(For PCT applications(s) - not to be used with micro-organisms)

AUSTRALIA

PATENTS ACT 1990

NOTICE OF ENTITLEMENT

(To be filed before acceptance)

I/We, CURA NOMINEES PTY LTD

of 772 Upper Brookfield Road, Upper Brookfield, Queensland, 4069, Australia.

being the applicant(s) in respect of Application

No....., state the following:-

PART 1. The person(s) nominated for the grant of the patent:

~~*is/are the actual inventor(s)~~

~~or~~

~~*has~~/*have entitlement from the actual inventor(s) by.....

Deed of Assignment

(eg by assignment, by mense assignment, as legal representative of...., etc)

PART 4. The person(s) nominated for grant of a patent:

~~*is~~/*are the applicant(s) of the application(s) listed in the declaration under Article 8 of the PCT.

~~or~~

~~*has/*have entitlement from the applicant(s) of the application(s) listed in the declaration under Article 8 of the PCT~~

~~by~~.....

(eg, by assignment, by mesne assignment, by consent, etc.)

The basic application~~(s)~~ listed in the declaration made under Article 8 of the PCT:

*is/~~*are~~ the first application~~(s)~~ made in a Convention country in respect of the invention

~~or~~

~~*was/*were not the first application(s) made in a Convention country in respect of the invention, and a request has been made under Section 96 of the Patents Act 1990 (or Section 142AA of the Patents Act 1952) to disregard the following application(s)~~.....

CURA NOMINEES PTY LTD;

BY:

..... (Signature)

14-7-92 (Date)

BILL ELLIOT CHAM (Name in block capitals)

Director (Position in company)

*Omit/Delete if not appropriate

GRANT ADAMS & CO 6-4-92 noecaic

AU9171594

(12) PATENT ABRIDGMENT (11) Document No. AU-B-71594/91
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 654474

(54) Title
GLYCOALKALOIDS

International Patent Classification(s)

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	A61K 037/02	A61K 037/26	A61K 037/36	A61K 049/02
	C07J 021/00	C07J 043/00	C07J 071/00	C12Q 001/02
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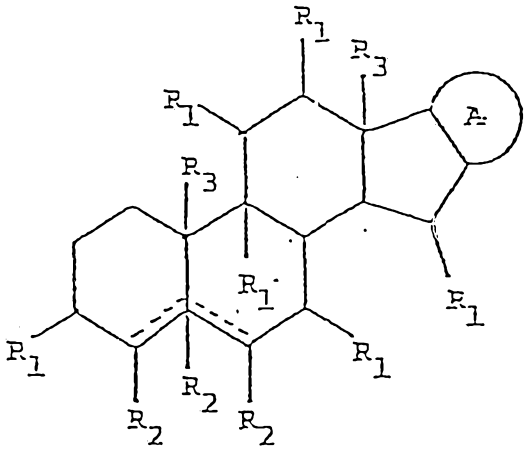
(56) Prior Art Documents
CA 1151549
DE 2519261
DE 1900060

(57) Claim

1. A method for identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells,

said compound being of the general formula (1):



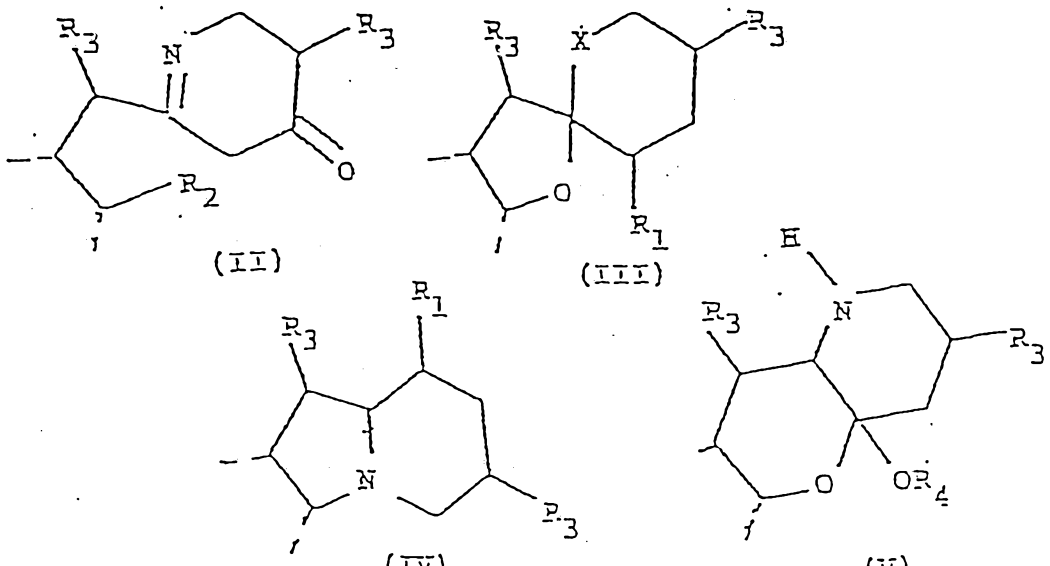
wherein:

either one of the dotted lines represents a double

.../2

bond, and the other a single bond, or both represent single bonds;

"A" represents a radical selected from the following radicals of general formulae (II) to (V):



(II) (III) (IV) (V) ;

each of R^1 is a radical separately selected from the group comprising hydrogen, amino, oxo and OR^4;

each of R^2 is a radical separately selected from the group comprising hydrogen, amino and OR^4;

each of R^3 is a radical separately selected from the group comprising hydrogen, alkyl and R^4O-alkylene;

each of R^4 is a radical separately selected from the group comprising hydrogen, carbohydrate and a carbohydrate derivative;

"X" is a radical selected from the group comprising $-CH_2-$, -O- and -NH-;

said method comprising:

(a) identifying receptor sites for said compound on the surface of a target cell which are

either not present or only present in significantly reduced numbers on the surface of a non-target cell; and

(b) selecting said compound which will selectively bind to said receptor sites of said target cell and which will exert said control.

3. A method for the treatment of cancer, contraception, termination of pregnancy, removal of pathogenic organisms or removal of abnormal cellular growth, said method comprising identifying a compound by the method as defined in Claim 1 and administering said compound to a patient requiring treatment.

5. A method of marking and identifying a target cell said method comprising:

(a) identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells by the method as defined in Claim 1;

(b) further modifying said compound to enable detection of the thus modified compound;

(c) inducing cell autophagy, cell agglutination or cell immobilization; and

(d) detecting said modified compound by any appropriate means.

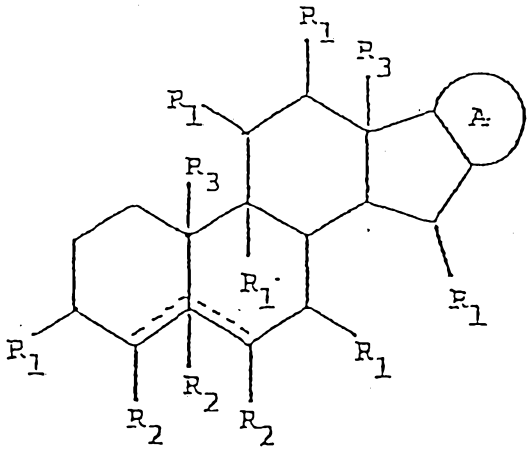
9. A method as defined in any one of Claims 1 to 8 wherein said radical R^4 is selected from the group comprising glyceric aldehyde; glycerose; erythrose; threose; ribose; arabinose; xylose; lyxose; altrose; allose; gulose; mannose; glucose; idose; galactose; talose; rhamnose; dihydroxyacetone; erythrulose; ribulose; xylulose; psicose; fructose; sorbose; tagatose; and other hexoses ($C_6H_{12}O_6$), heptoses ($C_7H_{14}O_7$),

.../4

octoses ($C_8H_{16}O_8$), nanoses ($C_9H_{18}O_9$) and decoses ($C_{10}H_{20}O_{10}$); deoxysugars with branched chains (such as, apiose, hamamelose, streptose, cordycepose, mycarose and cladinose); compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (such as with N-acetyl, acetyl, methyl, replacement of CH_2OH); sugar alcohols; sugar acids; benzimidazoles; the enol salts of the carbohydrates; saccharinic acids; and sugar phosphates.

10. A method as defined in any one of Claims 1 to 9, wherein said compound is selected from the group comprising solasodine, solanidine, diosgenin, solangustidine, leptinidine, solacongestidine, solafloridine, demissidine, soladulcidine, tomatidenol, paniculidine, jurjubidine, tigogenin, yamogenin, neotigogenin, solanocapsine, 26-aminofurostane, and a glycoside of any one thereof.

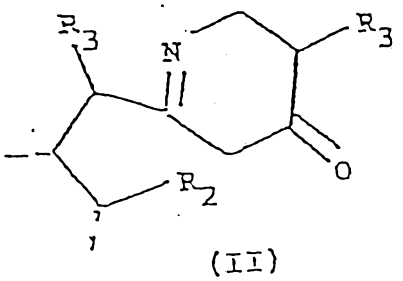
13. Compounds of the general formula (1):



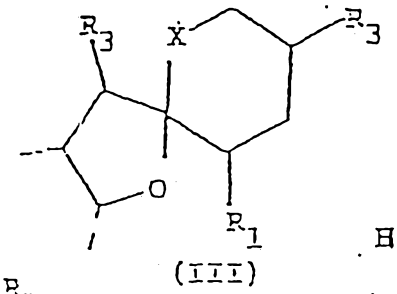
wherein:

either one of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

"A" represents a radical selected from the following radicals of general formulae (II) to (V):



(II)



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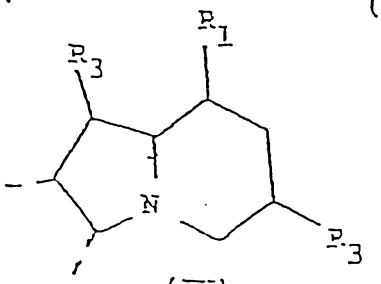

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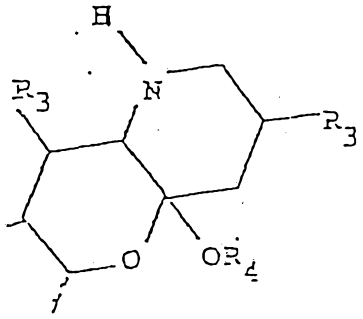

(V) ;

each of R^1 is a radical separately selected from the group comprising hydrogen, amino, oxo and OR^4;

each of R^2 is a radical separately selected from the group comprising hydrogen, amino and OR^4;

each of R^3 is a radical separately selected from the group comprising hydrogen, alkyl and R^4O-alkylene;

each of R^4 is a radical separately selected from the group comprising hydrogen, carbohydrate and a carbohydrate derivative;

"X" is a radical selected from the group comprising $-CH_2-$, -O- and -NH-;

when used in a method as defined in any one of Claims 1 to 12.

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(71) Applicant *(for all designated States except US)*: CURA NOMINEES PTY LTD [AU/AU]; 772 Upper Brookfield Road, Upper Brookfield, Brisbane, QLD 4069 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : CHAM, Bill, Elliott [NL/AU]; 772 Upper Brookfield Road, Upper Brookfield, Brisbane, QLD 4069 (AU). DAUNTER, Brian [AU/AU]; 2 Mazzard Street, Bellbowrie, Brisbane, QLD 4067 (AU).

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(54) Title: GLYCOALKALOIDS

(57) Abstract

The invention is directed to the control of cellular autophagy, cellular agglutination and the immobilization of motile cells. Such control is useful in, for example, the treatment of cancer, contraception, termination of pregnancy, removal of pathogenic organisms and removal of any abnormal cellular growth (malignant or otherwise); as a diagnostic and analytical tool whereby cell structure can be studied and testing could be undertaken for the presence (and subsequent analysis) of pathogenic and non-pathogenic organisms; and in the manufacture of biochemicals whereby certain cells must be destroyed or otherwise contained. From surface analysis of normal and abnormal cells, specific receptors on abnormal cells which are either not present on normal cells or are only present insignificantly reduced numbers can be identified. Alkaloids and other pharmaceutically acceptable compounds are preferentially recognised by the abnormal cells, and which bind thereto and subsequently destroy.

TITLE: GLYCOALKALOIDS

TECHNICAL FIELD

THIS INVENTION is directed to cellular autophagy. In particular, it is directed to the control of cellular autophagy, cellular agglutination and the immobilization of motile cells.

BACKGROUND ART

Controlled autophagy and/or agglutination or immobilization of cells should be very useful for a wide variety of therapeutic and other medical and non-medical uses.

Before describing the present invention in detail, it is first important to appreciate the differences between autophagy, cell lysis and apoptosis.

Autophagy (or self digestion) is the result of disrupting the cellular organelle - the lysosome- directly or indirectly, for example, by inhibition of mitochondrial activity. The cell thus digests itself from the inside, including digesting the plasma membrane of the cell, to leave behind the nucleus that is also partly digested and fragments.

Cell lysis, however, is cell death due to leakage of material from the cell and can be induced by changing the osmotic strength of the media surrounding the cell. Cell lysis can be induced by causing damage to the plasma membrane of the cell, for example with enzymes or antibiotics, to induce osmotic shock. Also in haemolysis, which is the specific lysis of red blood

cells, haemoglobin is extruded by the cell to leave behind a damaged red blood cell membrane.

Finally, apoptosis is the fragmentation of the nucleus and the encapsulation of these fragments and cell organelles in plasma membrane fragments - this does not involve cell lysis or autophagy.

Traditional treatment for an infected host is the destruction of the invading virus or the like, leaving the cells of the host intact. Similarly, if abnormal cell growth of the host is responsible for the malady, then treatment, obviously, must only target the abnormal cells, leaving the normal cells intact.

Treatment of the latter type of malady, eg cancer and related diseases, has been the subject of much research and a large range of chemical compounds have been investigated with mixed results.

Preference for treatment with naturally occurring compounds is increasing and of the many alkaloids currently used or tested in medicine, many have been extracted from plants. In particular, the use of extracts of the plant species Solanum as an effective treatment of certain cancers has been known since at least 1825. Research into these extracts from 1965 onwards has established that the antineoplastic compound(s) was most likely a glycoalkaloid(s). Examples include B-solamarine, a glycoalkaloid extracted from Solanum dulcamara as a tumour inhibitor, and other glycoalkaloids extracted from Solanum sodomaeum L. which possess antineoplastic activity both in mice and humans. Another example is Solaplumbin - which is rhamnosyl [4→3] Solasodine - obtained from Nicotiana

plumbaginifolia which has been shown to have anticancer properties in rats.

