(51) International Patent Classification A61K 31/40

(11) International Publication Number: WO 93/11763

(41) International Publication Date: 24 June 1993 (24.06.93)

(21) International Application Number: PCT/CA92/00526

(22) International Filing Date: 8 December 1992 (08.12.92)

(30) Priority data:
2,057,289 9 December 1991 (09.12.91) CA
07/939,317 2 September 1992 (02.09.92) US

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(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF CYTOCHALASINS FOR INHIBITING VIRAL REPLICATION

(57) Abstract

The invention provides for both a pharmaceutical preparation and a method for inhibiting in vivo the replication of viruses. In one embodiment the pharmaceutical preparation is a complex of dextran and cytochalasin-D which may be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from a viral disease such as AIDS.
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USE OF CYTOCHALASINS FOR INHIBITING VIRAL REPLICATION

This invention relates to a pharmaceutical preparation and method for inhibiting replication of a family of viruses known as human immunodeficiency virus HTLV-III (AIDS) virus.

BACKGROUND TO THE INVENTION

Certain retrovirus infections have been known to depress immune functions in animals. In recent years, it has been discovered that a family of T-lymphotropic retroviruses causes T-cell proliferation leukemia, helper T-cell depletion, and immunosuppression in humans infected by these viruses. These viruses have become known as the HTLV family of retroviruses. A group of these viruses designated as HTLV-III has been isolated from patients with Acquired Immune Deficiency Syndrome (AIDS) and has become considered to be responsible for the development of this condition in humans. These are also known as HIV, particularly HIV-1 and HIV-2.

A cell line representative of this group has been deposited under ATCC No. CRL 8543 by an agency of the U.S. Department of Health and Human Services.

Since the epidemic was first recognized in 1981, rapidly increasing numbers of cases of AIDS have been diagnosed in the United States. Significant progress has resulted from research in selected areas including identification of the populations at risk for AIDS, the methods of transmission of the causative agent, isolation and characterization of the virus that causes AIDS and development of a serologic test that identifies most infected individuals.

On the other hand, little progress has been made toward effective treatment of AIDS. Azidothymidine (AZT), a drug that inhibits reverse transcriptase prolongs the lives of patients with AIDS and many patients who receive AZT have temporary
increases in the numbers of circulating helper (CD4\(^+\)) T-lymphocytes. However, the drug has significant adverse effects and HIV has been isolated from the blood of patients even while they are under treatment with AZT.

**SUMMARY OF THE INVENTION**

The invention provides for a pharmaceutical preparation for inhibiting in vivo the replication of viruses which comprises a complex of dextran-cytochalasin D in an appropriate pharmaceutical dosage form. The invention also provides a method for inhibiting in vivo the replication of HIV viruses comprising the administration of a dextran-cytochalasin D complex in a pharmaceutically appropriate dosage form and quantity.

**BRIEF DESCRIPTION OF THE DRAWINGS**

In the figures which disclose example embodiments of the invention,

- figure 1 is a graph indicating the anti-HIV effects of cytochalasin-D,
- figure 2 is a schematic diagram of a cell with a dextran sulphate-cytochalasin-D complex,
- figure 3 comprises graphs of FPLC and HPLC of dextran and dextran-dialdehyde,
- figure 4 comprises graphs of FPLC and HPLC of dextran sulphate and dextran sulphate-dialdehyde,
- figure 5 comprises graphs of FPLC and HPLC of a coupling sample, and
- figure 6 is a graph of a complex of dextran and cytochalasin-D in different coupling times.

**DESCRIPTION OF THE INVENTION**

It is clear that the first step in the infection of human T-lymphocytes by human immunodeficiency virus Type 1 (HIV-
1) is attachment to the target cell receptor, the CD4 antidote. The cell-to-cell transmission of HIV represents a major obstacle in the development of effective anti-viral therapy. This method of provirus spread bypasses the requirements of reverse transcription, thereby rendering inhibitors of reverse transcriptase, such as the nucleoside analogs, inactive. The level of CD4+ T-cell depletion and the amount of HIV production could be reduced if the cell-to-cell spread of HIV was blocked by inhibiting conjugate formation.

Dextran sulphate has now been shown to inhibit syncytia formation and exert a potent inhibitor effect against HIV-1. The cytochalasins, especially cytochalasin-D, have now been shown to possess anti-HIV effects. The cytochalasins are a class of mold metabolites which have now been shown to exhibit inhibitory effects on cell processes. Six cytochalasins have been isolated. Major biological effects are (1) blockage of cytoplasmic cleavage resulting in multinucleate cell formation; (2) inhibition of cell movement; (3) induction of nuclear extrusion; (4) inhibition of glucose transport, thyroid secretion, growth hormone release, phagocytosis, as well as platelet aggregation and clot contraction.

