present invention. According to one embodiment of the invention, the method is applied for the purpose of treating a human patient. In a specific embodiment of the invention, the patient is one diagnosed as having a herpes simplex virus infection, especially an HSV-1 infection. In accordance with another embodiment of the present invention, the mammal is a cow, pig, sheep or other livestock infected with a herpesvirus infection. Suitable treatment regimens are those which maintain at the desired site e.g. in the infected tissue or at a localized skin surface, an amount of the compound sufficient to control herpesvirus replication. The precise dosage sizes appropriate for treatment can readily be established in appropriately controlled trials. It is anticipated that an effective treatment regimen for patients infected with herpesvirus will involve the systemic administration of dosage sizes in the range from 0.01mg to about 10mg per kg, e.g., between about 0.1mg/kg to about 5mg/kg. It will be appreciated however, that effective dosage sizes will vary according to the route of administration, and the frequency of administration. For example, topical formulations, which remain localized at the infected site, may be administered less frequently. Suitable topical formulations are expected to contain the anti-herpetic compound at a concentration of from about 0.1 to 50mg/ml, e.g. about 1-10mg/ml.

Example 1

Oligopeptides of series 1 noted below were synthesized using the solid phase peptide synthesis approach, and in accordance with protocols conventional thereto. More particularly, synthesis was performed on a Beckman 990 synthesizer, using chloromethyl-polystyrene as solid support, and Boc-based protocols and protecting groups, to generate the following compounds;

- 1A) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gly-Arg-Arg-Arg-Pro
- 1B) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Cys-Arg-Arg-Arg-Pro
- 1C) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Ser-Arg-Arg-Arg-Pro
- 1D) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-His-Arg-Arg-Arg-Pro
- 1E) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Lys-Arg-Arg-Arg-Pro
- 1F) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Asn-Arg-Arg-Arg-Pro
- 1G) Tyr-Gly-Arg-Lys-Lys-Arg-Arg-homoGln-Arg-Arg-Arg-Pro

Oligopeptides in series 2 as noted below were also synthesized by BOC chemistry and purified using conventional procedures, to yield the acetate salt of the following compounds:

- 2A) Arg-Gln-Arg-Arg-Arg-Arg-Arg-Arg-Arg
- 2A.5) Arg-Arg-Gln-Arg-Arg-Arg-Arg-Arg
- 2B) Arg-Arg-Arg-Gln-Arg-Arg-Arg-Arg
- 2B.5) Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg
- 2C) Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg
- 2D) Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gln-Arg

Synthesis and evaluation of an L-Arg nonamer was also undertaken. Nona-L-arginine, (L-Arg), was prepared by the BOC solid-phase synthesis method. Synthesis was performed by The American Peptide Company using a Beckman 990 synthesizer and chloromethylpolystyrene resin as solid support.

The tert-butyloxycarbonyl group (BOC) was used to protect the α -NH₂ function of L-arginine during the synthesis. The guanidino function was protected with the para-toluenesulfonyl group (Tos). Couplings were carried out using excess hydroxybenzotriazole (HOBt)-activated ester of BOC-L-Arg(Tos). Removal of the BOC protecting group after each cycle was effected with TFA. The final peptide, (L-Arg), was cleaved from the polymer resin and the Tos protecting groups removed via standard HF treatment. After removal of HF, the peptide + resin mixture was washed with diethyl ether and extracted with aqueous acetic acid.

The crude peptide was lyophilized, then fractionated by RP-HPLC on a C₁₈ silica column using a gradient of 2-40% acetonitrile in 0.1% TFA. Fractions were collected and checked by analytical RP-HPLC. Those containing $\geq 95\%$ of the major product were combined. High resolution mass spectrometry showed the product to be the expected L-(Arg),.