However, specificity remains a problem and it is not always possible to target solely the invading virus or the like or to only affect abnormal cells of the host.

DISCLOSURE OF THE INVENTION

The present inventors' studies of normal and abnormal cells have discovered specific receptors on abnormal cells which are either not present on normal cells or are only present in significantly reduced numbers such that certain compounds are preferentially recognised by the abnormal cells, and which bind thereto and subsequently destroy. Once these receptors have been identified it has also been discovered that certain alkaloids and other pharmaceutically acceptable compounds can be used to control cellular autophagy, cellular agglutination and immobilization of motile cells.

With such control, it would be far easier to target a particular cell for destruction or some other modification and would be useful in, for example, the treatment of cancer, contraception, termination of pregnancy, removal of pathogenic organisms and removal of any abnormal cellular growth (malignant or otherwise). It would also be useful as a diagnostic and analytical tool whereby cell structure could be studied and testing could be undertaken for the presence (and subsequent analysis) of pathogenic and non-pathogenic organisms. This control of cellular function would also be useful in the manufacture of biochemicals whereby certain cells must be destroyed or otherwise contained.

The present inventors have discovered that, by identifying a particular receptor site of the target cell and coupling a suitable compound thereto, the required control of cellular function can be achieved.

Thus, according to one aspect of the present invention, there is provided a method for identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells, said method comprising:

(a) analyzing the surface of each of a target cell and of a non-target cell;

(b) identifying receptor sites on said surface of said target cell which are either not present or only present in significantly reduced numbers on said surface of said non-target cell; and

(c) selecting a compound which will selectively bind to said receptor sites of said target cell and which will exert said control.

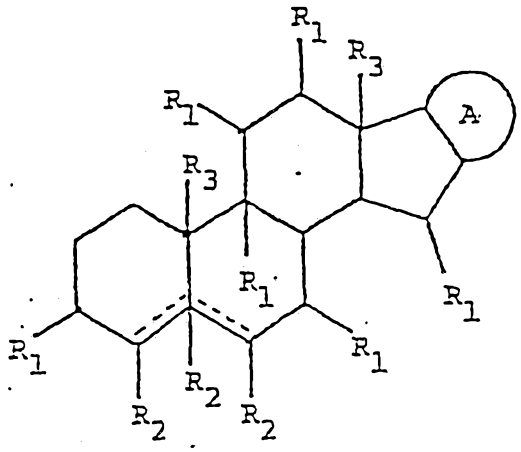
As a second aspect of the present invention, there is provided a method to control cell autophagy, cell agglutination or immobilization of motile cells, said method comprising:

(a) identifying a compound suitable for the required control by the method as hereinbefore defined; and

(b) binding said compound to said receptor sites of said target cell.

A third aspect of the present invention is the provision of a compound which is suitable for the control of cell autophagy, cell agglutination or immobilization of motile cells by the method as hereinbefore defined.

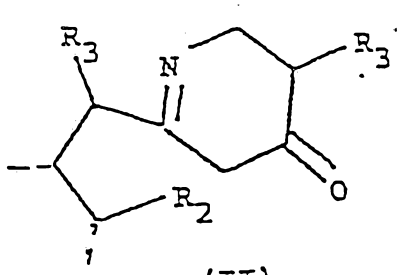
Preferably, the control of cellular function by the present invention is by using compounds of the general formula (1):



wherein:

either one of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

"A" represents a radical selected from the following radicals of general formulae (II) to (V):



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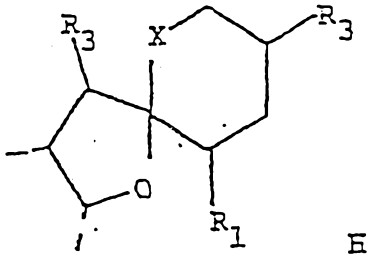

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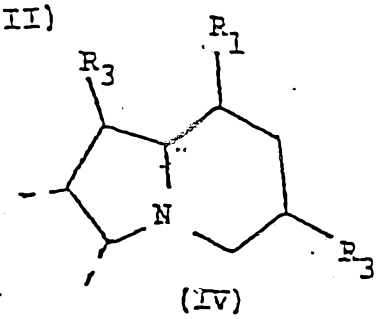

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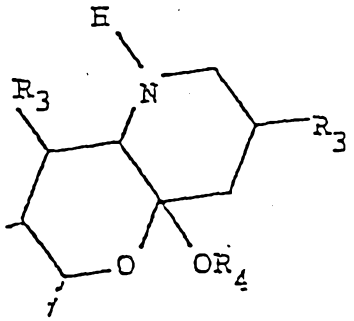


(V) ;

each of R^1 is a radical separately selected from the group comprising hydrogen, amino, oxo and OR^4;

each of R^2 is a radical separately selected from the group comprising hydrogen, amino and OR^4;

each of R^3 is a radical separately selected from the group comprising hydrogen, alkyl and R^4O-alkylene;

each of R^4 is a radical separately selected from the group comprising hydrogen, carbohydrate and a carbohydrate derivative;

"X" is a radical selected from the group comprising $-CH_2-$, -O- and -NH-.

For non-carbohydrate compounds of general formula (1), the preferred compounds are solasodine, solanidine, diosgenin, solangustidine, leptinidine, solacongestidine, solafloridine, demissidine, soladulcidine, tomatidenol, paniculidine, jurjubidine, tigogenin, yamogenin and neotigogenin.

The more preferred compounds are solasodine and solanidine.

When the compounds of general formula (1) represent a carbohydrate (such as a mono, oligo or polysaccharide) or a derivative thereof, the preferred radicals R^4 are glyceric aldehyde; glycerose; erythrose; threose; ribose; arabinose; xylose; lyxose; altrose; allose; gulose; mannose; glucose; idose; galactose; talose; rhamnose; dihydroxyacetone; erythrulose; ribulose; xylulose; psicose; fructose; sorbose; tagatose; and other hexoses ($C_6H_{12}O_6$); heptoses

($C_7H_{14}O_7$); octoses ($C_8H_{16}O_8$); nanoses ($C_9H_{18}O_9$); decoses ($C_{10}H_{20}O_{10}$); deoxysugars with branched chains (eg apiose, hamamelose, streptose, cordycepose, mycarose and cladinose); compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (eg N-acetyl, acetyl, methyl, replacement of CH_2OH); sugar alcohols; sugar acids; benzimidazoles; the enol salts of the carbohydrates; saccharinic acids; sugar phosphates.

The more preferred compounds are solasonine, solamargine and solanine.

Other preferred compounds of the general formula (1) are solanocapsine and 26-aminofurostane.

It will be appreciated that the various compounds referred to throughout this specification may be chiral and the present invention relates both to the individual stereoisomers and to any mixtures thereof including mixtures of enantiomers and/or diastereoisomers.

Although not wishing to be bound by theory, the proposed mechanism of autophagy induction by the preferred compounds of the present invention wherein all of the radicals R^1 represent hydrogen is by diffusion through the plasma membrane of the cell to interact either directly with the lysosome causing its disruption or/and indirectly by inhibition of mitochondrial activity. When R^1 is other than hydrogen, the mechanism of entry into the cell is by receptor mediated endocytosis. In the case of R^1 representing carbohydrate (ligand), the receptors are endogenous lectins.

This receptor mediated endocytosis is important because, as different cells express different receptors for various compounds, it is thus possible to couple any ligand (which interacts with a specific cell-surface receptor) to specifically induce autophagy and/or cell agglutination or immobilization of different cell types.

Suitable ligands other than carbohydrates include the steroid and non-steroid hormones (eg, progesterone, insulin, oestrogen, growth hormone), growth factors, polyamines, cytokines, lymphokines, lymphotoxins, chalones, fatty acids and cholesterol - ie, essentially any chemical messenger required for endocytosis.

Once such a ligand has been identified and coupled to the appropriate steroid derivative or other compound, administration to the cell-containing host should induce cell autophagy and/or cell agglutination or immobilization.

It should be noted that it is not essential that the aforesaid ligand be directly coupled to the said steroid or other compound. For example, the said steroid or other compound could be attached to one end of a suitable organic or inorganic carrier, such as a polymer, with the ligand coupled to the other end of the carrier. This indirect coupling of the ligand to the steroid or the like could provide a convenient delivery system for the present invention when it is inappropriate, for whatever reason, to directly couple the ligand to the active component.

This ability to control cellular function at will is not evident from the current prior art. Different diseases require different treatment - a treatment effective against skin cancer is unlikely to be as

effective against, for example, ovarian cancer as the malignant cells have different receptors. For example, although it is known that certain glycosides are effective against certain cancers, these same glycosides have not been assessed for their potential use against other cancers nor has their mode of action been studied. It is believed that the present inventors are the first to have studied this mode of action, identified those receptor sites on "abnormal" cells which differ from those on "normal" cells and by a suitable selection of an appropriate compound, achieved a required control on cellular function.

For their use in practice, the compounds of the present invention are not generally employed by themselves. Preferably, they are used in a composition containing one or more of the compounds, in association with any pharmaceutical-type carrier or diluent which is suitable for its administration.

As used throughout the specification, the term "carrier of diluent" denotes an organic or inorganic, natural or synthetic material with which the active ingredient is combined in order to facilitate the administration of the invention. This carrier or diluent is, therefore, generally inert and it must be pharmaceutically acceptable.

With the cellular control available from the present invention, it should be possible to target a particular cell for destruction or modification necessary in, for example, the treatment of cancer, contraception, termination of pregnancy, removal of pathogenic organisms and removal of abnormal cellular growth.

Thus, as a fourth aspect of the present invention, there is provided a method of inducing cell autophagy, cell agglutination or immobilization within an animal body (including humans), said method comprising administering to said animal a compound or a composition, both as hereinbefore defined.

The present invention should also be useful as a diagnostic and analytical tool whereby a compound found to selectively bind to a target cell could be initially further modified to enable its detection by suitably available techniques, thus acting as a "marker" identifying the target cell.

Thus, according to a fifth aspect of the present invention, there is provided a method of marking and identifying a target cell said method comprising:

(a) identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells by the method as hereinbefore defined;

(b) further modifying said compound to enable detection of the thus modified compound;

(c) inducing cell autophagy, cell agglutination or cell immobilization by the method as hereinbefore defined; and

(d) detecting said modified compound by any appropriate means.

Preferably, said compound is modified by further conjugation with another compound, this other compound being detectable by its fluorescence or radioactivity.

For example, a compound of the general formula (1) as hereinbefore defined could be reacted with the fluorescent reagent dansyl chloride, or said compound could be modified to incorporate the usual radiolabel(s) known in the art. The thus modified compound could be purified before its use in the induction of cell autophagy, agglutination or immobilization and the progress of the binding followed under a fluorescence microscope or radioactivity counter as applicable. If the binding is undertaken at a low temperature, the internalization of the marked compound will be slow allowing the binding to be followed.

DETAILED DESCRIPTION OF EMBODIMENTS

Specific details of the compounds and methods of the present invention will now be illustrated. The technical terms identified have the usual meaning in the art.

GENERAL

Solasodine glycosides can be isolated and purified as a mixture (known as BEC) comprising solamargine (33%), solasonine (33%) together with di- and mono-glycosides (34%) or as separate components (Cura Nominees Pty Ltd, Australia) and these compounds have been investigated for the inhibition of cell replication of human cells, ovarian cancer cells, melanoma cells, cells infected with viruses, normal fibroblasts, normal bone marrow cells, leukemic cells and normal lymphocytes. The inhibition of the glycosides of solasodine have been demonstrated with the appropriate "free" sugars and covalently bound to bovine serum albumin. The sugars that have been investigated and shown to have inhibitory effects on the action of the mixture of solasodine

glycosides are as follows: glucose α and ß, NAc glucosamine NAc glucose, mannose α and ß, NAc mannosamine NAc mannose, galactose α and ß and the disaccharide lactose.

Chlorambucil (Sigma Chemical Co., USA), 1-2mg was dissolved in 50µl of DMSO and diluted immediately before use in TCM + 10% FCS. Cis-platinum was supplied as a saline solution (David Bull Laboratories, Australia), and was diluted with TCM + 10% FCS. Vinblastine (Sigma Chemical Co., USA) was dissolved in TCM + 10% FCS. Solasodine glycosides, 100mg were dissolved in 5ml DMSO and diluted with TCM + 10% FCS to give a 5% solution of DMSO, and further diluted before use. Appropriate DMSO cytotoxicity studies were also conducted.

Peripheral blood human lymphocytes were isolated using an Isopaque-Ficoll gradient and cultured in replicates of 10 in microwell titre plates. $2x10^5$ lymphocytes/well ($1x10^6$/ml) in CO_2 1640 tissue culture media supplemented with 10% fetal calf serum containing 20µg/ml of photohaemagglutinin (PHA), concanavalin A (Con A) or pokeweed nitrogen (PWM). This was cultured for 48 hours and pulsed with tritiated thymidine for a further 24 hours. In the inhibition studies, the cell concentration was $8x10^5$/ml.

IN VITRO PREFERENTIAL CYTOTOXICITY FOR HUMAN CANCER CELLS

The inhibition of cell replication by steroid alkaloids is determined by the inhibition of the incorporation of ^3H thymidine into DNA. Using this technique, it should be possible to monitor any preferential inhibition of uptake of ^3H thymidine by cancer cells.

Below it is shown that solamargine, a glycoside of solasodine, preferentially inhibits the uptake of tritiated thymidine by cancer cells. In contrast, solamargine at equivalent concentration, and the mono- and di-glycosides of solasodine had a limited effect on the uptake of tritiated thymidine for other cell types, including unstimulated lymphocytes and lymphocytes stimulated with Con A. Also, the solasodine glycosides do not inhibit the uptake of tritiated thymidine by lymphocytes stimulated with PHA or PWM. The inhibition of tritiated thymidine uptake by solamargine and the mono- and di-glycosides of solasodine are dependent upon their cellular uptake by endogenous endocytic lectins (EELs). The mode of action of the solasodine glycosides, in particular solamargine, appears to be the induction of cell lysis, as determined by morphological examination.