On the cytochalasins, cytochalasin D (C₃₀ H₃₇ NO₅) a polycyclic hydroxylated hydrocarbon appears to be the preferred compound as regards its cytotoxicity by inhibition of cell processes.

Although cytochalasin-D has previously been recognized to have some biological effects, its application in clinical treatment has not been developed probably due to its high toxicity and metabolic instability. The inventors herein have now discovered that cytochalasin-D may be used in vivo to inhibit replication of viruses. Cytochalasin-D at non-cytoxic concentrations using a 4-day syncytium inhibition assay, infection of MT-2 cells with cell free HIV-1 (MOI = 0.001) followed by immediate exposure to cytochalasin D at non-cytotoxic concentrations (< 1 ug/ml), resulted in a potent anti-HIV effect (effective dose 50% = 10 ng/ml: Fig. 1). In contrast, when cell
lines that grow as single cells, such as CEM or SUPTI, were used as targets in the infectivity assays, no antiviral activity was detected when using HIV p24 antigen-positive cell counts or cell death as end-points of viral expressions.

The anti-HIV effects of cytochalasin, especially cytochalasin-D may be noted from Figure 1. MT-2 cells were infected with HIV-1 at a multiplicity of infection of 0.001 for 1 h followed by exposure to cytochalasin-D. On day 4 of the culture, coinciding with the peak of cytopathic effects, the number of syncytium forming units (SFU) and HIV p24-positive cells (indirect immunofluorescence) was determined and compared to controls. In Figure 1, 0 represents the number of HIV infected cells: [] represents the percentage inhibition of HIV induced syncytium formation. Infected cultures without drug exposure had 74 +/- 3 SFU and 72 +/- 5% of the cells had staining for intracellular HIV p24 antigen (> 30 cells/slide were counted). Uninfected controls had no SFU and < 1% cellular staining. Results represent the mean of triplicate determinations from six sets of experiments. Cytochalasins, other than cytochalasin-D are likely to exhibit similarly useful properties.

It has been found by Dr. Lionel Resnick of the Mount Sinai Medical Center in Miami, that the transmission of HIV requires the interaction of the cell-surface CD4 receptor and the viral envelope glycoprotein. Experiments were performed to determine the role of other cell-surface molecules in the development of HIV-induced syncytia. Although CEM and MT-2 cells had similar cell-surface CD4 receptor densities, < 1% of CEM cells and > 95% of MT-2 cells formed syncytia with H9 cells chronically infected with HIV-1 (H9-111B). When compared with CEM cells, MT-2 cells exhibited a 10 fold and threefold greater capacity to form homotypic and heterotypic conjugates with H9 cells, respectively. Increasing the conjugate formation capacity of CEM cells with the lectin wheat germ agglutinin led to a > 30 fold increase in the formation of syncytia with H9/111B cells. The formation of syncytia between MT-2 and H9/111B cells was
magnesium, energy, temperature, and actin-cytoskeleton dependent, and could be inhibited (65%) by an anti-LP-1 monoclonal antibody. The combination of anti-leucocyte function associated antigen-1 (LFA-1) and anti-CD2 monoclonal antibodies resulted in a synergistic inhibition (89%) of syncytium formation. These results indicate that integrins and other cell-surface adhesion molecules regulate HIV-induced syncytium formation.

The HIV envelope glycoprotein contains fusogenic domains that are important for virus infection and syncytium formation.

Experiments using site directed mutagenesis have revealed that the NH2 terminus of the transmembrane HIV envelope glycoprotein (gp41) is essential for the fusion which occurs after the binding of the external envelope glycoprotein (gp120) to the cell surface CD4 receptor. Although these events are required for virus to cell and cell to cell fusion, the fusion of HIV infected to uninfected cells may involve the interaction of other cellular components, as seen with ortho and paramyxoviruses.

The initial event in cell to cell interactions usually involves the formation of cellular conjugates. The integrins are a family of cell-surface structures that mediate conjugate formation and facilitate the development of specific cellular interactions, such as cell mediated cytolysis and antigen presentation. Leucocyte function associated antigen-1 (LFA-1), an integrin molecule that mediates cell to cell binding, has been reported to have a role in HIV induced syncytium formation. The capacity to form syncytia may not, therefore, be solely dependent on the density of CD4 receptors of HIV envelope glycoprotein expressed on cell surfaces. In these studies, experiments were performed to determine the role of cell surface molecules, other than CD4 and the HIV envelope glycoprotein, in the development of HIV induced syncytium formation.