Example 2

A number of compounds containing D-amino acids were also synthesized for analysis, as outlined below:

4A) D-(Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)

The named D-peptide, designated 4A, is readily prepared by the Fmoc solid-phase synthesis method and an automated synthesizer, e.g. Applied Biosystems 430A. α -Amino groups of the D-amino acids are protected with the base-labile fluorenylmethyloxycarbonyl group (Fmoc). The lysine and arginine side-chains are protected via acid-labile protecting, e.g. BOC an methoxytrimethylbenzenesulfonyl (Mtr), respectively. The C-terminal Fmoc-D-Arg(Mtr) residue is double-coupled to a suitably derivatized polystyrene resin, e.g. HMP-polystyrene, via the symmetric anhydride. Removal of the Fmoc group is carried out in 20%

piperidine. Addition of amino acid residues to the peptide-resin is effected via their activated HOBt esters. Cleavage and deprotection of the final peptide is carried out by treatment with TFA. The crude peptide is purified by RP-or ion exchange HPLC. The purified product is characterized by standard amino acid analysis and/or mass spectrometry and/or sequence analysis.

4C) acetyl-[D-Arg],-NH₂

The title compound, designated compound 4C, was synthesized using p-methylbenzhydrylamine (MBHA) resin as solid support, to provide the C-terminal blocking amine on the resultant peptide. Synthesis proceeded using D-arginine residues in which the amino function was blocked with the t-BOC group, and the guanidino function was blocked with the Tos group. Coupling cycles and deprotection were performed as described for the L-Arg nonamer. When coupling cycles were completed, the resin-bound peptide was treated with 20% acetic anhydride in acetonitrile, to incorporate an acetyl protecting group at the N-terminus thereof. Liberation of peptide from the resin, and removal of Tos groups, were achieved by treatment with hydrofluoric acid, yielding the C-terminally amidated, title compound. After removal of hydrofluoric acid, the resin/peptide mixture was washed with diethyl ether and extracted with aqueous acetic acid. The crude peptide was lyophilized, and then purified by RP-HPLC fractionation as described for the L-Arg nonamer. High resolution mass spectrometry showed the product to be the desired compound.

Using synthesis protocols just described for synthesis of compound 4C, to incorporate an amidated C-terminus and an acetylated N-terminus, the following additional oligopeptides consisting essentially of D-amino acids were synthesized and purified for testing in the HIV inhibition

assay:

- 4G) acetyl-[D-(Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)]-NH₂;
- 4H) acetyl-(D-Arg)₃-(D-Gln)-(D-Arg)₅-NH₂;
- 4J) acetyl-[D-(Arg)₈]-NH₂
- 4K) acetyl-[D-(Arg)₇]-NH₂

Example 3 - Inhibition of HSV replication

Selected compounds were first formulated as 10 mM stocks in water for in vitro and cell culture procedures. The stocks are then diluted into buffers used for specific assays, or into cell culture media. For animal studies peptides are diluted into phosphate buffer saline.

The following procedures were then used to determine inhibitory effects of compounds on the replication of HSV. First, confluent monolayers of Vero cells (continuous passage cell line derived from African Green Monkeys) in 24 well cell culture plates were treated with specified concentrations of peptide for 25 hours. This was accomplished by diluting the stock peptide solution in growth medium used to overlay the monolayers, i.e. growth medium containing 10% fetal bovine serum and 10 ug/ml gentamicin in Dulbecco's MEM (DMEM).

After pretreatment with peptide, monolayers were overlaid with 0.1 ml log₁₀ dilutions of virus (HSV-1 F) ranging from 10⁻¹ to 10⁻⁶. Virus was then allowed to adsorb for 1 hour at 37°C. The virus inoculum was removed and the monolayers are overlaid with DMEM containing 2% FBS, 10 ug/ml gentamicin and specified concentration of peptide. Virus was next allowed to replicate for 2 to 3 days until the plaques were judged to be well developed, and then the monolayers were fixed and stained with a solution of 1% crystal violet in 1% formaldehyde, 70% ethanol. Finally, plaques (each representing a single viable virion) were

counted and checked microscopically.

