



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 39/395, 37/50, C12N 11/02 C12N 9/96, 9/04	A1	(11) International Publication Number: WO 91/00108 (43) International Publication Date: 10 January 1991 (10.01.91)
(21) International Application Number: PCT/US90/01527 (22) International Filing Date: 21 March 1990 (21.03.90) (30) Priority data: 374,048 30 June 1989 (30.06.89) US (71) Applicant: BRUNSWICK CORPORATION [US/US]; One Brunswick Plaza, Skokie, IL 60077 (US). (72) Inventors: DUNCAN, John, D. ; 8208 Torrell Way, San Diego, CA 92126 (US). HOFMANN, Frieder, K. ; 2630 Autumn Drive, Oceanside, CA 92056 (US). (74) Agent: WALDRON, James, S.; Waldron & Associates, 2120 L Street, N.W., Suite 200, Washington, DC 20037 (US).		(81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent)*, DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (Eu- ropean patent), SE (European patent). Published <i>With international search report.</i> <i>With amended claims and statement.</i>
(54) Title: ANTIBODY-OXIDASE CONJUGATES WITH NON-SYSTEMIC SUBSTRATES (57) Abstract Enzyme-immunoconjugates which can be used for therapeutic purposes without significant patient side effects due to their administration. The general type of enzyme-immunoconjugate is composed of any antibody or fragment thereof linked to an oxidase whose natural substrate is not normally present in the body fluids or otherwise available in the extracellular spaces of the body. The therapeutic action of such oxidases lies in their ability to produce the cytotoxic yet biologically rapidly degrading agent hydrogen peroxide. Such oxidase-immunoconjugates would be specifically targeted to the desired tissues by use of the antibody component but would not be active until such time as a substrate of exogenous source is administered to the patient, whereupon hydrogen peroxide would be produced by the enzymatic process wherever the oxidase-immunoconjugate is present.		

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UNIQUEMENT A TITRE D'INFORMATION

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

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ANTIBODY-OXIDASE CONJUGATES WITH NON-SYSTEMIC
SUBSTRATES

TECHNICAL FIELD

The technical field of the present application relates to conjugates of antibodies and enzymes, linked together by linker molecules, which are designed for therapeutic purposes.

More specifically, it relates to therapeutic preparations of enzyme-immunoconjugates composed of any antibody or fragment thereof linked to an oxidase for which the natural substrate is not normally present in the body fluids or otherwise available in the extracellular spaces of the body.

BACKGROUND ART

It has long been recognized that therapeutic agents which specifically target invading organisms or diseased cells are highly desirable. Such specific targeting by the biologically active agent allows lower doses to be given and reduces the side effects created by non-specific action of the agent. Specific targeting has previously been restricted to drugs which act by exploitation of biochemical differences between the host and the disease organism. However, the development of techniques for the selection of specific hybridoma cell lines which produce specific monoclonal antibodies, and the subsequent technologies developed for mass production of such monoclonal antibodies, has brought the therapeutic use of targeted drugs in situations where the biochemical properties of the diseased cells are nearly identical to those of healthy cells to the threshold of reality.

Monoclonal antibodies specific for epitopes unique to certain types of cancer cells, T cell, B cells, and the like have been identified and are proposed for delivery of drugs directly and specifically to the target cells. Specificity, and thus amelioration of side effects, depends upon two factors: the specificity and tenacity of attachment to the target; and the effects of freely circulating (not attached) agent on non-target tissues in the body. These two factors are interrelated due to the fact that any binding agent, regardless of how strong the bond is, is in some state of equilibrium with the lymph

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and blood. Also, during administration, either intraperitoneally or intravenously, there will be a high concentration of freely circulating toxic agent until it is bound to the target cells or cleared by the liver and/or kidneys or the lymphocytes. This clearing process relieves the burden of freely circulating toxic agent, but also concentrates the toxic agent in the organs involved.

Various therapeutic approaches have been proposed involving targeted toxic agents. The glucose oxidase immunoconjugate, for example, has been described in the prior art, particularly in PCT Application No. PCT/GB86/00711, November 21, 1986, published June 4, 1987, Publication No. WO 87/03205, Starkie - inventor. The major problem of all the therapeutic approaches to date is the unspecific killing of non-target cells. The present invention proposes a solution to this problem.