Cells were maintained as monolayers in RPMI-1640 tissue culture media (Grant Island Biological Co) buffered with N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid pH 7.5 and supplemented with 10% heat inactivated fetal calf serum (FCS): (i) a human ovarian cancer cell line (C180-135), (ii) HeLa cells, (iii) human fibroblasts, and (iv) as a cell suspension of lymphoblastoid cells (EBV transformed lymphocytes). Monolayers were trypsinized to form a cell suspension. All cell suspensions were passed through a 21 gauge syringe needle to remove cell clumps (95% viability). Cell concentration was adjusted to $2x10^5$cells/ml, and $2x10^4$ cells were added in replicates of 10 to the wells ($1x10^5$/ml well) of HA microtitre plates (Millipore Corp., USA). The cells were preincubated at 37.5°C for 7 hours prior to the addition of 50µl of the cytotoxic drug followed by the addition of 50µl 5uCi/ml of ^3H thymidine, in the same tissue culture media 21 hours

later. Incubation was continued for a total of 24 hours, which includes the pre-incubation time.

All cells were harvested by vacuum filtration by washing in 250µl of each of phosphate buffered saline, 5% trichloroacetic acid, 1.0M NaCl and 95% ethanol and subjected to ß-scintillation counting. Similarly, peripheral human blood lymphocytes were isolated and cultured $2x10^5$ lymphocytes in CO_2 1640TCM 10% HIFCS. Lymphocytes were stimulated with 20µg/ml PHA, Con A or PWM, and cultured for 48 hrs followed by a 24 hr pulse. Inhibition of cytotoxicity was carried out with lactose, lactosyl-albumin, glucose, galactose or rhamnose (Sigma Chemical Co., USA). Cell concentrations were $8x10^4$/well total volume 250µl. The dpm of the experimental replicates were expressed as a percentage of the mean value of the controls, and the mean value of the experimental replicates calculated. The SD of the controls did not exceed 10% of their mean value.

Cytotoxic drugs studied included chlorambucil (Sigma Chemical Co., USA))1-2mg diluted in 50µl of DMSO immediately before use), cisplatinum (in saline solution) (David Bull Laboratories, Australia), vinblasine (Sigma Chemical Co., USA), solasodine glycosides, 100mg in 5ml DMSO and diluted to give a 5% solution of DMSO. Appropriate DMSO cytotoxicity studies were also conducted. Solasodine glycosides were supplied as a mixture (BEC), and as separate components, solamargine, solasonine, a mixture of di- and mono-glycosides and the aglycone solasodine. All cytotoxic drugs were further diluted with HIFCS/TCM before testing. $5x10^4$ ovarian cancer cells (200µl/chamber of a chamber of microscope slide (Lab Tek Miles Scientific) were used. Controls received 50µl HIFCS/TCM and experimental chambers 50µl of solasodine glycosides

(BEC) 1.5-3.8 µM/L after 7 hrs preincubation, and incubated for a further 3 hrs. Similarly, the cells were treated with the aglycone solasodine 19.4-96.8 µM/L. The cells were fixed and examined by the Papanicolaou method.

Solasonine at 11.3µM/L was ineffective in inhibiting the uptake of tritiated thymidine by the various cell types relative to solamargine at 11.5µM/L (Table 1). The mixture of di- and mono-glycosides at 14.45µM/L were also ineffective for lymphoblasoid cells and HeLa cells, whereas they caused approximately 30% inhibition for ovarian cancer cells and fibroblasts (Table 1). The highest concentration of BEC used contained 6µM/L of di- and mono-glycosides and this would account for 10%-12% inhibition for susceptible cells. In order that comparisons could be made with previous studies, and because of the limited availability of the individual glycosides, the mixture of solasodine glycosides BEC was used for further investigations and the molar concentration expressed in terms of the most cytotoxic component solamargine.

In contrast to the other cells that have been investigated, solamargine has limited cytotoxicity for unstimulated lymphocytes and lymphocytes stimulated with Con A, and an absence of cytotoxicity when lymphocytes are stimulated with PHA or PWM (Table 2). Solasonine was also found to be ineffective (Table 2). The composition BEC has been used in this investigation and the cytotoxicity of BEC expressed in terms of the most active components, in this case, the di- and mono-glycosides (DMG).

From the dose response curves (Figs. 1a,b,c,d), the lethal dose (LD) of cytotoxics required to inhibit 50% (LD_{50}) uptake of tritiated thymidine was determined. The LD_{50} of the various cell types can be expressed as a ratio relative to the LD_{50} of ovarian cancer cells, to give a thymidine uptake ratio (TR_{50}). Therefore, TR_{50} values greater than 1.0 indicate that more ovarian cancer cells are killed relative to the other cell type (Table 3). From Table 3, other TR_{50} can be calculated. The TR_{50} for fibroblasts/lymphoblastoid cells in the presence of vinblastine is 0.73, which demonstrates its known cytoxicity to normal cells. Similarly, the TR_{50} for chlorambucil (alkylating) and cis-platinum (DNA binding) which are used in the treatment of chronic lymphatic leukemia (CLL) and ovarian cancer respectively, also reflects their known attendant toxicity, although that of chlorambucil is not reflected by fibroblasts. However, in the case of vinblastine or cis-platinum, the low TR_{50} for fibroblasts relative to ovarian cancer cells is evident (Table 3). In contrast, solamargine gives a TR_{50} greater than 2 for fibroblasts and lymphoblastoid cells relative to ovarian cancer cells. This suggests that solamargine has an acceptable degree of specificity for ovarian cancer cells, and under these conditions is superior to the other cytotoxic drugs. The specificity of solamargine for ovarian cancer cells is also reflected by the TR_{50} relative to HeLa cells (Table 3). In addition, the molar concentration of solamargine that is required to achieve an LD_{50} for ovarian cancer cells is 6-40 times less than that of the other cytotoxics investigated (Table 3).

The LD_{50} from the dose response curves (Figs. 8a,b,c,d) for lymphocytes have been calculated and are presented in Table 4. The LD_{50} for DMG indicates that they are equivalent or more cytotoxic than the other anti-neoplastic drugs investigated except in the case of PWM stimulated lymphocytes. The LD_{50} concentration for the di- and mono-glycosides of solasodine (DMG) is increased with stimulated lymphocytes, in particular with PWM stimulation. Stimulated lymphocytes undergo a number of changes, which includes and increase in plasma membrane permeability, and this may account for the variable LD_{50} for some of the cytotoxic drugs investigated. However, this situation does not appear to apply to DMG. Firstly, PHA and PWM stimulation of lymphocytes negates, rather than enhances the effect of DMG. Similarly, this applies to the effect of solamargine and the absence of any effect of solasonine on unstimulated and stimulated lymphocytes (Table 2). Secondly, the only difference between the glycosides are their carbohydrate moieties (glycone), because they all contain the same aglycone solasodine. This suggests that the different effects of the glycosides on unstimulated lymphocytes are due to the presence or absence of different EELs.

CELLULAR UPTAKE BY ENDOCYTIC ENDOGENOUS LECTINS

A mixture of solasodine glycosides (BEC), of which the active compound is solamargine and from which the aglycone solasodine is derived was prepared for use in cytotoxicity assays as determined by the uptake of tritiated thymidine. Inhibition studies were carried out with lactose, lactosyl-albumin, galactose or rhamnose (Sigma Chemical Co., USA). All experimental replicates were related to their own controls. Total volume of replicates was 250µl (carbohydrates added in

50µl tissue culture media + 10% fetal calf serum), and the cell concentrations were $8x10^4$/ml.

A human ovarian cancer cell line (C180-135) was grown as mono layers and trypsinized to form a cell suspension. The cells were adjusted to a concentration of $1x10^5$/ml (viability ≥95%) and 200µl placed in each of the eight chambers of microscope chamber slides (Lab Tek, Miles Scientific). After 21 hours preincubation at 37.5°C, 50µl of supplemented tissue culture media were added to the control chambers. To the other chambers were added 50µl of solasodine glycosides (BEC) to give final concentrations of 3, 4.8, 6.7, 7.7, 9.6, 11.1 and 15.3 µM/L. Similarly, to a separate chamber slide was added the aglycone solasodine (19.4 - 96.8 µM/L). The slides were incubated at 37.5°C for a further 3 hours, and the cells fixed in 95% V/V alcohol and stained by the Papanicolaou method. Two other chamber slides were prepared in a similar manner, but the solasodine glycosides and the aglycone were added at a final concentration of 0.77, 1.5, 2.3, 3.1 and 3.8 µM/L after 7 hours preincubation, and then incubated for a further 17 hours.

As the ovarian cells (CI80-135) are more susceptible to the cytotoxic effects of solamargine in the BEC mixture of solasodine glycosides, these cells were selected as a representative example of the response of susceptible cells. Lysosomotropic drugs are weakly basic amines, like solamargine, which can be trapped and accumulate as the protonated (acidic) form in the lysosomes. This results in the rupture of the lysosomal membranes and the release of their proteolytic enzymes.

In order to observe any immobilization of spermatozoa, sperm ($10x10^6$/ml) (50µl) were placed on a microscope

slide separated from 50µl of solamargine (1 mg/ml) by 5µl of eosin. A cover slip was then placed over the slide. The drops spread out but do not mix with each other to any great extent. This is a standard method known in the art. It was noted that sperm was not present on the side of the slide where the drop of solasodine glycosides was placed.

The results are presented in Tables 5 and 7 Figs. 5, 6 and 7. The legend for Figs. 6 and 7 is as follows:

a = ovarian cancer cells (Fig. 6 and Fig. 7)

a_1 = ovarian cancer cells in the presence of lactose 1.1µM/L (Fig. 6).

a_2 = ovarian cancer cells in the presence of lactosyl-albumin 1.1µM/L lactose (Fig. 6).

bcd = HeLa cells, lymphoblastoid cells and fibroblasts respectively (Fig. 6 and Fig. 7).

Fig. 6: Top curve - effect of the aglycone solasodine.

Partial inhibition of solamargine cytotoxicity by lactose and lactosyl-albumin is demonstrable for ovarian cancer cells (Fig. 6). The lactosyl-albumin inhibitory effect is approximately 4 times that of lactose at equivalent lactose concentration (Fig. 6). This is because glycoconjugates have an increased affinity for their corresponding lectin relative to unconjugated carbohydrates. Similarly, lactose and galactose cause partial inhibition of solamargine cytotoxicity for both ovarian cancer cells and lymphoblastoid cells (Table 5). In contrast, lactose and galactose completely inhibit solamargine cytotoxicity in the case of fibroblasts, and

inhibition by rhamnose is also demonstrable (Table 5). Rhamnose is not found in mammalian glycoconjugates, but under certain conditions, can be identified by galactose reactive lectins. Therefore, the EEL expressed by cells susceptible to solamargine has specificity for Gal(1→4)Glu(2→1)Gal. It is therefore possible that lactose may compete for this EEL, in terms of the lactose group (Gal(1→4)Glu) and galactose/rhamnose for the terminal galactose ((2→1)Gal).

Similarly, these carbohydrate moieties of solamargine may be identified by their corresponding EEL's. This latter situation appears to apply to fibroblasts, as solamargine cytotoxicity was completely inhibited by lactose and galactose (Table 5). However, in the case of HeLa cells, solamargine cytotoxicity is not inhibited by lactose and galactose. Further, solasonine, a glycoside of solasodine, with a glycone moiety Glu(1→3)Gal(2→1)Rh is not cytotoxic. This gives further support for the presence of an EEL specific for Gal(1→4)Glu(2→1)GAl. Whether or not the partial inhibition of solamargine cytotoxicity by lactose and galactose, in the case of ovarian cancer cells and lymphoblastoid cells (Table 5), is the result of competitive inhibition or the presence of lactose and galactose EELs, has not been elucidated.

The aglycone solasodine does not exert a cytotoxic effect at equivalent concentrations of solamargine (Fig. 6). However, at higher concentrations, there is an increase in cytotoxicity, but this is more apparent with ovarian cancer cells (Fig. 7). This may be explained by the changes in membrane permeability of cancer cells. It is possible that solasodine, which is a very hydrophobic molecule, undergoes enhanced protein-binding

and thus reduces its bioavailability. Cytotoxic effects therefore become apparent at higher concentrations. Nevertheless, collectively, the results demonstrate the presence of EELs for solamargine.

The aglycone solasodine does not exert a cytotoxic effect at equivalent concentrations of DMG. However, at much higher concentrations, there is a substantial effect on unstimulated lymphocytes, which is significantly reduced in the case of stimulated lymphocytes (Fig. 8e). This suggests that membrane permeability to the aglycone is decreased in stimulated lymphocytes. However, this would not account for the differential effect of the glycosides on unstimulated and stimulated lymphocytes (Table 2, Figs. 8a,b,c,d), and inhibition by carbohydrates (Table 7). Thus, collectively the results support the active uptake of solasodine glycosides by EELs.

Glucose or rhamnose inhibit the action of DMG with Con A stimulated lymphocytes (Table 7). Since these carbohydrates do not inhibit DMG with unstimulated lymphocytes, this suggests that Con A stimulation results in the expression of EELs that react with glucose and rhamnose. Thirdly, Con A stimulation gives rise to a subpopulation of T suppressor cells (TS) that inhibit helper T cells (TH) within the total population. The TS cells produce soluble factors that inhibit TH cell functions but these suppressor factors can be inhibited by N-acetylglucosamine or rhamnose by complexing the TH cell receptors. Therefore, one of the soluble TS cell factors and/or Con A may be involved in receptor (EEL) induction on TH cells.

The aglycone solasodine did not have any observable effect on the ovarian cancer cells at any of the

concentrations investigated (Fig. 2A). In contrast, with increasing concentration of the solasodine glycosides (expressed as concentration of solamargine) over 3 hours, the cytoplasm of the cancer cells undergo dissolution, the nuclei contract and become dark staining (Fig. 2B), nuclei then enlarge (Fig. 3A), the chromatin clumps (Fig. 3B), and finally the nuclei disintegrate (Fig. 4A). Fig. 4B represents the effect of the solasodine glycosides over 17 hours, in which cellular debris is left. It therefore appears that the inhibition of thymidine uptake by solamargine is the result of cell lysis.

Solamargine was also found to inhibit human spermatozoal motility (Fig. 5) as a red line of demarcation between the sperm in tissue culture media and the solamargine aliquot became visible as sperm penetrated the boundary and then became immobilized within 15 sec.

The immobilization of spermatozoa can result from the inactivation of their mitochondria, and in the case of cells that contain lysosomes, this could lead to rupture of the lysomal membranes.