Results of the plaque reduction assay performed with various peptides, expressed in terms of IC_{50} are tabulated below:

Compound	IC_{50} (μM)
[D-Arg] ₉	5
acetyl-[D-Arg] ₇ -NH ₂	slight
acetyl-[D-Arg] ₈ -NH ₂	20
acetyl-[D-Arg] ₉ -NH ₂	2
acetyl-[D-Arg] ₁₀ -NH ₂	2
acetyl-[D-Arg] ₁₁ -NH ₂	2
acetyl-[D-(RRRQRRRR)]-NH ₂	>50
acetyl-[D-(RKKRRQRR)]-NH ₂	>20
acetyl-[L-(FIAIA)]-[D-(RKKRRQRR)]-NH ₂	10

The results show that incubation of the cells with oligopeptide in a concentration from 2 to 50 μM induces significant reduction in the herpesvirus replication. There was no apparent inhibition of host cell replication at the concentrations tested. In separate experiments, a 100uM concentration of peptide 4C (the protected D-arginine nonamer) was found to have no significant detrimental effect on cell replication, although some reduction was noted at 500uM. This indicates that 4C can be formulated and used at therapeutic, non-toxic doses.

A representative formulation consists of 1.70mg of the selected compound, dissolved in neutral phosphate buffer (pH 6.5-7.5) and packaged in sterile-filled vials containing 17mg of compound in 10mL of solution (1.7mg/ml).

Example 4 - Peptide distribution within tissue

The distribution and localization of a representative compound,

N- α -[^{14}C -acetyl]-nona-D-arginine amide acetate (a radiolabelled form of compound 4C), was determined following administration by intravenous and sub-cutaneous injection. Ten mice were injected intravenously in the tail vein with 0.25mL of a solution of 26 μg of ^{14}C -labelled compound in 10mL PBS, and ten mice were given the same dose by subcutaneous injection in the abdomen. One mouse from each group was sacrificed at the time points noted in Figures 1 and 2, and the noted organs were weighed and digested to homogeneity for scintillation counting. Counts were measured and used to calculate the amount and concentration of drug in each organ. The results, plotted on a logarithmic scale, are illustrated in Figure 1 (i.v.) and Figure 2 (s.c.). It will be noted that both i.v. and s.c. injection bring about rapid distribution of drug to tissues. The highest and most prolonged levels are attained in the liver, followed by the kidneys and spleen.

Blood levels in mice resulting from single subcutaneous injection of a 53 μg dose of the labelled compound were also evaluated. It was revealed that near-peak blood levels around 1 $\mu\text{g}/\text{ml}$ were reached 5 minutes following injection. Peak levels were maintained for about 20 minutes. At 40 minutes and 2 hours, blood levels had declined to about 80% of peak values. A rapid decline in blood levels followed, so that the 4 hour level was about 10% of the peak, and blood levels further declined to the threshold of detection at 8 hours and 24 hours. Evidently, the compound 4C reaches the bloodstream rapidly. Near peak levels are maintained for 20-120 minutes, and the drug is then cleared rapidly from blood.

Example 5 - In vivo efficacy

The efficacy of a representative peptide, that designated 4C, was next evaluated *in vivo*. Mice received a one week pretreatment consisting of three foot-pad injections of peptide 4C in PBS at the doses noted in Figure 3. After pretreatment, the left footpad was inflamed by injecting about 5 μ L of 1M NaCl. After about 6 hours, the same footpad was abraded and a drop of HSV-1 strain F (2×10^3 pfu/ml) was then added deposited on the inflamed footpad. Treatment with the peptide 4C continued three times weekly until termination of the experiment. The results are shown in Figure 3, which illustrates clearly an anti-herpetic result particularly at the doses of 5 and 50 μ g/mouse.

WE CLAIM:

1. A pharmaceutical composition useful to treat herpesvirus infection, comprising a pharmaceutically acceptable carrier and, in an amount effective to treat said infection, a compound of the formula:



wherein

R1 is H or an N-terminal protecting group;

R2 is OH or a C-terminal protecting group;

X represents an anti-herpetic oligopeptide consisting of 'n' amino acids and having a net positive charge of $n \geq 2$, wherein n is an integer from 6 to 12;

y is 0 or 1;

z is 0 or 1; and

A and B each independently represent from 1 to 20 independently selected amino acids that retain the anti-herpetic activity of oligopeptide X.