DISCLOSURE OF INVENTION

The present invention relates to a novel approach to the problem of targeting toxic agents to cancer cells or other pathogenic cells without a significant degree of side effect on healthy tissue. The present invention involves a class of conjugates for therapeutic use which consist of an enzyme attached to a target cell binding protein. The enzyme can be any which produces a freely diffusible cytotoxic agent when positioned on the exterior of a target cell or organism. The target cell binding protein can be an antibody, either mono- or polyclonal, a fragment of an antibody, or any other molecule with specificity for a specific type of cell, e.g. a tumor cell, or organism. The enzyme can be attached either directly to the target cell binding protein or via a linker molecule designed to provide adequate spacing to prevent steric hindrance. The attachment of the enzyme to the target cell binding protein is preferably stable to all conditions of administration to a patient and to conditions present in the microenvironment at the site of action.

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The novelty of the present invention lies in the fact that each of the enzymes chosen acts on substrates which are not normal constituents of the environment of the site in the body in which it is expected to function nor through which it passes on its way to that site. Thus the
5 enzyme will be exposed to no substrate and produce no cytotoxic products while circulating in the body enroute to its target.

MODES FOR CARRYING OUT THE INVENTION

10 The conjugates of the present invention utilize an oxidase enzyme, attached to tumor-specific monoclonal antibodies or other target binding proteins, to produce hydrogen peroxide as the toxic agent. Hydrogen peroxide is a highly toxic compound capable of causing rapid cell
15 death through membrane disruption. Hydrogen peroxide is freely diffusible in aqueous solution and will rapidly diffuse to the surface of cells adjacent to the enzyme. In addition to being highly water-soluble, hydrogen peroxide also has lipophilic characteristics which give it
20 an affinity for cell membranes which is probably a significant factor in its high toxicity to cells. Hydrogen peroxide has an advantage in therapy over other cytotoxic agents as it is fairly unstable and will be decomposed to water and oxygen by erythrocytes or catalase
25 released from lysed target cells. Therefore, no side reactions from activated agents diffusing away from the generation site are expected. This is in sharp contrast to the pro-drug approach where a modified drug (pro-drug) is rendered active again by action of an enzyme bound to
30 an antibody. Even after cell death, the cell membrane bound enzyme will continue to convert pro-drugs to drugs.

One such conjugate is an ethanol oxidase conjugate. Ethanol oxidase is not found in mammals and its substrate, ethanol, is not normally present anywhere in the mammalian
35 body. However, even 0.1% ethanol concentration is sufficient to give good enzymatic turnover. The toxic product of the enzymatic reaction is hydrogen peroxide.

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Another conjugate is a galactose oxidase conjugate. Galactose oxidase is not found in mammals and its substrate, galactose, can easily be eliminated from normal circulation by normal metabolic pathways. Again, the
5 toxic agent produced is hydrogen peroxide.

Another conjugate is a D-amino acid oxidase conjugate. This enzyme and its substrates, the D-amino acids, are not found in mammals. The toxic agent produced is hydrogen peroxide.

10 Another conjugate is an α -glycerol phosphate oxidase conjugate. This enzyme is not found in mammals and its substrate, L- α -glycerol phosphate, is found only inside of cells, not outside them. This particular conjugate most likely functions by attachment to the exterior of a cell
15 acting upon exogenously added substrate, which does not cross cell membranes.