These results indicate that the interpretation of HIV biologic phenotypic data from human cell based \textit{in vitro} assay systems should take into account the host cell potential for
conjugate formation. For example, antiviral evaluations should use cells with different capacities for conjugate formation. The importance of comparing the anti-viral activity of drugs by different in vitro methods is supported by the results with cytochalasin, especially cytochalasin-D, an agent which specifically inhibits the cell to cell spread of HIV. Cytochalasin, especially cytochalasin-D was a potent inhibitor of HIV induced MT-2 syncytium formation but had no effect in preventing the cell free transmission of HIV in CEM or SUPTI cells.

Excluding the HIV infected microglial multinucleated giant cells of the brain, the significance of in vivo syncytium formation is suspect. However, the cell to cell transmission of HIV is likely to be a major contributor to virus spread and CD4+ T-cell depletion within the host. Factors that lead to an increase in the level of expression of adhesion molecules would be expected to favour the cell to cell spread of HIV. For example, opportunistic infections can function as cofactors in the progression of HIV disease by their effect on the activation of T-lymphocytes, leading to an up regulation in the expression of LFA-1 on cell surfaces. Furthermore, the coinfection of HTLV and HIV retroviruses accelerates the progression of HIV disease. The common feature of HTLV-1 transformed cell lines (MT-2 and C-8166) is their enhanced expression of integrins and conjugate formation. This results in a mechanism whereby the coinfection of HTLV and HIV retroviruses favours the cell to cell spread of HIV.

Our invention is that we have discovered that a specifically prepared (1) cytochalasin, especially cytochalasin-D, (2) a complex of cytochalasin, especially cytochalasin-D and dextran 40,000 molecular weight (blood plasma extender FDA approved), (3) a complex of cytochalasin, especially cytochalasin-D and dextran sulphate (usherdex 8,000 molecular weight) are all effective and relatively nontoxic for the treatment in vivo of various viruses including the HTLV-III AIDS virus. Dextrans of any molecular weight may be used in the
preparation of a dextran-cytochalasin-D complex having therapeutic value. Their efficacy in the treatment of the former virus will apply to other viruses such as herpes, chicken pox, influenza, etc. Having demonstrated on mammals the lack of toxicity, by intravenous injection, any of these products would be effective when used for oral, nasal, inter-or intradermal patches for the diffusion of drugs, or intramuscular application. The usefulness of the instant invention includes topical application for the treatment of ulcers and viruses including genital ulcers and viruses, and mouth ulcers. The invention is also useful in the treatment of Kaposisarchoma and other tumours. The invention promotes inactivation of such tumours. Similar polysaccharides having the property of slow release mechanism such as dextrin, cyclodextrin etc. may also be as effective as those already above mentioned.

By complexing or coupling cytochalasin-D with dextran, dextran sulphate or other suitable polysaccharides the cytotoxicity of the cytochalasin-D to the host is reduced due to its slow release from the complex. As a result, not only will the complex of dextran and cytochalasin-D be useful in inhibiting virus replication in vivo, but in addition such a complex will also be useful in the treatment (in vivo) of a variety of different types of cancers.

These skilled in the art will be aware of pharmaceutically appropriate dosage forms for the complex of dextran and cytochalasin-D or dextran sulphate and cytochalasin-D, as well as the manner in which a suitable dosage quantity and regimen may be derived in respect of a particular patient.

The demonstrated absorption qualities of dextran sulphate or dextran when complexed with cytochalasin, especially cytochalasin-D, will bring this complex into the endothelium system with a consequently improved effect in the treatment of viruses.

As cytochalasin-D couples with dextran, the following advantages will be exhibited.

(1) Dextran-cytochalasin-D as a macromolecular
compound has excellent metabolic stability, resulting in a more effective treatment.

(2) Because the complex is a large molecule and consists of polysaccharide, it is easy to be received and combined by receptors of the cell which consist of polysaccharide-protein.

(3) Dextran is useful not only as a "transfer weapon", but also as an immunologically active material which has now been used in the treatment study of AIDS.

(4) The complex provides an effective weapon which will be able to attack and kill the harmful cells of several severe diseases including AIDS and carcinoma.

This is in contrast to cytochalasin-D which is insoluble in water, has a high toxicity and is not easily received by receptors of cells. Figure 2 illustrates the effect of dextran sulphate-cytochalasin-D complex 10 on an abnormal cell 12, having a receptor 14.