2. A pharmaceutical composition according to claim 1, wherein said compound is one in which y and z are both zero.

3. A pharmaceutical composition according to claim 2, wherein said compound is one in which R1 is an N-terminal protecting group and R2 is a C-terminal protecting group.

4. A pharmaceutical composition according to any one of claims 1 to 3, wherein said compound is one in which X represents an oligopeptide comprising at least one D-amino acid.

5. A pharmaceutical composition according to any one of claims 1 to 3, wherein said compound is one in which X

represents an oligopeptide consisting of D-amino acids.

6. A pharmaceutical composition according to any one of claims 1 to 5, wherein said compound is one in which X is an oligopeptide selected from among the group consisting of (i) an oligopeptide consisting of from 7 to 9 basic amino acids and one amino acid other than a basic amino acid; and (ii) an oligopeptide consisting essentially of from 8 to 10 basic amino acids.

7. A pharmaceutical composition according to claim 6, wherein said compound is one in which X is an oligopeptide consisting of from 8 to 10 D-arginine residues.

8. A pharmaceutical composition according to claim 1, wherein said compound is selected from acetyl-[(D-Arg)₆]-NH₂; acetyl-(D-Arg)₃-(D-Gln)-(D-Arg)₅-NH₂; and acetyl-[D-(Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg)]-NH₂.

9. A pharmaceutical composition useful to inhibit herpesvirus replication according to claim 8, wherein said compound is acetyl-[(D-Arg)₆]-NH₂.

10. A pharmaceutical composition useful to inhibit herpesvirus replication according to any one of claims 1 to 9, in a form suitable for topical administration.

11. A pharmaceutical composition useful to inhibit herpesvirus replication according to claim 10, wherein said compound is acetyl-[(D-Arg)₆]-NH₂.

12. A pharmaceutical composition useful to inhibit herpesvirus replication according to any one of claims 1 to 9, in a form suitable for injection.

13. A pharmaceutical composition useful to inhibit

herpesvirus replication according to claim 12, wherein said compound is acetyl-[(D-Arg)_n]-NH₂.

14. A method for treating a mammal having a herpesvirus infection, which comprises the step of administering to said mammal a pharmaceutical composition as defined in any preceding claim.

15. A method for treating a mammal having a herpesvirus infection according to claim 14, wherein said composition is administered topically to a herpesvirus-induced skin lesion.