Attachment of the various enzymes to the target cell binding protein may be achieved by a variety of means. PCT Application No. PCT/GB86/00711, filed November 21,
20 1986, published June 4, 1987, Publication No. WO 87/03205, Starkie - inventor and European Patent No. 0 088 695, to Cytogen, both disclose such means. However, the preferred aspect of such attachment is that it is preferably stable to the physiological conditions present during
25 administration to a patient and transport to the site of action. In addition, the attachment is preferably stable to the conditions present at the site of action, particularly to the concentration of hydrogen peroxide present in the immediate vicinity when the enzyme is fully
30 functional. Covalent attachment of the enzyme to a linker molecule is preferable for such stability. Such linking reactions are preferably achieved at a position on the enzyme and under conditions which do not affect the function of the catalytic site of the enzyme and on the
35 antibody at a site which maintains the specificity and affinity of the antigen binding site. The linker molecule may be of a variety of types depending upon the specific sites of attachment to the enzyme and the antibody. The

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preferred features are that the bond formed is preferably stable to all conditions which the therapeutic agent encounters and that the linker molecule itself preferably does not have reactive groups which would result in its degradation *in vivo*. The order of reaction would be determined by the specific chemistries of bonding used and could either follow the sequence of attaching the linker to the enzyme and finally to the antibody, or attaching the linker to the antibody followed by attachment to the enzyme.

The enzyme-antibody ratio is preferably about 1:1. Excess enzyme is generally to be avoided.

An enzyme-linker-antibody conjugate meeting the requirements described above would produce hydrogen peroxide in close proximity to the targeted cell. In addition, the hydrogen peroxide produced would be free to diffuse to all adjacent cells. Given the known heterogeneity of tumor cells, this would enhance the effectiveness of such a conjugate as a therapeutic agent. Even highly efficacious, specifically targeted cytotoxic agents which act directly on cells have the shortcoming that they will kill only those cells to which they attach. Surrounding cells which may be neoplastic, but lack the specific epitope targeted, will not be killed. The only solution to this problem using this specific targeting technique is to create a bank of antibodies which will locate all tumor cells. This is a more complex solution and may not be possible when small tumor cell populations are involved. The present invention would solve the problem of tumor cell heterogeneity by killing all cell adjacent to targeted cells. Some normal cells may also be killed, but this would be a very minor side effect compared to thorough destruction of all tumor cells.

The conjugates are preferably targeted to sites which are not rapidly internalized, since this would not allow for killing of adjacent heterogenous tumor cells and would very likely result in inactivation of the oxidase. These conjugates, once attached on the exterior of the cells,

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preferably utilize substrates which are not normally found in the body fluids filling the extracellular spaces but can diffuse to the site of attachment of the conjugate. Such substrates can include molecules normally found
5 within cells, but not external to them, molecules not normally found in the body, or molecules found in the body *in vivo* but normally in low enough concentrations that no toxicity is produced by reaction with the oxidases.

A corollary to the requirement that the substrate be
10 not normally found external to cells in the body is that the specific enzyme itself preferably does not occur naturally in humans.

For each of the specific conjugates described previously, the enzyme portion of the conjugate would not
15 produce toxic agent (hydrogen peroxide) until desired. For therapeutic treatments involving these conjugates, the procedure would involve administering the conjugate into the blood, allowing ample time for the conjugate to seek and attach to its specific target and for excess or
20 unattached conjugate to be cleared from the general circulation by action primarily of the liver and kidneys. After such time, a dose of the enzyme's substrate would be injected into the blood at a concentration adequate for enzyme activity without side effects (if any) in the body
25 from the presence of the substrate.

Glycerol phosphate and galactose are normal metabolites and can be administered at a fairly high dose without showing side effects. Although ethanol is not a normal metabolite, it will be metabolized to fat and its
30 side reactions are generally regarded as pleasant. D-amino acids do not normally occur in humans. If at all, only a slight inhibitory reaction of the normal human enzymes which act on the L-amino acid form of the D-amino acid might be expected. This inhibitory reaction could
35 possibly be overcome by simultaneous administration of a higher dose of the equivalent L-amino acid.

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Treatment regimes can be determined based on the life of the enzyme conjugate, the concentration of the substrate, the reaction rate of the enzyme, and the like.

Since hydrogen peroxide is a general cytotoxic agent, meaning that it will kill all cell types, the generating enzyme could be linked to any target cell binding protein. Thus, an enzyme-linker conjugate could be prepared as a general agent for attachment to any antibody or antibody fragment of the desired specificity. Polyclonal antibodies to viruses or bacteria, for example, could also be attached in like manner. Specific enzyme-linker conjugates could also be prepared for attachment to other types of target cell binding proteins, such as hormones, growth factors, binding proteins of various types, and the like. It would therefore be possible to use the present invention for destroying a certain population of T cells or B cells in the body, for example, or to "purify" a culture of cells by killing a contaminating population having a specific affinity which can be exploited. Such conjugates, using a variety of linkers for various purposes is also an aspect of the present invention.