Modification of the dextran sulphate molecule for coupling may be achieved as follows.

(1) Principle

Because the cytochalasin-D molecule contains a -NH group, it suggests that coupling will be possible if dextran is modified to dextran dialdehyde. The principle of coupling of dextran and cytochalasin-D by dialdehyde may be represented by the following formula:
(2) Method

i. 1 ml of 12% sodium periodate was added to 10 ml of 10% dextran solution, then left the mixture overnight in the dark at 4°C.

ii. A 3% solution of sodium bisulfite was added until the mixture turned brown and then, once again colourless.

iii. The mixture was dialysed against distilled water for more than 24 hours.

iv. Concentrate the dextran-dialdehyde solution to a proper volume.
(3) Quality Control

Figure 3 illustrates the FPLC and HPLC of dextran and dextran-dialdehyde and figure 4 illustrates the FPLC and HPLC of dextran sulphate and dextran sulphate-dialdehyde.

Coupling of dextran and cytochalasin-D

(1) Method

i. Make a certain concentration of cytochalasin-D in 0.3 M sodium Bicarbonate buffer, pH 9.5 depending on the purpose.

ii. Take one volume of dextran-dialdehyde solution and two volume of cytochalasin-D solution for coupling overnight at 4°C.

(2) Experiments

i. Primary Study on Coupling

Procedure: 5 mg of cytochalasin-D as dissolved in 0.4 ml of dextran-dialdehyde solution which contains about 5 mg of dextran then add 0.2 ml of distilled water and 7.0 mg NaHCO3 with good mixing overnight at 4°C (total volume = 0.6 ml).

Result: Take 2.0 ml of coupling sample for injection of FPLC the result is shown in Figure 5. Then collect peak A and B portions for HPLC (see figure 5).

ii. Study on the relationship between formation of the complex and coupling time.

Procedure: 2.5 ml of dextran-dialdehyde solution (contains 850 mg of dextran) was added in 5.0 ml of 0.1% of cytochalasin-D solution (5 mg in 5 ml of 0.3 M of NaHCO3 pH 9.5) that means: dextran : cytochalasin-D = 350 mg : 5 mg.

Coupling was performed at 4°C and samples were taken at 4 hours, 9 hours and 24 hours (for result: see Figure 6).

The results show that the amount of complex of dextran-cytochalasin-D increases with coupling time. The area rate of A to G is increased from 0.55 at 4 hours to 0.96 at 24 hours.

Figure 6 shows the FPLC of complex of dextran and cytochalasin-D in different coupling times.

iii. Chemical coupling of cytochalasin-D to dextran and activation of dextran and coupling to E-Aminocaproic Acid.

Dextran (200 mg) in IM sodium carbonate (20 ml) was
treated with cyanogen bromide (0.4 ml from 2 g CNBr in 1 ml of acetonitrile) and mixed vigorously for 1 min. at room temperature.

E-Aminocaproic acid (200 mg) was added and the solution stirred with a magnetic bar for 24 hr at room temperature. The product was dialysed against three changes (12 hours each) of 0.1 M sodium carbonate.

To the dialysed product was added cytochalasin D (10 mg) dissolved in ethanol (1.5 ml) or dimethyl sulfoxide (1.5 ml). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (400 mg) was added with vigorous stirring. The pH was adjusted to 6.0 by addition of 2N hydrochloric acid and stirring was continued for 24 hr. The product was dialysed exhaustively against water and freeze-dried.

Two sets of experimental conditions were compared.
1. The dextran had M.W. 10,000 and in the coupling with cytochalasin D, the solvent was ethanol. In addition, [\(^{3}\)H]cytochalasin B (2.5 uCi) was present as a marker to permit estimation of the extent of coupling by measurement of \(^{3}\)H in the product.

The recovery of dextran was quantitative. The recovery of \(^{3}\)H indicated that 3.0% (0.30 mg) of the cytochalasin had been chemically coupled to the dextran.

2. The dextran had M.W. 40,000 and the solvent was dimethyl sulfoxide. The recovery of dextran was quantitative. The recovery of \(^{3}\)H indicated 7.5% (0.75 mg) coupling of the cytochalasin.

It is not considered that the different in molecular weights of the dextrans contributed to the different degrees of coupling of cytochalasin. Rather the difference is considered to reside in the use of dimethyl sulfoxide, rather than ethanol, as the solvent for the cytochalasin.