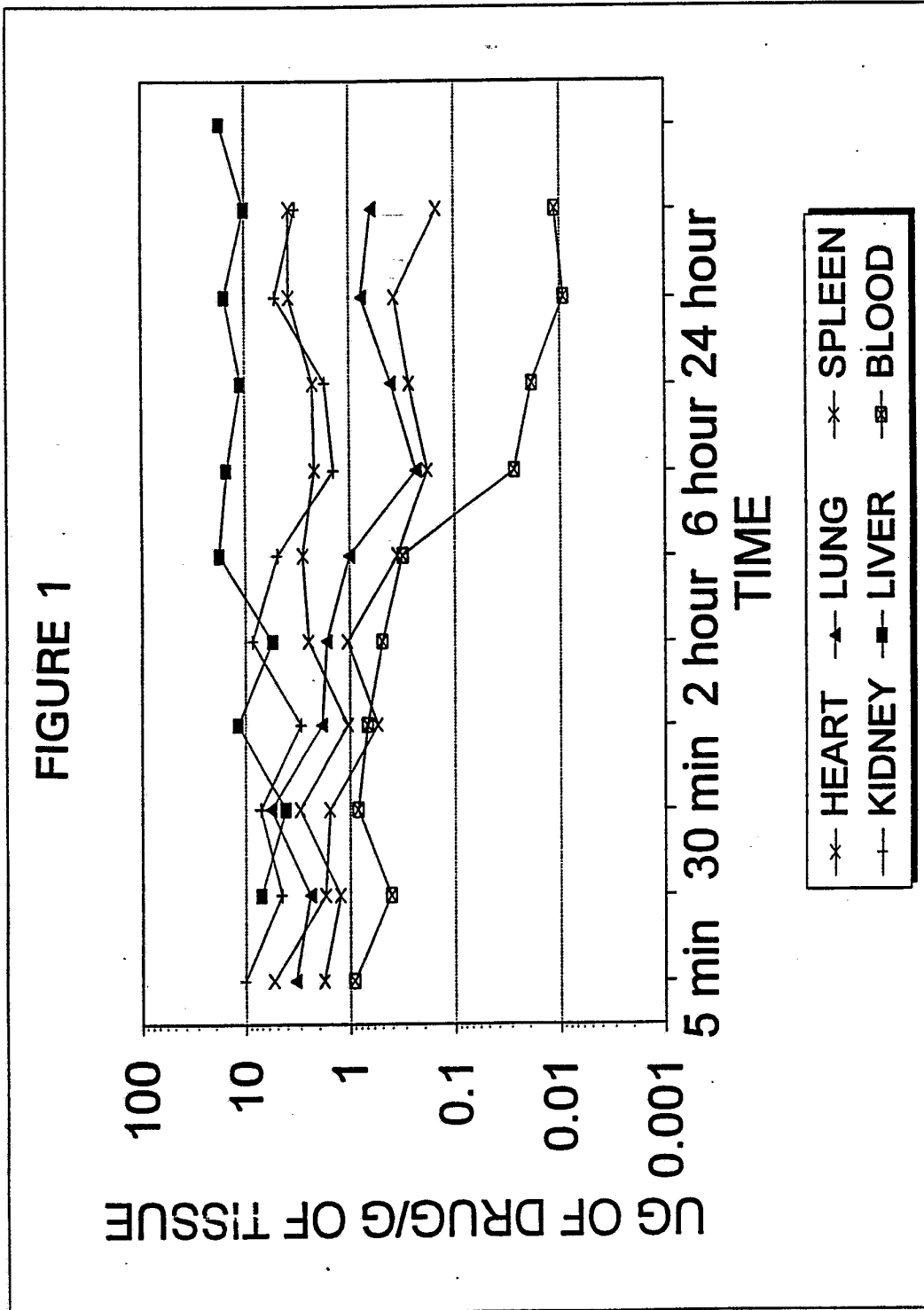
16. A method for treating a mammal having a herpesvirus infection according to claim 17, wherein said composition comprises the compound acetyl-[(D-Arg)_n]-NH₂.