EXAMPLES

Example 1 - Preparation of Oxidase-Antibody Conjugates

Each of ethanol oxidase, galactose oxidase, D-amino acid oxidase, and α -glycerol oxidase is conjugated to monoclonal antibody 9.2.27 by the following procedure.

Thiolation of the Oxidase

To a solution containing 48 nmol of the oxidase in 0.75 ml of 10 mM triethanolamine, 0.2 M NaCl (pH 8.0) is added 7.5 μ l of 0.1 M 2-iminothiolane (in 0.5 M triethanolamine, pH 8.2). The reaction is run on ice under N₂ for two hours. The reaction mixture is gel filtered on a PD-10 column (Pharmacia) equilibrated in 0.1 M potassium phosphate, 1 mM EDTA (pH 7.3). The solution is kept on ice under N₂ and is reacted with SMCC derivatized monoclonal antibody as described below.

SMCC Derivatization of Antibody

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To 75 nmol (11.3 mg) of antibody 9.2.27 in 1 ml phosphate buffered saline (pH 7.0) is added 15 μ l of a 25 mM solution of SMCC (succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate). The reaction is allowed to proceed for one hour at room temperature. The mixture is centrifuged and then gel filtered on a PD-10 column equilibrated in 0.1 M potassium phosphate (pH 6.0).

Preparation of conjugate

To 34 nmol of the thiolated oxidase is added 34 nmol of the SMCC-derivatized monoclonal antibody. The reaction is allowed to proceed on ice under N₂ for 2 hours and then overnight at 4°C. The reaction is stopped by the addition of excess (700 nmol) of 2-mercaptoethanol.

The conjugate mixture is gel filtered on a PD-10 column equilibrated in 10 mM MES (pH 6.0). The void volume eluate is centrifuged and applied to a column (4 x 1 cm) of S-Sepharose equilibrated in 10 mM MES (pH 6.0). The column is washed with equilibration buffer and the conjugate is eluted with an appropriate eluent depending on the oxidase, e.g. 10 mM MES, 0.2 M NaCl (pH 6.0). If necessary, to the conjugate eluate is added 200 μ l of a 1 mM solution of cofactor for the particular oxidase in the conjugate.

The conjugate is further purified by chromatography on a column (60 x 2.6 cm) of Sephacryl S-300 HR equilibrated in 0.1 M potassium phosphate, 0.05 M NaCl (pH 7.0). Those fractions which contained 1:1 conjugate are pooled and concentrated by ultrafiltration. Concentration and activity will be different for each conjugate.

After purification, each conjugate is assayed and found to be a 1:1 adduct of the enzyme and antibody.

Example 2

Each of the conjugates prepared in Example 1 is evaluated for *in vitro* cytotoxic activity using the following procedure.

10⁴ M21-UCLA melanoma cells per well are allowed to grow in 96-well plates in a 10% CO₂ atmosphere at 37°C in

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100 μ l of RPMI 1640, 10% FBS medium. After 24 hours, the nutrient is taken off and replaced by 100 μ l of RPMI 1640, free of the substrate for the conjugate oxidase, containing varying concentrations of the immunoconjugate and a 10-fold activity excess of catalase. Catalase is an enzyme which acts to break down hydrogen peroxide into oxygen and water and serves to prevent cytotoxic effects during the binding step. The immunoconjugates are formed using enzyme and 9.2.27 monoclonal antibody, as indicated in Example 1. The immunoconjugate and catalase containing nutrient is removed 30 minutes later and the wells are rinsed three times with the original medium and the cells are allowed to continue to grow in the original medium, to which 2mM of substrate for the conjugate oxidase is added. After 24 hours, 10 μ l of 1 μ Ci ^{3} H-thymidine containing medium is added in order to measure thymidine uptake. Thymidine is incorporated into DNA and thymidine uptake is used to measure DNA synthesis which relates to cell viability. After another day of growth the plates are shock frozen, then thawed and the individual well contents passed through glass fiber filters. The radioactivity is determined and taken as a measure of cell viability.