The preferential cancer cells cytotoxicity (lysosomotropic/mitochondrial inhibition) of solamargine appears to be effective against both proliferating and resting cancer cells, as evidenced by the absence of cancer cells upon cytological examination (Fig. 4B). In addition, the lack of lysosomotropic/mitochondrial inhibition by the aglycone solasodine, the triglycoside solanine and the di- and mono-glycosides of solasodine indicates that the cellular uptake of solamargine is possibly mediated by plasma membrane endocytic

endogenous lectins (EELs), specific for the carbohydrate moiety of solamargine.

FLUORESCENCE ACTIVATED CELL SORTER ANALYSIS (FACS)

To verify the inhibition studies of the "free" sugars and sugars covalently bound to albumin (uptake of ^{3}H-thymidine) were due to competition for endogenous lectin receptors, FACS analysis was used. This involves the use of sugar(s) covalently bound to bovine serum albumin and the fluorescent compound FITC (S-Alb-FITC). To $1x10^6$ cells, washed twice in phosphate buffered saline pH 7.2 containing 10mg/ml of bovine serum albumin, 1mM $CaCl_2$ and 0.5mM $MgCl_2$ was added 1.0ml of S-Alb-FITC 200µg/ml in the same buffer and incubated for 1 hour. This experiment was conducted at two different temperatures, 4°C and ambient temperature (RT). At 4°C the endogenous lectins would undergo endocytosis (internalization by the cells) at a slower rate when complexed to S-Alb-FITC relative to that at RT. Thus a greater percentage of fluorescence should be expressed at RT, unless the cell were expressing excessive amounts of endogenous lectins.

The data for the lactose specific lectin receptor is presented in Table 6 for a number of cell types.

INHIBITION OF CYTOTOXICITY BY RHAMNOSE IN MICE WITH SARCOMA 180

Rhamnose is not found in mammalian glycoconjugates but forms part of solasonine, solamargine and diglycosides of solasodine in BEC. If specific receptors for this sugar are present on cancer cells (absolutely or in greater abundance) relative to normal cells, then rhamnose would be expected to inhibit the cytotoxic effects of BEC.

Below it is shown that rhamnose inhibits the efficacy of BEC, and that the aglycone solasodine is not effective against murine S180. It is also demonstrated that mice in their terminal stage with S180 can tolerate and become symptom-free of cancer by a large single dose of BEC. The mice tolerate BEC at concentrations which are equivalent to 3 times the LD_{100} of control normal mice.

Herston White mice with a body weight of approximately 30g and aged 8-10 weeks served as recipients. Twelve mice were randomly chosen for each experimental group. Sarcoma 180 tumour cells ($5x10^5$) were inoculated intraperitoneally into mice. This caused a mortality of 100% with a median survival time of 20 days in the control groups. A standard mixture of glycoalkaloids (BEC) was dissolved in dimethylsulfoxide at a concentration of 0.5g BEC/100ml dimethylsulfoxide. Similar solutions were made up but also contained 0.3125, 0.625 and 0.9375g of rhamnose. These solutions were administered intraperitoneally in concentrations of 8mg/kg animal weight for BEC (Fig. 9) without (-▲-), and with 5mg (-□-), 10mg (-•-) and 15mg (-Δ-) rhamnose/kg animal weight. The first dose was given 0.5 hours after administration of the Sarcoma 180 tumour cells. The remaining three doses were given at daily intervals. Dimethylsulfoxide and rhamnose had no effect on Sarcoma 180 activity in the absence of BEC (-o-).

Using similar conditions to those described in Fig. 9, single high doses of BEC (Fig. 10) 25mg/kg (-o-), 50mg/kg (-▼-), and 100mg/kg (-■-) were administered intraperitoneally 12 days after inoculation of the Sarcoma 180 tumour cells (arrow), that is, one day before the animals enter into the terminal stage. Dimethylsuphoxide had no effect on Sarcoma 180 activity and all animals died in 20 days (-•-).

Fig. 9 illustrates that the survival of mice with S180 treated with 4 doses of 8mg BEC/kg was dependent on given doses of rhamnose. Mice inoculated with S180 cells alone died in 2-3 weeks. When four doses of BEC at 8mg/kg were given on consecutive days, complete inhibition of S180 activity was achieved and all the animals survived. The number of survivals was decreased with increasing concentrations of rhamnose. Five mg rhamnose/kg decreased the survival to 75%, whereas 10mg rhamnose/kg decreased the survival to 50% and 15mg rhamnose/kg decreased the survival to 42%. This indicates that rhamnose may competitively inhibit the efficacy of BEC. Similar concentrations of rhamnose or glucose have no effects on S180 activity in the absence of BEC. These observations suggest that the binding of solasodine glycosides on tumour cells may be mediated through the monosaccharide rhamnose, which forms part of solasonine, solamargine and diglycosides of solasodine in BEC.

In all reported in vivo studies with S180, BEC was injected before the terminal phase. Fig. 10 illustrates the effect of single doses of varying concentrations of BEC on the absolute survival of mice which had the S180 tumour for 12 days, that is, one day before the animals enter into the terminal stage. All animals inoculated with S180 and not treated with BEC died. The survival time is increased with a dose of 25mg/kg. However, at day 30, all the mice had died. The survival time and number of survivors were increased with increasing concentrations of BEC, and 17% were symptom-free with a given dose of 50mg/kg, whereas 42% were symptom-free with a given dose of 100mg/kg.

There are two important observations to note from these results.

The first is that animals which are in their terminal stage can be rendered symptom-free of S180 by BEC therapy.

The second is that the animals can tolerate very high doses of BEC. It is known that the LD_{50} (intraperitoneal) of BEC in mice is 30mg/kg for single doses and the LD_{100} is 35mg/kg. Thus, in the present studies, it is shown that if the mice suffered from advanced S180 activity, virtually three times the LD_{100} of BEC for normal mice can be tolerated. This important observation has not been reported with other antineoplastic drugs.

This lack of toxicity may be due to increased plasma or tissue enzymatic activity, resulting in hydrolysis of the sugars from the solasodine. Solasodine is relatively non-toxic in mice (100mg solasodine/kg- which is equivalent to approximately 200mg BEC/kg - does not produce any deaths in mice). However, this is unlikely, since solasodine at similar concentrations (100mg/kg) is not effective in inhibiting S180 activity in mice, and Fig. 10 shows clearly that S180 activity was inhibited by the equivalent concentration of BEC. Alternatively, and a more likely explanation, is that the S180 cells which are in great abundance in the ascitic fluid of the mice 12 days after inoculation of S180 cells, recognize and bind BEC by means of specific receptors (endogenous lectins), reducing the bioavailability of BEC to normal cells, which in turn reduces the toxicity of BEC. Furthermore, this explanation is supported by the fact that BEC inhibits S180 activity even though the animals are suffering from advanced S180 activity. At this advanced stage, BEC, at concentrations less than 25mg/kg is not effective in inhibiting S180 activity. These results provide

evidence that BEC selectively destroys tumour cells relative to normal cells and the mode of entry of BEC into tumour cells appears to be mediated by the sugar moiety of the solasodine glycosides.

Table 1

Percentage Cell Survival in the Presence of Solasodine Glycosides

	Percentage Survival: Cell Type			
	OvCa	HeLa	LCL	FB
BEC 12.42µM/L	0.6 ± 0.1	27 ± 7	54 ± 4	48 ± 6
Solamargine 11.5µM/L	7 ± 0.8	2.6 ± 0.5	35 ± 4.2	23 ± 1.7
Solasonine 11.3µM/L	93 ± 8.0	105 ± 9.5	117 ± 8.0	96 ± 8.3
di- & mono-glycosides 14.45µM/L	71 ± 9.0	94 ± 8.3	97 ± 11.0	76 ± 5.7

OvCa = ovarian cancer cells;
HeLa = HeLa cells;
LCL = lymphoblastoid cells;
FB = fibroblasts.

Table 2

Percentage Survival of Unstimulated and Stimulated Lymphocytes in the Presence of Solasodine Glycosides

	Percentage Survival			
S.G.	US	PHA	Con A	PWM
BEC 12.42µM/L	63±7	95±4	90±8	100±7.5
Solam 11.5µM/L	76±9.6	93±4.4	79±4	93±9.4
Solas 11.3µM/L	97±11.8	103±5.7	97±7	110±3.8
DMG 14.45µM/L	40±4.6	93±4.2	57±5	103±9.7

n = 10

SG = solasodine glycosides
BEC = Solam, Solas and DMG
Solam = Solamargine
Solas = solasonine
DMG = di- and mono-glycosides
US = unstimulated lymphocytes
PHA, Con A and PWM = stimulated lymphocytes

Table 3.

Dose of Cytotoxic (μM/L) to Inhibit 50% Tritiated Thymidine Uptake LD_{50}) and Expressed as Thymidine Ratio (TR_{50}) Relative to Ovarian Cancer Cells

Cytot	OvCa	HeLa	TR	LCL	TR_{50}	FB	TR_{50}
Solam	1.55 μM/L	2.5 μM/L	1.6	3.95 μM/L	2.5	3.65 μM/L	2.3
V.B.	9.5 μM/L	2.7 μM/L	0.28	2.6 μM/L	0.27	1.9 μM/L	0.2
C.P.	63 μM/L	49.5 μM/L	0.78	50 μM/L	0.79	95.5* μM/L	1.5
CB	60 μM/L	47.5 μM/L	0.79	27 μM/L	0.45	NSE μM/L	-

Cytot = cytotoxic;
V.B. = vinblastine;
CB = chlorambucil;
HeLa = HeLa cells;
FB = fibroblasts
* = extrapolation;
NSE = no significant effect

Solam = solamargine;
C.P. = cis-platinum;
OvCa = ovarian cancer cells;
LCL = lymphoblastoid cells

Table 4

LD_{50} Concentration of Cytotoxic Drugs for Unstimulated and Stimulated Lymphocytes

	CB µM/L	CP µM/L	VB µM/L	DMG µM/L
US	33.0	21.0	11.0	6.4
PHA	24.6	17.0	19.2·	14.8*
Con A	33.0	11.0	7.8··	11.2**
PWM	22.0	9.0	10.8··	196.0***

US = unstimulated lymphocytes
PHA, Con A and PWM = stimulated lymphocytes
CB = chlorambucil
CP = cis-platinum
VB = vinblastine
DMG = di and mono-glycosides of solasodine

* extrapolation r = -0.86 p <0.05 >0.02 (from Fig.1a)
** extrapolation r = -0.91 p = 0.01 (from Fig. 1a)
*** extrapolation r = -0.89 p = <0.02 >0.01 (from Fig. 1a)
· extrapolation r = -0.811 p = 0.05 (from Fig. 1c)
·· extrapolation r = -0.94 p = <0.01 >0.001 (from Fig. 1c)

Table 5.

Effect of Solamargine: Percentage Cell Survival in the Presence and Absence of Lactose, Galactose or Rhamnose

		Percentage Survival			
Cells	Solamargine Concn.	No Carbohydrate	1.1µM/L Lactose	1.1µM/L Galactose	1.1µM/L Rhamnose
OvCa	1.54µM/L	62±7(9)	75±*11(7)	79±**10(10)	67±7.5(9)
HeLa	2.4µM/L	25±2.6(8)	24±2.9(10)	24±2.2(10)	
		24±4(8)			26±2.1(7)
LCL	3.1µM/L	34±3(10)	47***±4.8(10)	42***±3(10)	36±4.5(10)
Fb	3.1µM/L	58±9.6(7)	100***±11(7)	101***±10(10)	87***±6.7(10)

n = 10

OvCa = Ovarian cancer cell line

HeLa = HeLa cells

LCL = Lymphoblastoid cells

Fb = Fibroblasts

Significant inhibition of solamargine

* = p <0.02 >0.01

** = p = 0.001

*** = p <0.001

Table 6

FACS analysis of percentage of cells with lactose lectin receptors

CELL TYPE	4°C	RT
Viral infected cells	49%	55%
Ovarian cancer cells	67%	83%
Fibroblasts	29%	33%
Lymphocytes	27%	43%
Lymphocytes stimulated	52%	72%

Table 7

Percentage Survival of Unstimulated and Con A Stimulated Lymphocytes in the Presence of Solasodine Glycosides and Carbohydrates

	DMG Conc.	Percentage Survival				
			1.1µM/L Lactose	1.1µM/L Galactose	1.1µM/L Glucose	1.1µM/L Rhamnose
US	4.81µM/L	72±12	69±12	77±8.6	76±15	85±12
Con A	4.81µM/L	85±11	75±5.5	84±11.8	109±15*	102±11**

n = 7

* p <0.01 >0.001 ** p <0.02 >0.01

DMG = di- and mono-glycosides

US = unstimulated, Con A = stimulated

It has been demonstrated that alkaloids will induce cellular autophagy (self-digestion) and/or cell agglutination or immobilization. These effects of such alkaloids are greatly enhanced when conjugated to certain ligands, particularly carbohydrates (glycoconjugates). The invention is particularly effective if the cell to be targeted possesses receptors that recognise a rhamnose - or a rhamnose-like - residue. By conjugating different ligands to these alkaloids, it is possible to induce autophagy and/or cell agglutination or immobilization of specific cell types.

The most cytotoxic compound is solamargine, the dose required to inhibit DNA synthesis by 50% (LD_{50}) of an ovarian cancer cell line and HeLa cells being 1.5 - 3.3 times less than that required for lymphoblastoid cells and fibroblasts. The effective LD_{50} of solamargine is 6-40 times less than that of vinblastine, chlorambucil or cis-platinum, compounds that are equally or more cytotoxic to normal cells relative to cancer cells.

Although it is known that neoglycoprotein conjugates of cytotoxic drugs may be suitable for targeting of cells via EELs, this known art has only used mono- or disaccharide conjugates for this targeting of EELs which are also expressed by various normal cells.

Further, such prior art drugs are limited to the treatment of one type of malady and it is not possible to predict the effectiveness of such drugs in the treatment of a different type.

In contrast, the present invention demonstrates, inter alia, a more complex EEL on a ovarian cancer cell line and HeLa cells for the trisaccharide

Gal(1 4)Glu(2 1)Gal, as well as EELs for lactose and galactose.

The presence of an EEL for a trisaccharide such as rhamnose is surprising as rhamnose is a plant sugar and is not generally known to occur in mammalian cells.