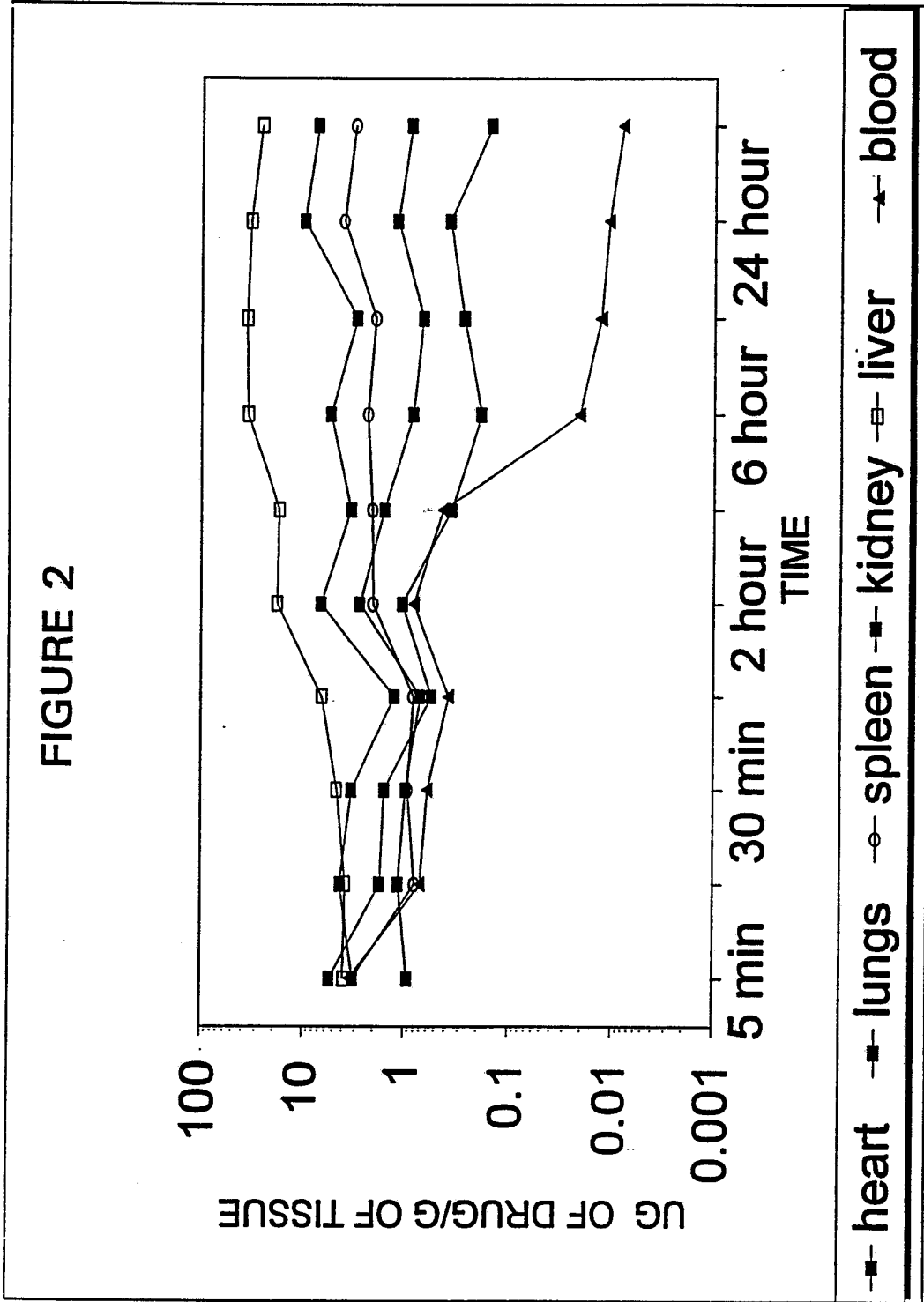
17. A method for treating a mammal having a herpesvirus infection according to claim 14, wherein said composition is administered topically to a herpesvirus-induced skin lesion and said composition comprises the compound acetyl-[(D-Arg)_n]-NH₂.