The results of the *in vitro* assay show a high level of cytotoxicity activity for each conjugate.

25 Example 3

Each of the conjugates prepared in Example 1 is evaluated for *in vivo* binding specificity and affinity by the following procedure.

Thymus deficient BALBc (nude/nude) mice are subcutaneously injected with 2×10^6 M21-UCLA melanoma cells. After two weeks, 3 μ Ci of I^{125} iodinated immunoconjugate is injected into the tail vein. After 48 hours the animals are sacrificed and the radioactivity in individual organs is determined. The immunoconjugates are formed using enzyme and 9.2.27 monoclonal antibody following the procedures discussed in Example 1.

The *in vivo* biodistribution data obtained with tumor bearing nude mice also shows that each conjugate has a

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high degree of binding specificity and affinity. Further, the data indicates that for each conjugate, any unbound conjugate is cleared from the body as indicated by the low levels of conjugate found in the blood, liver, kidney, spleen and intestine.

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CLAIMS

What is claimed is:

1. A composition for producing localized cytotoxic effects on targeted cells comprising:

5 a conjugate of oxidase bound to a target cell binding protein,

said conjugate substantially retaining the binding specificity and binding affinity characteristics of said target cell binding protein and at least about 50% of the catalytic activity of said oxidase, and

10 said oxidase having a substrate which is not normally present in the surroundings of said targeted cells.

2. The composition of claim 1, wherein said oxidase is a member of the group consisting of ethanol oxidase, galactose oxidase, D-amino acid oxidase, and α -glyceryl phosphate oxidase.

3. The composition of claim 1, wherein said target cell binding protein is a member of the group consisting of monoclonal antibodies, polyclonal antibodies, antibody fragments, hormones, growth factors, and binding proteins.

4. The composition of claim 1, wherein said target cell binding protein is a monoclonal antibody.

5. The composition of claim 1, wherein said target cell binding protein is a fragment of a monoclonal antibody.

6. The composition of claim 1, wherein said oxidase is bound to said target cell binding protein via a linker molecule.

7. A method of producing localized cytotoxicity on targeted cells which comprises:

30 combining an effective amount of an agent for producing localized cytotoxic effects on targeted cells and a pharmaceutically acceptable carrier therefor, said agent comprising

35 a conjugate of oxidase bound to a target cell binding protein,

said conjugate having substantially the binding specificity and binding affinity characteristics of

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said target cell binding protein and the catalytic activity of said oxidase,

5 said oxidase having a substrate which is not normally present or otherwise available in the extracellular spaces of said target cells,

 said oxidase catalyzing a reaction, which results in the formation of hydrogen peroxide in the vicinity of said targeted cells,

10 said hydrogen peroxide causing the destruction of said targeted cells and cells in the vicinity of said targeted cells,

 administering said combined agent and vehicle;

 allowing sufficient time for said target cell binding protein of said agent to seek and bind to its target cell;

15 permitting or causing unbound amounts of said agent to be cleared; and thereafter

 administering a dose of said oxidase substrate which is not normally present or otherwise available in the extracellular spaces of said target cells in an amount
20 sufficient to produce cytotoxic concentrations of hydrogen peroxide adjacent said target cells.

8. The method of claim 7, wherein said targeted cells are hosted in a mammalian host.

9. The method of claim 7, wherein said agent is
25 administered by a technique selected from the group consisting of topical administration, intravenous administration, subcutaneous administration and intraperitoneal administration.

30 10. The method of claim 8, wherein unbound amounts of said agent are cleared by a mammalian host.

11. The method of claim 7, wherein said targeted cells are in a cell culture in vitro.

35 12. The method of claim 7, wherein said dose of said substrate is an effective concentration adequate for oxidase activity while limiting side effects from the presence of said substrate.