The chemical equations for the synthesis of the cytochalasin D-dextran adduct follow.
Synthesis of Cytochalasin D-Dextran Adduct

Activation of Dextran with Cyanogen Bromide

Coupling of ε-Aminocaproic Acid
Alternative Structures for the Cytochalasin D-Dextran Adduct

\[
\text{NH} \cdot (\text{CH}_2)_4 \cdot \text{CH}_2 \cdot \text{CO} \quad \text{CYTOCHALASIN D}
\]

\[
\text{N} \cdot (\text{CH}_2)_4 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CYTOCHALASIN D}
\]
WHAT IS CLAIMED IS:

1. A pharmaceutical preparation for inhibiting in vivo the replication of various viruses including the various HIV viruses which comprises a compound selected from the class consisting of cytochalasins.

2. The pharmaceutical preparation of claim 1 wherein the cytochalasin is cytochalasin-D.

3. A pharmaceutical preparation for inhibiting in vivo the replication of various viruses including the various HIV viruses which comprises a complex of a compound selected from the class consisting of cytochalasins and a dextran sulphate or dextran of any molecular weight.

4. The pharmaceutical preparation of claim 3 wherein the cytochalasin is cytochalasin-D and the molecular weight of the dextran sulphate is 40,000.

5. The pharmaceutical preparation of claim 3 wherein the cytochalasin is cytochalasin-D and the molecular weight of the dextran sulphate or dextran is 8,000.

6. The pharmaceutical preparation of claim 3 or 4 wherein the dextran sulphate or dextran is substituted by a polysaccharide exhibiting a slow release mechanism.

7. The pharmaceutical preparation of claim 6 wherein the polysaccharide is dextrin or cyclodextrin.

8. The pharmaceutical preparation of the preceding claims including a pharmaceutically acceptable carrier.

9. A pharmaceutical preparation for inhibiting in vivo the replication of viruses which comprises a complex of dextran
sulphate or dextran and cytochalasin D in an appropriate pharmaceutical dosage form.

10. The pharmaceutical preparation of claim 9 wherein the viruses are HIV viruses.

11. A method for inhibiting in vivo the replication of HIV viruses comprising the administration of a complex dextran or dextran sulphate and cytochalasin D in a pharmaceutically appropriate dosage form and quantity.

12. A process for coupling dextran sulphate to cytochalasin-D comprising modifying the dextran sulphate to dextran dialdehyde by reacting the dextran sulphate with periodate, and thereafter reacting the dextran dialdehyde with cytochalasin-D.

13. A complex of dextran and cytochalasin-D when prepared by the process of claim 12.
Figure 3
**Figure 4**

**SUBSTITUTE SHEET.**
INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 92/00526

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 A61K31/40

II. FIELDS SEARCHED

Minimum Documentation Searched7

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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched8

III. DOCUMENTS CONSIDERED TO BE RELEVANT9

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<td>AIDS vol. 5, no. 12, December 1991, pages 1425 - 1432 BUSSO, MARIANO E. ET AL 'HIV-INDUCED SYNCTIUM FORMATION REQUIRES THE FORMATION OF CONJUGATES BETWEEN VIRUS- INFECTED AND UNINFECTED T-CELLS IN VITRO.' see page 1430 figure 5; table 2 see the whole document</td>
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IV. CERTIFICATION

Date of the Actual Completion of the International Search 27 APRIL 1993

Date of Mailing of this International Search Report 12.05.93

International Searching Authority EUROPEAN PATENT OFFICE

Signature of Authorized Officer MAIR J.
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<td>ANTIMICROBIAL AGENTS AND CHEMOTHERAPY&lt;br&gt;vol. 34, no. 10, October 1990,&lt;br&gt;pages 1991 - 1995&lt;br&gt;BUSSO, MARIANO E. ET AL 'ANTI-HUMAN IMMUNODEFICIENCY VIRUS EFFECTS OF DEXTRAN SULFATE ARE STRAIN DEPENDENT AND SYNERGISTIC OR ANTAGONISTIC WHEN DEXTRAN SULFATE IS GIVEN IN COMBINATION WITH DIDEOXYNUCLEOSIDES'&lt;br&gt;see the whole document</td>
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<td>US,A,4 855 416 (THOMAS C. USHER)&lt;br&gt;8 August 1989&lt;br&gt;see the whole document&lt;br&gt;especially column 1 lines 15-21</td>
<td>3-13</td>
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**INTERNATIONAL SEARCH REPORT**

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

   **ALTHOUGH CLAIM 11 IS CHAPTED AS A METHOD OF TREATMENT OF THE HUMAN BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOSITIONS.**

2. [ ] Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)
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. 27/04/93

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