It is believed that the present inventors are the first to demonstrate that an EEL for a trisaccharide occurs on cancer cells relative to normal cells such that the difference in EEL expression can be exploited for increased specific targeting of cytotoxic glycoconjugates.

In this regard, given the lack of toxicity of solasodine glycosides relative to other cytotoxic drugs, the unique mode of action, the ability to be targeted via EELs and the potential to produce synthetic glycoconjugates of solasodine with enhanced specificity, the present invention should be of particular importance in cancer chemotherapy.

The present invention is expected to be of great value in the control of cellular function in all types of vertebrates or invertebrates, including bacteria, viruses, protozoa and fungi - for example, in the control of pathogenic organisms in blood, lymph and tissue; any new growth of tissue or tissue that is in an ectopic site; embryonic cells; non-malignant and malignant cells; spermatozoa and semen; ova; and for the control of biochemical manufacturing processes.

Further, it follows that the present invention can be used to prevent cell autophagy and/or cell agglutination or immobilization but change cellular metabolism - for example, the production of a tumour necrosis factor by

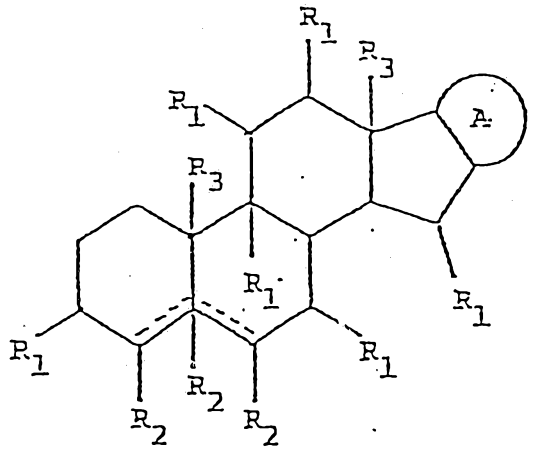
cells, such as macrophages, and/or inhibition of cellular metabolism and catabolism.

It will be appreciated that the above experimental results are given by way of exemplification of the invention only and that changes may be made to the details set out therein without departing from the inventive concept as defined in the following claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells,

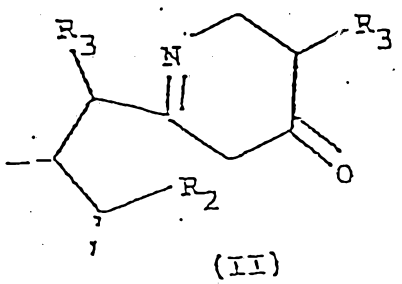
said compound being of the general formula (1):



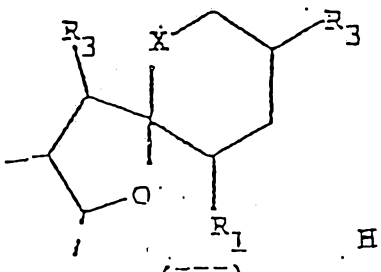
wherein:

either one of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

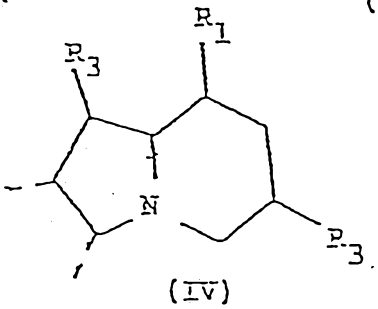
"A" represents a radical selected from the following radicals of general formulae (II) to (V):



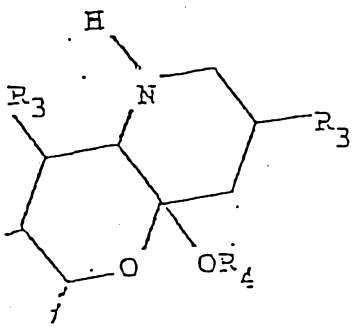
(II)



(III)



(IV)



(V) ;

each of R^1 is a radical separately selected from the group comprising hydrogen, amino, oxo and OR^4;

each of R^2 is a radical separately selected from the group comprising hydrogen, amino and OR^4;

each of R^3 is a radical separately selected from the group comprising hydrogen, alkyl and R^4O-alkylene;

each of R^4 is a radical separately selected from the group comprising hydrogen, carbohydrate and a carbohydrate derivative;

"X" is a radical selected from the group comprising $-CH_2-$, -O- and -NH-;

said method comprising:

(a) identifying receptor sites for said compound on the surface of a target cell which are either not present or only present in significantly reduced numbers on the surface of a non-target cell; and

(b) selecting said compound which will selectively bind to said receptor sites of said target cell and which will exert said control.

2. A method to control cell autophagy, cell agglutination or immobilization of motile cells, said method comprising:

(a) identifying a compound suitable for the required control by the method as defined in Claim 1; and

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(b) binding said compound to said receptor sites of said target cell.

3. A method for the treatment of cancer, contraception, termination of pregnancy, removal of pathogenic organisms or removal of abnormal cellular growth, said method comprising identifying a compound by the method as defined in Claim 1 and administering said compound to a patient requiring treatment.

4. A method of inducing cell autophagy, cell agglutination or immobilization within an animal body (including humans), said method comprising identifying a compound by the method as defined in Claim 1 and administering said compound to a patient requiring treatment.

5. A method of marking and identifying a target cell said method comprising:

(a) identifying a compound suitable to control cell autophagy, cell agglutination or immobilization of motile cells by the method as defined in Claim 1;

(b) further modifying said compound to enable detection of the thus modified compound;

(c) inducing cell autophagy, cell agglutination or cell immobilization; and

(d) detecting said modified compound by any appropriate means.

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6. A method as defined in Claim 5 wherein said compound is modified by further conjugation with another compound, this other compound being detectable by its fluorescence or radioactivity.

7. A method as defined in Claim 6, wherein said compound is reacted with a fluorescent reagent, or wherein said compound is modified to incorporate a radiolabel(s).

8. A method as defined in Claim 1 or Claim 2 wherein said compound is selected from the group comprising progesterone, insulin, oestrogen, growth hormone, growth factors, polyamines, cytokines, lymphokines, lymphotoxins, chalones, fatty acids and cholesterol.

9. A method as defined in any one of Claims 1 to 8 wherein said radical R^4 is selected from the group comprising glyceric aldehyde; glycerose; erythrose; threose; ribose; arabinose; xylose; lyxose; altrose; allose; gulose; mannose; glucose; idose; galactose; talose; rhamnose; dihydroxyacetone; erythrulose; ribulose; xylulose; psicose; fructose; sorbose; tagatose; and other hexoses ($C_6H_{12}O_6$), heptoses ($C_7H_{14}O_7$), octoses ($C_8H_{16}O_8$), nanoses ($C_9H_{18}O_9$) and decoses ($C_{10}H_{20}O_{10}$); deoxysugars with branched chains (such as, apiose, hamamelose, streptose, cordycepose, mycarose and cladinose); compounds wherein the aldehyde, ketone or hydroxyl groups have been substituted (such as with N-acetyl, acetyl, methyl, replacement of CH_2OH); sugar alcohols; sugar acids; benzimidazoles; the enol salts of the carbohydrates; saccharinic acids; and sugar phosphates.

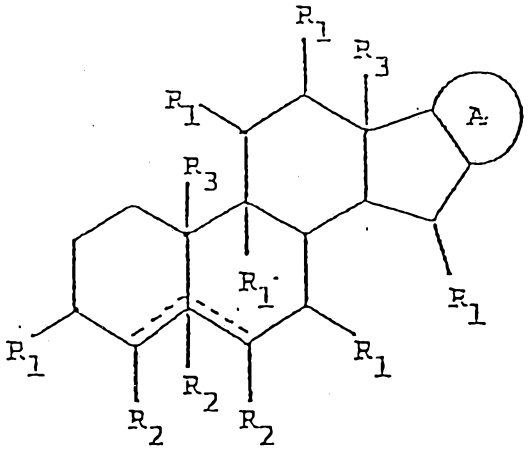


10. A method as defined in any one of Claims 1 to 9, wherein said compound is selected from the group comprising solasodine, solanidine, diosgenin, solangustidine, leptinidine, solacongestidine, solafloridine, demissidine, soladulcidine, tomatidenol, paniculidine, jurjubidine, tigogenin, yamogenin, neotigogenin, solanocapsine, 26-aminofurostane, and a glycoside of any one thereof.

11. A method as defined in Claim 10, wherein said compound is selected from the group comprising solasodine, solanidine, solasonine, solamargine and solamine.

12. A method as defined in Claim 11, wherein said compound is solamargine.

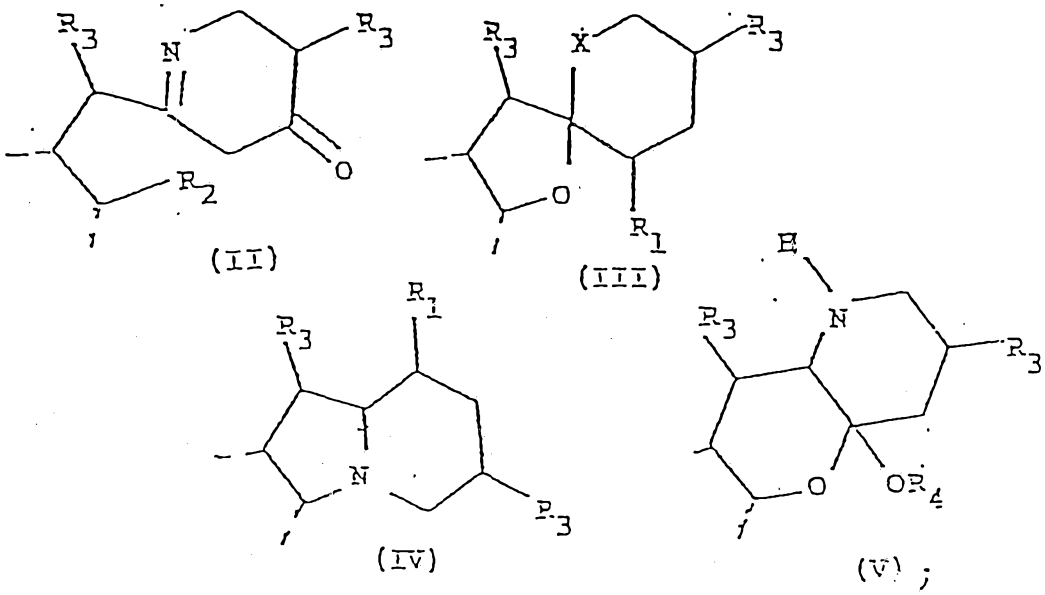
13. Compounds of the general formula (1):



wherein:

either one of the dotted lines represents a double bond, and the other a single bond, or both represent single bonds;

"A" represents a radical selected from the following radicals of general formulae (II) to (V):



(II) (III) (IV) (V);

each of R^1 is a radical separately selected from the group comprising hydrogen, amino, oxo and OR^4;

each of R^2 is a radical separately selected from the group comprising hydrogen, amino and OR^4;

each of R^3 is a radical separately selected from the group comprising hydrogen, alkyl and R^4O-alkylene;

each of R^4 is a radical separately selected from the group comprising hydrogen, carbohydrate and a carbohydrate derivative;

"X" is a radical selected from the group comprising $-CH_2-$, -O- and -NH-;

when used in a method as defined in any one of Claims 1 to 12.

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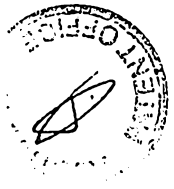
14. A method as defined in Claim 1 substantially as hereindescribed with reference to the Examples.

15. A method as defined in Claim 2 substantially as hereindescribed with reference to the Examples.

DATED this Thirty-First day of August 1994.

CURA NOMINEES PTY LTD
(ACN 010 391 974)
By its Patent Attorneys
GRANT ADAMS & COMPANY



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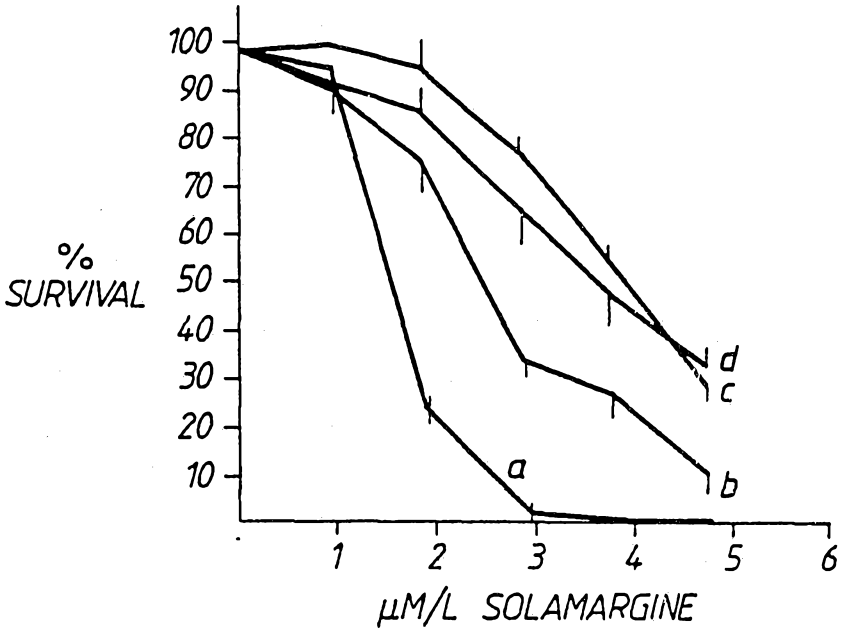

Fig.1a.