AMENDED CLAIMS

[received by the International Bureau on 29 October 1990 (29.10.90);
original claims 3 and 7 amended; other claims unchanged (3 pages)]

1. A composition for producing localized cytotoxic effects on targeted cells comprising:

5 a conjugate of oxidase bound to a target cell binding protein,

said conjugate substantially retaining the binding specificity and binding affinity characteristics of said target cell binding protein and at least about 50% of the catalytic activity of said oxidase, and

10 said oxidase having a substrate which is not normally present in the surroundings of said targeted cells.

2. The composition of claim 1, wherein said oxidase is a member of the group consisting of ethanol oxidase, galactose oxidase, D-amino acid oxidase, and α -glyceryl phosphate oxidase.

3. The composition of claim 1, wherein said target cell binding protein is a member of the group consisting of monoclonal antibodies, polyclonal antibodies, antibody fragments, hormones, and growth factors, and binding proteins.

4. The composition of claim 1, wherein said target cell binding protein is a monoclonal antibody.

5. The composition of claim 1, wherein said target cell binding protein is a fragment of a monoclonal antibody.

6. The composition of claim 1, wherein said oxidase is bound to said target cell binding protein via a linker molecule.

30 7. A method of producing localized cytotoxicity on targeted cells which compromises:

combining an effective amount of an agent for producing localized cytotoxic effects on targeted cells and a pharmaceutically acceptable carrier therefor, said agent compromising

35 a conjugate of oxidase bound to a targeted cell binding protein,

said conjugate having substantially the binding specificity and binding affinity characteristics of said targeted cell binding protein and at least about 50% of the catalytic activity of said oxidase,

5 said oxidase having a substrate which is not normally present or otherwise available in the extracellular spaces of said targeted cells,

 said oxidase catalyzing a reaction, which results in the formation of hydrogen peroxide in the
10 vicinity of said targeted cells,

 said hydrogen peroxide causing the destruction of said targeted cells and cells in the vicinity of said targeted cells,

 administering said combined agent and vehicle;

15 allowing sufficient time for said targeted cell binding protein of said agent to seek and bind to its targeted cell;

 permitting or causing unbound amounts of said agent to be cleared; and thereafter

20 administering a dose of said oxidase substrate which is not normally present or otherwise available in the extracellular spaces of said targeted cells in an amount sufficient to produce cytotoxic concentrations of hydrogen peroxide adjacent said targeted cells.

25 8. The method of claim 7, wherein said targeted cells are hosted in a mammalian host.

 9. The method of claim 7, wherein said agent is administered by a technique selected from the group consisting of topical administration, intravenous
30 administration, subcutaneous administration and intraperitoneal administration.

 10. The method of claim 8, wherein unbound amounts of said agent are cleared by a mammalian host.

35 11. The method of claim 7, wherein said targeted cells are in a cell culture in vitro.

 12. The method of claim 7, wherein said dose of said substrate is an effective concentration adequate for

oxidase activity while limiting side effects from the presence of said substrate.

STATEMENT UNDER ARTICLE 19

The claims 3 and 7, as amended, are now in conformity with the claims of corresponding U.S. Application Serial No.374,048, and are believed to be novel and involve inventive steps not shown in the references cited in the International Search Report.

It is respectfully submitted that the claims as amended in the present application are clearly patentable over Jansen, et al., Starkie, and Plotkin, et al.

A favorable examination report is accordingly solicited.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US90/01527

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5) : A61K 39/395, 37/50; C12N 11/02, 9/96, 9/04 U.S. Cl : 424/85.91, 943; 435/177, 188, 189, 190, 191, 192, 7		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
424/85.91, 94.3; 435/177, 188, 189, 190, 191, 192, 7		
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
CAS ONLINE, BIOSIS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US,A 4,762,707 (JANSEN ET AL) Issued 09 August 1988 See entire document.	1-12
Y	WO,A 87/03205 (STARKIE) Issued 04 June 1987 See entire document.	1-12
Y	US,A 4,496,539 (PLOTKIN ET AL) Issued 29 January 1985 See entire document.	1-12
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IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
01 August 1990	04 OCT 1990	
International Searching Authority ¹	Signature of Authorized Officer ¹⁰	
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