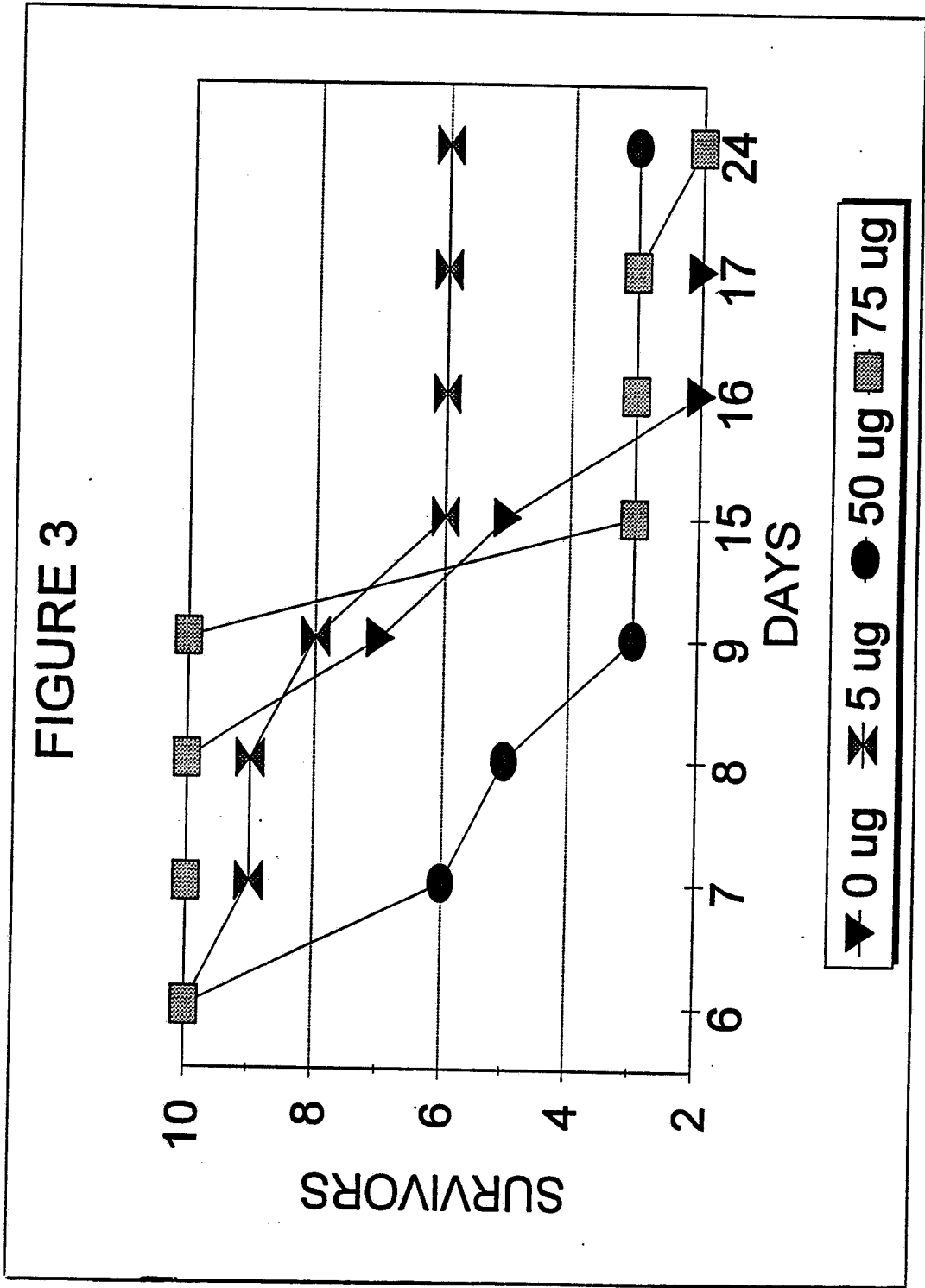
18. A method for treating a human having a herpes simplex virus infection, which comprises the step of administering thereto a composition comprising a physiologically tolerable carrier and an anti-herpetic amount of the compound acetyl-[(D-Arg)_n]-NH₂.

19. The method according to claim 18, wherein the herpes simplex virus infection is a herpes simplex virus type 1 infection.

20. The use of a composition according to any preceding claim for the treatment of herpesvirus infection.







INTERNATIONAL SEARCH REPORT

PCT/CA 93/00166

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K37/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	WO,A,9 207 871 (ALLELIX) 14 May 1992 * see claims 1-27, page 20 line 2 - page 21 line 18*	1-12
X	WO,A,8 912 461 (ST. LOUIS UNIVERSITY) 28 December 1989 * see claims 1-4, 9-11; page 5 line 4 - page 7 line 13, page 1 lines 17-20*	1, 12, 14, 15, 20
X	ANTIMICROBIAL AGENTS AND CHEMOTHERAPY vol. 31, no. 10, October 1987, pages 1562 - 1566 J.J. DOHERTY ET AL. 'Inactivation of herpes simplex virus types 1 and 2 by synthetic histidine peptides' * see the abstract, figure 2 *	1, 2, 12, 14, 20
<p>¹⁰ Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
03 AUGUST 1993	25 -08- 1993	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	B. ISERT	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9300166
SA 72886

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 03/08/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9207871	14-05-92	AU-A- 8725991	26-05-92
		CA-A- 2092075	25-04-92

WO-A-8912461	28-12-89	AU-A- 3756789	12-01-90

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82