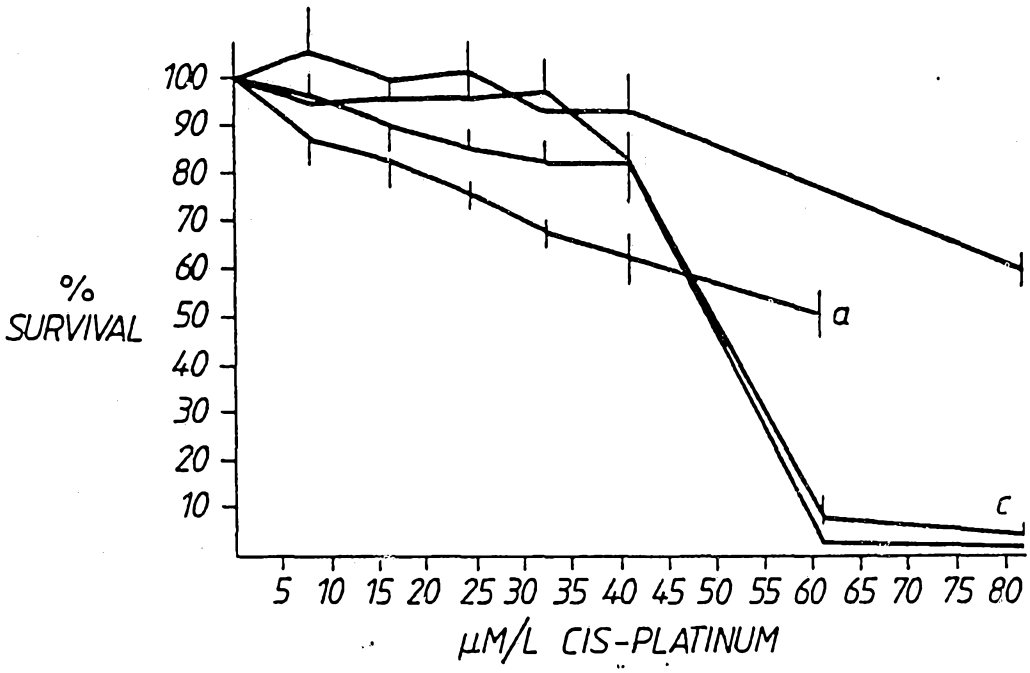

Fig.1b.

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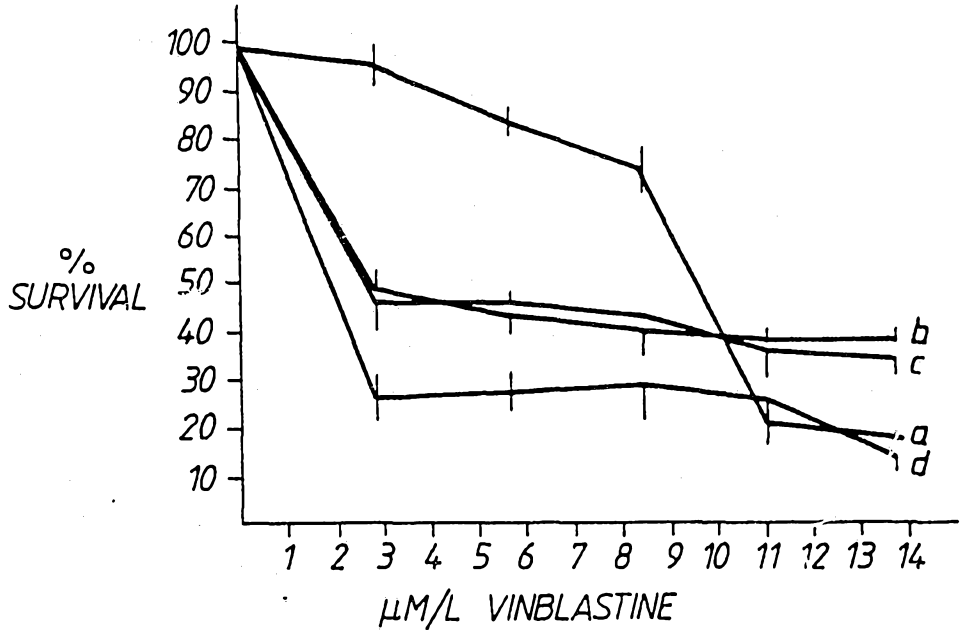

100
90
80
70
60
50
40
30
20
10
% SURVIVAL
1 2 3 4 5 6 7 8 9 10 11 12 13 14
µM/L VINBLASTINE
a
b
c
d

Fig.1c.

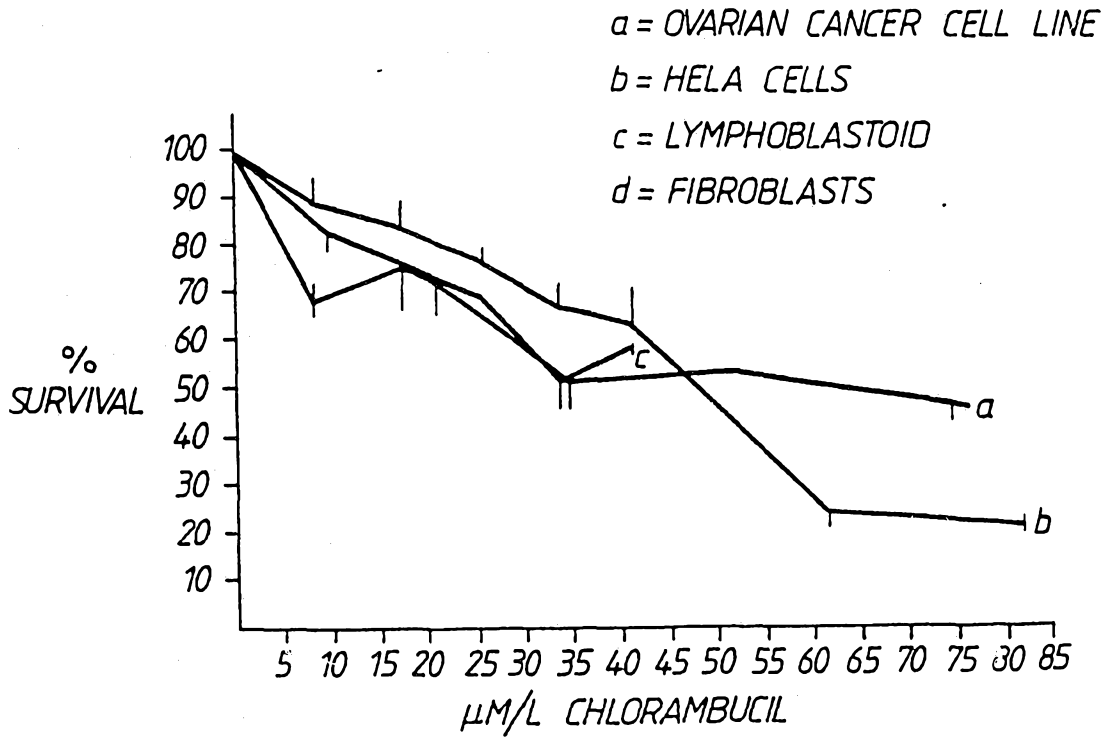
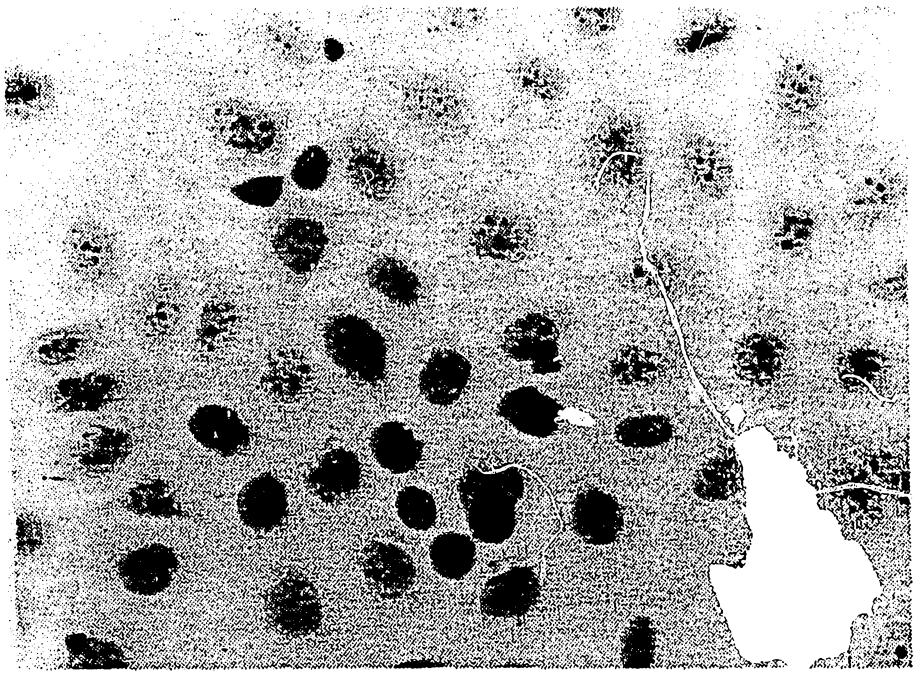
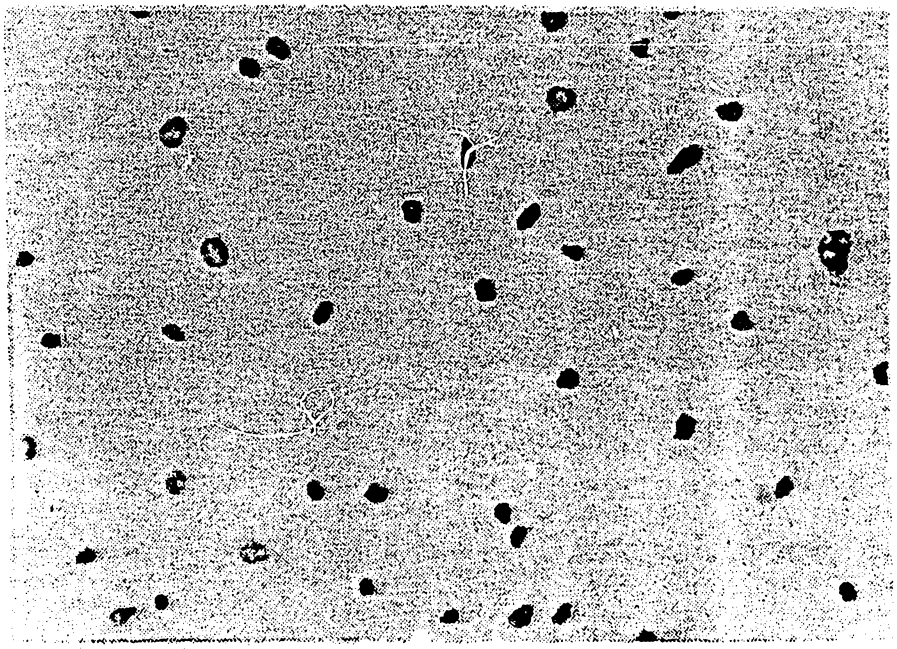

a = OVARIAN CANCER CELL LINE
b = HELA CELLS
c = LYMPHOBLASTOID
d = FIBROBLASTS
100
90
80
70
60
50
40
30
20
10
% SURVIVAL
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85
µM/L CHLORAMBUCIL
a
b
c

Fig.1d.



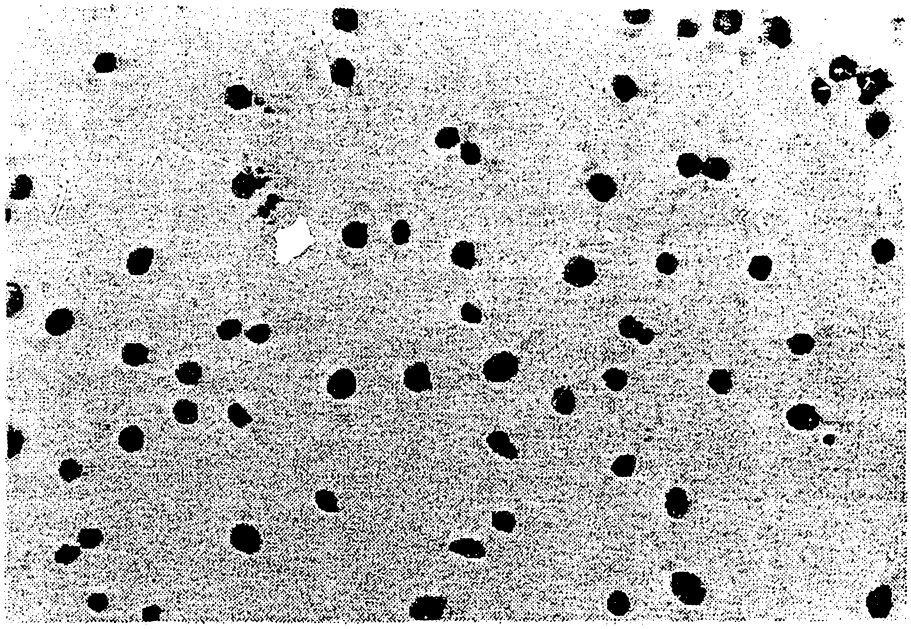
OVARIAN CANCER CELLS IN THE PRESENCE OF THE AGLYCONE SOLASODINE 96·8 μM/L 3 HOURS

Fig.2A. (×1000)



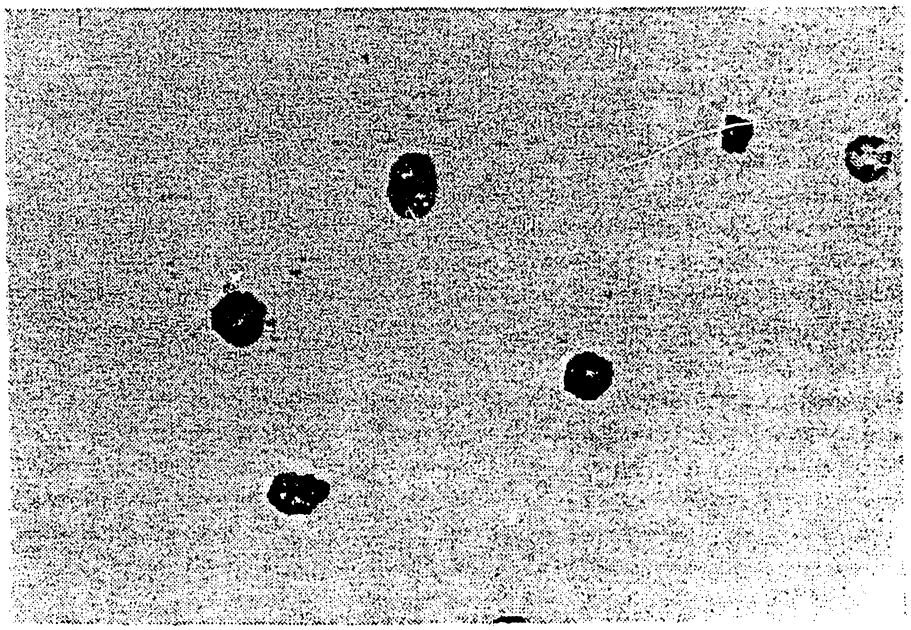
OVARIAN CANCER CELLS IN THE PRESENCE OF SOLASODINE GLYCOSIDES EXPRESSED IN TERMS OF SOLAMARGINE 6·1 μM/L 3 HOURS

Fig.2B. (×1000)



OVARIAN CANCER CELLS IN THE PRESENCE OF SOLASODINE GLYCOSIDES EXPRESSED IN TERMS OF SOLAMARGINE 9·6 μM/L 3 HOURS

Fig.3A. (×1000)



OVARIAN CANCER CELLS IN THE PRESENCE OF SOLASODINE GLYCOSIDES EXPRESSED IN TERMS OF SOLAMARGINE 11·1 μM/L 3 HOURS

Fig.3B. (×1575)

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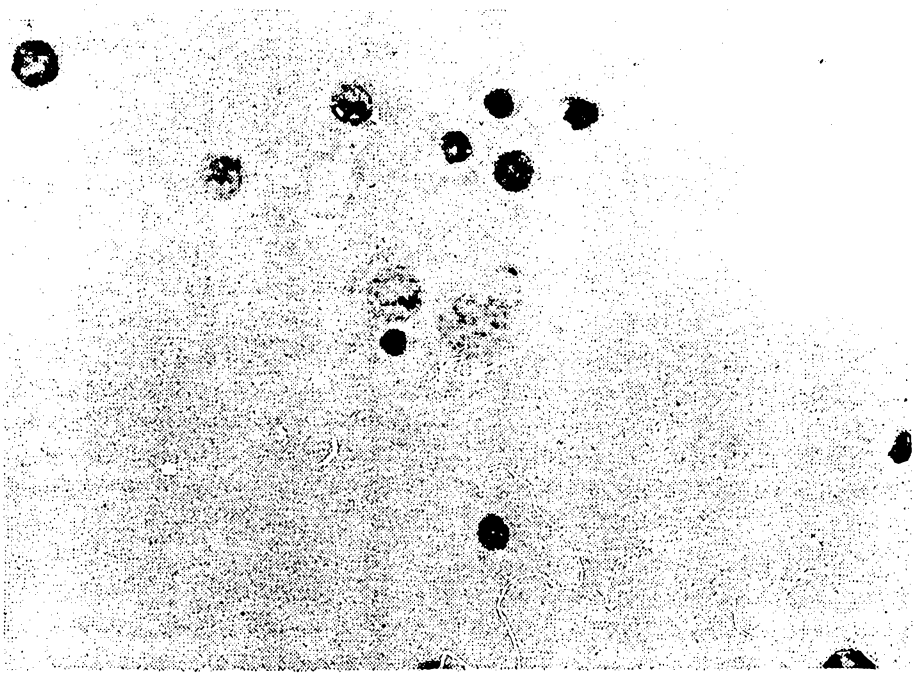
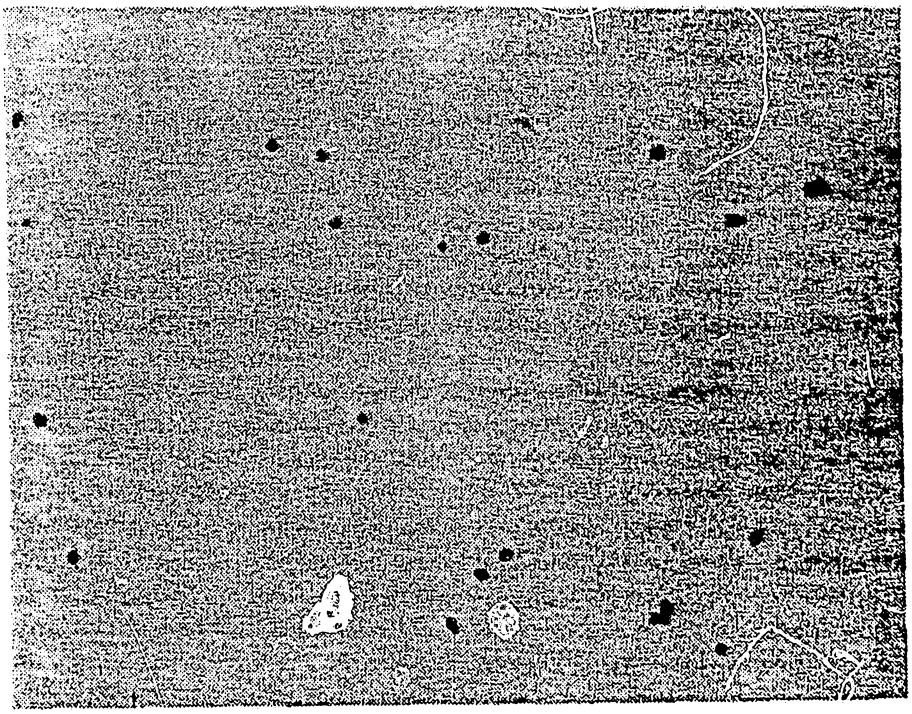


OVARIAN CANCER CELLS IN THE PRESENCE OF SOLASODINE GLYCOSIDES EXPRESSED IN TERMS OF SOLAMARGINE 15·3 μM/L 3 HOURS

Fig.4A. (×1575)



OVARIAN CANCER CELLS IN THE PRESENCE OF SOLASODINE GLYCOSIDES EXPRESSED IN TERMS OF SOLAMARGINE 3·1 μM/L 17 HOURS

Fig.4B.

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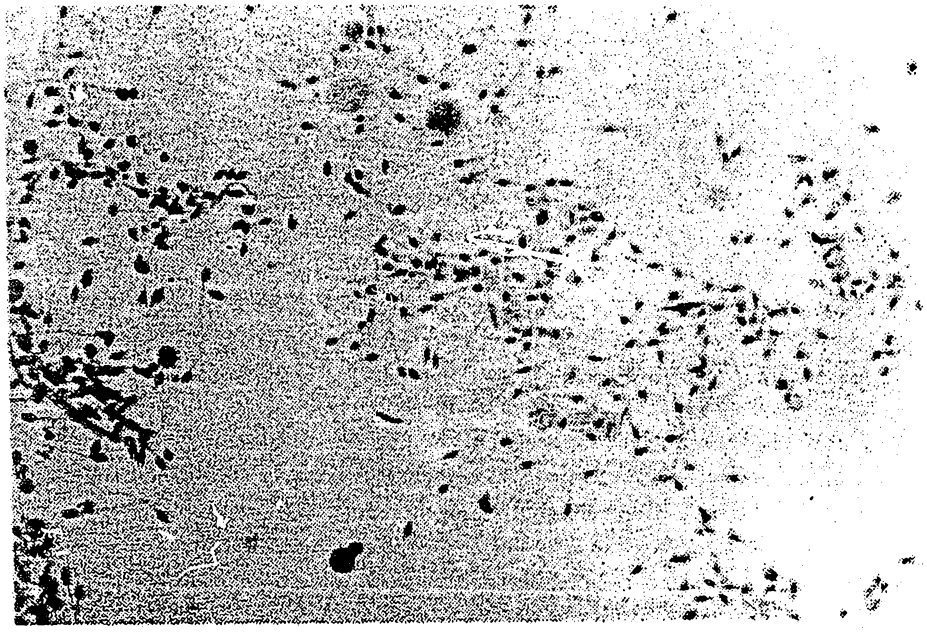

DEAD INTACT SPERMATOZOA IN THE ZONE OF SOLAMARGINE/EOSIN

Fig.5.

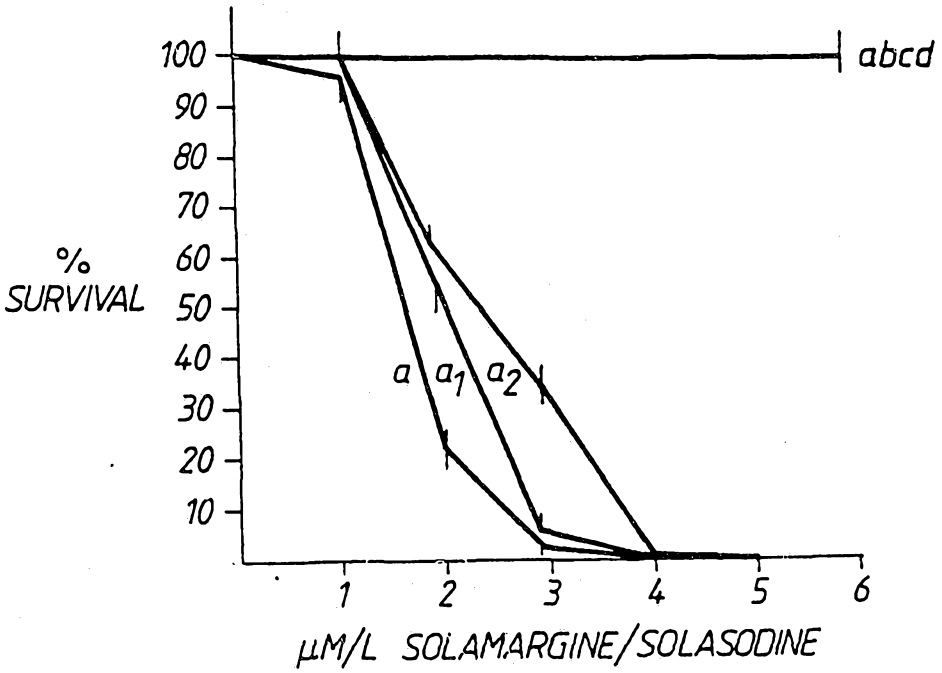

Fig.6.

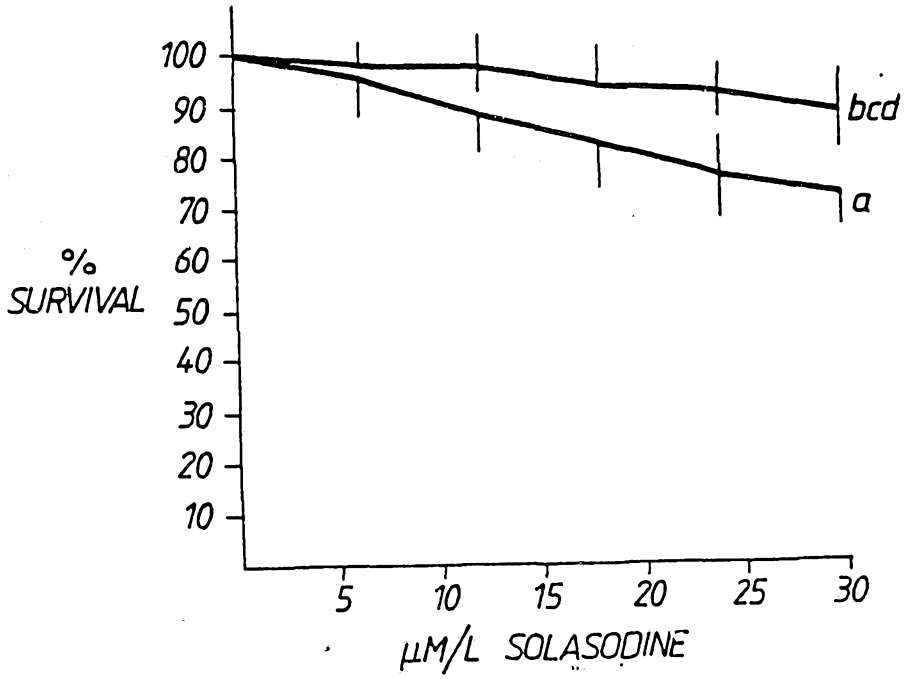

Fig.7.

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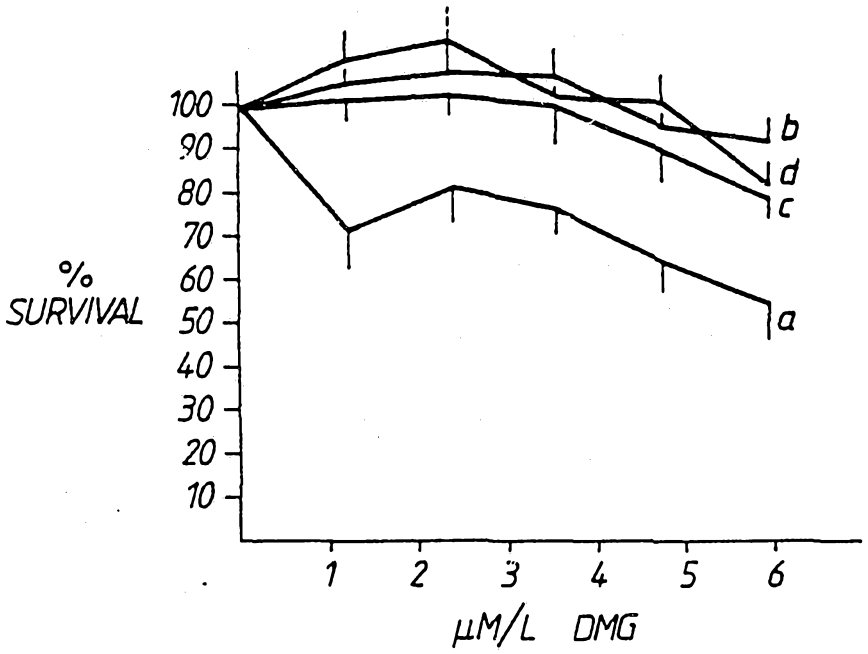
100
90
80
70
60
50
40
30
20
10
% SURVIVAL
1
2
3
4
5
6
μM/L DMG
a
b
c
d

Fig.8a.

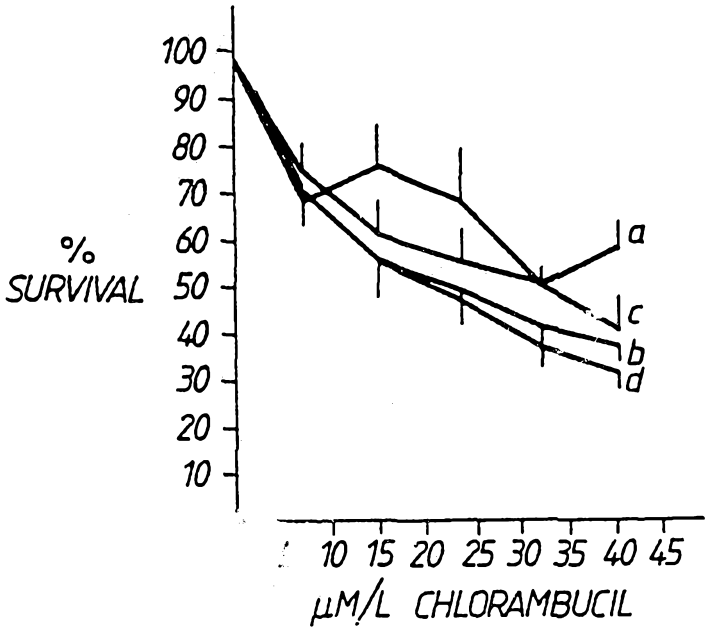
100
90
80
70
60
50
40
30
20
10
% SURVIVAL
10 15 20 25 30 35 40 45
μM/L CHLORAMBUCIL
a
c
b
d

Fig.8b.

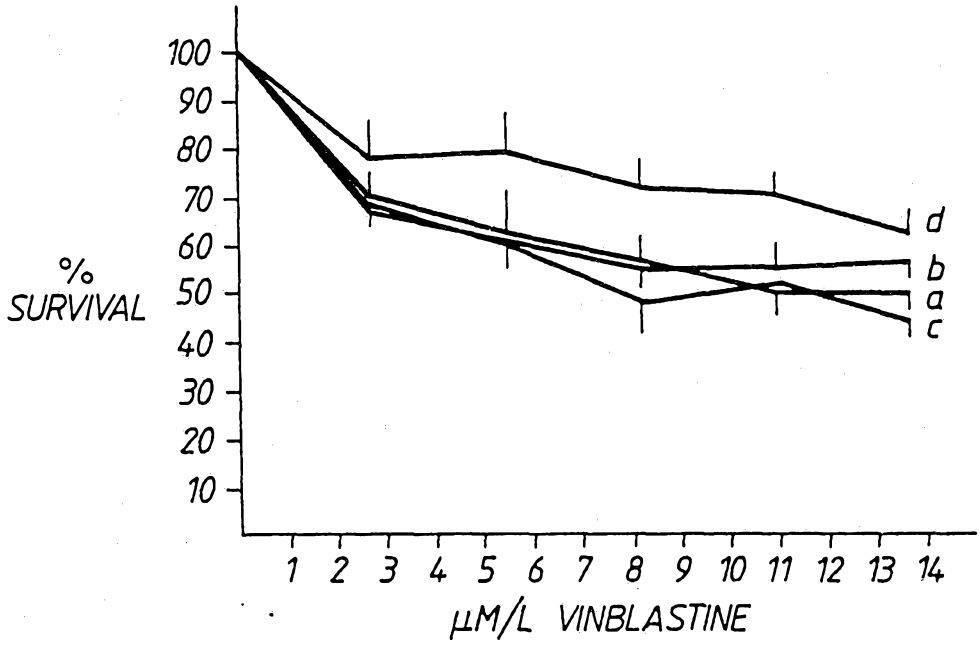

Fig.8c.

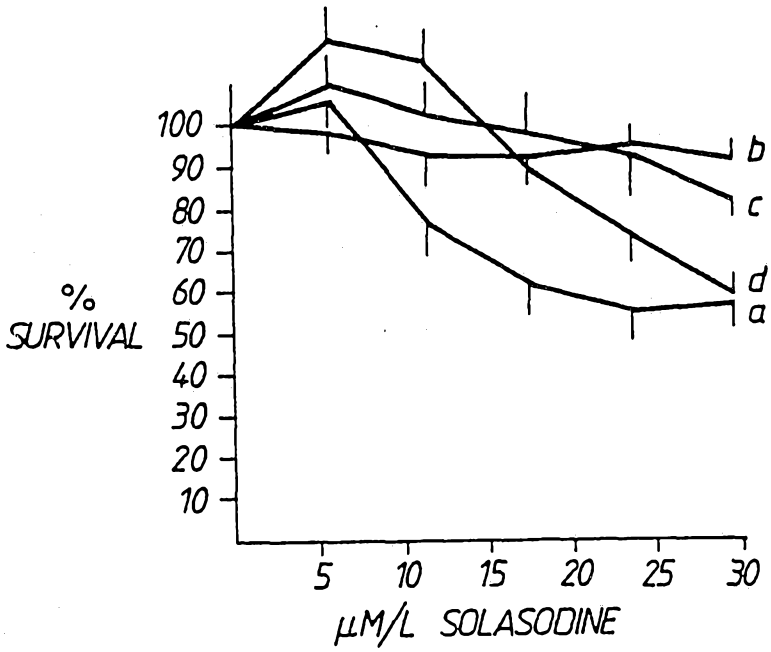

Fig.8d.

SUBSTITUTE SHEET

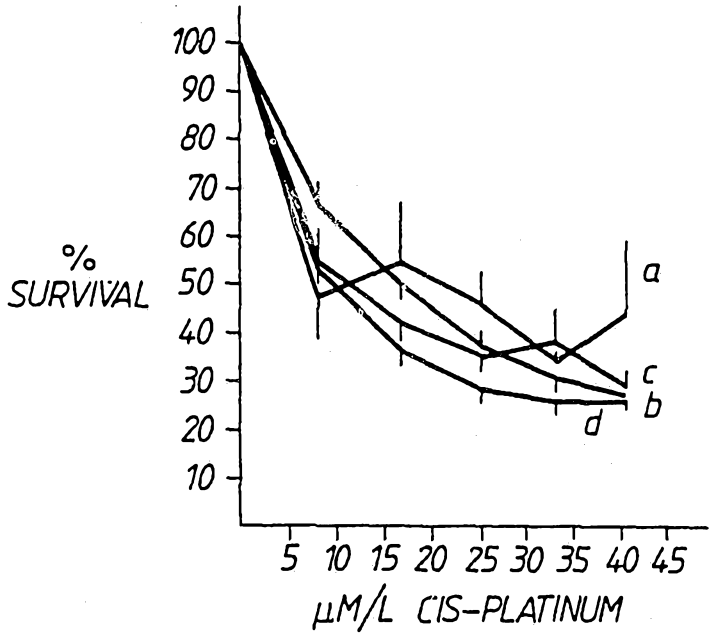
100
90
80
70
60
50
40
30
20
10
% SURVIVAL
a
c
b
d
5 10 15 20 25 30 35 40 45
μM/L CIS-PLATINUM

Fig.8e.

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PCT/AU 91 / 00020

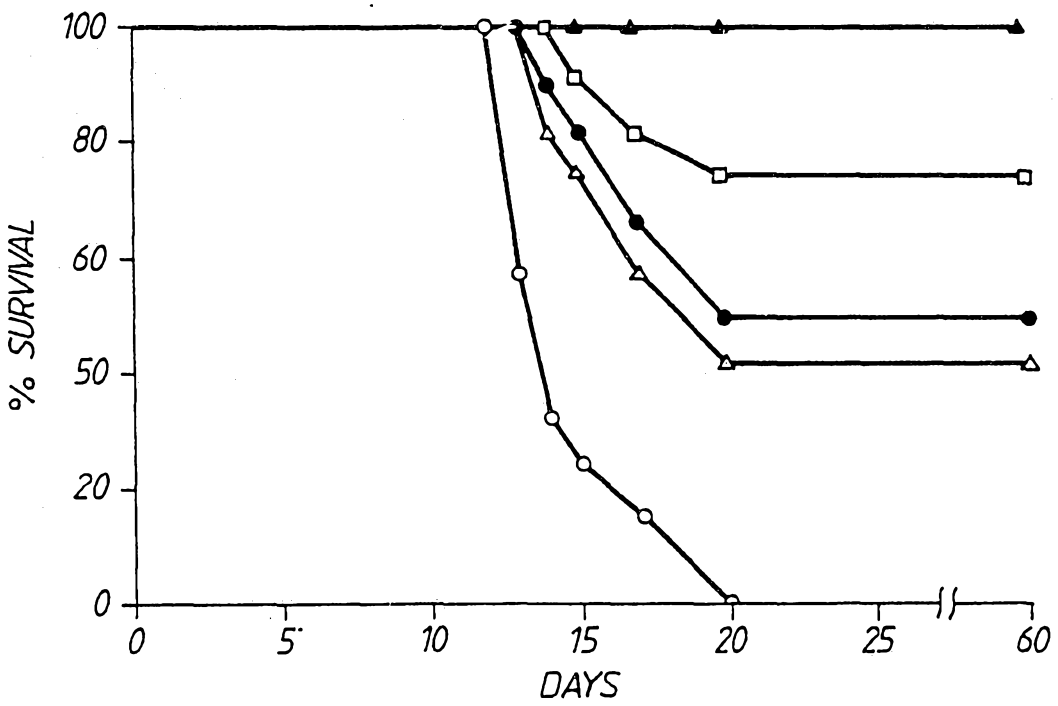

Fig.9.

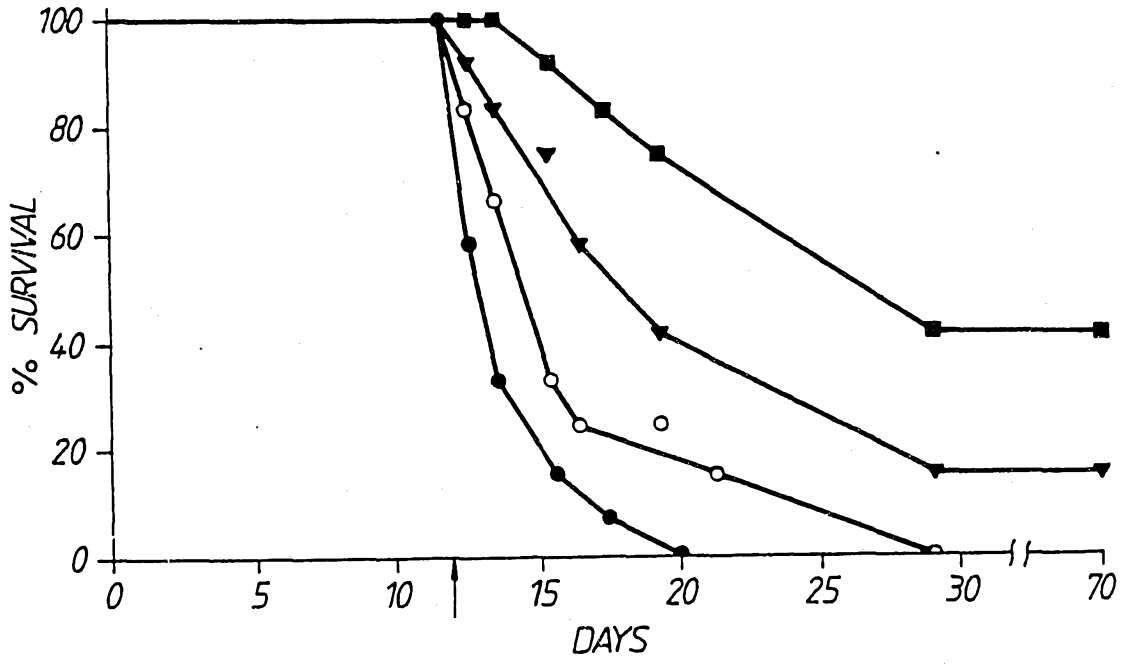

Fig.10.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00020

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] C12Q 1/02, A61K 31/58, 37/26, 37/36, 31/705, 49/02, C07J 21/00, 43/00, G01N 33/532 33/569 33/574 33/74

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	C12Q 1/02, A61K 31/58, C07J 71/00, 43/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document,[11] with indication,[12] where appropriate, of the relevant passages	Relevant to Claim No 13
X	AU,A, 74958/87 (Bristol-Myers Company) 7 January 1988 (07.01.88). See particularly pages 5-8	(1-5)
X	Martinez, O and Wofsy, L 1986 "Immunotoxins" in D M Weir (ed) Immunochemistry Volume I. Blackwell Scientific Publications (London) See entire document, particularly page 37.1, column 1	(1-7)
X	S Budavari (Ed), "The Merck Index" 11th Edition, published 1989, by Merck (Rahway, N J), Entries 8665, 8660, 3289, 9367, 8662	(8-14)
X	L F Fieser and M Fieser, "Steroids", published 1959, by Reinhold (New York), see pages 777, 819, 848	(8-14)
X	E Heftmann and E Mosettig, "Biochemistry of Steroids", published 1960, by Reinhold (New York), see pages 48, 55	(8-14)
X	Annu Rev Biochem, Volume 59, 1990, K Arai et al, "Cytokines: Co-ordination of Immune and Inflammatory Responses", see pages 783-836	(8-14)
X	Derwent Abstract Accession no. 35682D/20, Class B01, SU,A, 724521	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1 May 1991 (01.05.91)	3 May 1991
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	(AS MOLD MICROBIOL) 30 March 1980 (30.03.80)	(8-14)
X	Derwent Soviet Inventions Illustrated, issued November 1972, Pharmaceuticals, Photographic p.7. SU 336023 (TUROVA et al) 24 May 1972 (24.05.72)	(8-14)
X	Patent Abstracts of Japan, C280, page 73, JP,A, 60-1130 (TAKUO KOSUGE) 7 January 1985 (07.01.85)	(8-14)
X	Derwent Abstract Ascension no. 90-338476/45, class B01, JP,A, 2243631 (WAKUNGA SEIYAKU KK) 27 September 1990 (27.09.90)	(8-14)
X	CA,A, 1151549 (ARUBA (QLD) PTY LTD) 9 August 1983 (09.08.83)	(8-14)
X	GB,A, 1380253 (INVERNI DELLA BEFFA SpA) 8 January 1975 (08.01.75)	(8-14)
X	FR,A, 943800 (LABORATORIES JOUVEINAL) 2 November 1964 (21.11.64)	(8-14)
X	DE,A, 1804331 (CARBO ERBA SpA) 22 May 1969 (22.05.69)	(8-14)
X	DE,A, 1900060 (LENTA GmbH) 13 August 1970 (13.08.70)	(8-14)
X	DE,A, 2519261 (DEUTSCHE GOLD-UND SILBER SCHEINDEANSTALT VORMALS ROESSLER) 11 NOVEMBER 1976 (11.11.76)	(8-14)
X	US,A, 4937182 (HANCOCK et al) 26 June 1990 (26.06.90)	(1-7)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.[] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

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

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

2. [] Claim numbers ..., because they relate to parts of the international application that do comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

VI.[] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

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

3. [] 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 claim numbers:

4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

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

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