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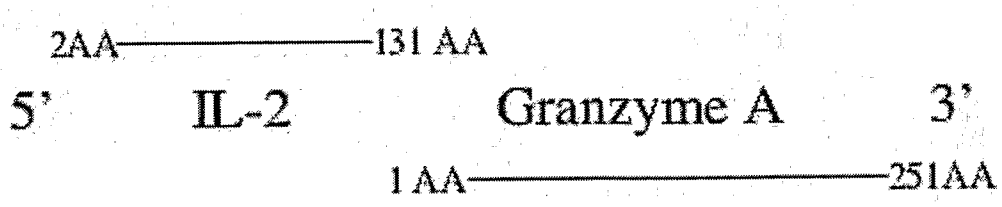

(57) **Abstract:** A chimeric protein comprising a cell targeting moiety and a cell killing moiety, wherein the cell killing moiety induces alternative programmed cell death. Also disclosed is a method for treating a subject suffering from a disease involving cells which are multi-drug resistant. The method comprises administrating to the subject a pharmaceutical composition comprising the aforementioned chimeric protein.

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TARGETED CHIMERIC PROTEINS

FIELD OF THE INVENTION

This invention relates to chimeric proteins which may be used for killing specific cells, particularly within the framework of a therapeutic treatment.

BACKGROUND OF THE INVENTION

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Caspase dependent cell death:

Apoptosis is a well-controlled programmed cell death (PCD) with specific morphological characteristics; condensation of chromatin, shrinkage of the cytoplasm, blebbing of the plasma membrane and formation of apoptotic bodies. In its classical form, apoptosis can occur only when caspases, especially caspase 3 are activated. Caspases are cystein proteases that exist as zymogens which are activated by proteolytic cleavage. Two separate pathways leading to caspase activation have been characterized. The extrinsic pathway is initiated by ligation of transmembrane death receptors (CD95, TNF and TRAIL receptors) to activate membrane proximal caspases (caspase 8 and 10) which in turn activate effector caspases 3 and 7. The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins including Smac/Diablo, HtRA2 and cytochrome c. Cytochrome c functions with Apaf-1 to induce activation of caspase 9, thereby initiating the apoptotic caspase dependent cascade, while Smac/Diablo and HtRA2 bind to and antagonize inhibitors of apoptosis proteins IAPs. Ultimately, caspase dependent DNase (CAD/DFF40) is activated and fragments chromosomal DNA into oligonucleosomes.

Recently, it was discovered that caspase-dependent apoptosis is not the only existing PCD. There is also PCD that doesn't depend upon caspases, but has many features resembling apoptosis. It is called the *alternative PCD* or *caspase independent cell death*. Granzyme A is one of many recently discovered executioners of this process.

The biology of Granzymes

Cytotoxic T and Natural Killer (NK) cells are the effector lymphocytes that defend the body from transformed or virus infected cells. They kill their target by two main mechanisms: (1) releasing the cytotoxic ganules` content into the intracellular space by exocytosis, and (2) engagement of cell surface killing receptors.

Cytotoxic granules are lysosomes that exist only in cells with cytolytic ability. Those granules contain the pore forming protein – Perforin, and a family of serine proteases called Granzymes. The Granzymes are first formed as inactive zymogens in the endoplasmic reticulum and are converted into active enzymes after a two step

process; cleavage of the leader peptide which leaves two amino acids attached at the mature amino terminal and then processing of the pro protein by Cathepsin C into an active form, taking place on the way or already in the granules (1). The Granzymes are inactive in the acidic pH of the granules and are non covalently bound to the negatively charged proteoglycan, Serglycin.

Once the effector lymphocytes recognize their target, an immunological synapse is formed. The granules are exocytosed and perforin enters the cell membrane and polymerizes in a Ca^{2+} dependent way to create a channel through which the granzymes pass.

Members of the granzyme family are known to be involved in alternative programmed cell death. Granzyme A is one of the most abundant granzymes known to be involved in cell death processes, using the alternative programmed cell death pathway (2).

Granzyme A

Granzyme A is composed of a disulfide linked homo dimer of 50 kDa with both active catalytic sites. In contrast to all the granzymes that contain six cysteine residues, which are involved in intramolecular disulfide bonding, granzyme A contains three additional cysteine residues; two form a fourth intramolecular disulfide linkage and the third is responsible for the disulfide linkage of the homodimer.

In the cell, Granzyme A targets a 270-420 kDa endoplasmic reticulum (ER) associated complex, called SET. It includes nuclear assembly protein, SET, DNA binding protein, HMG2, base excision repair pathway apurinic endonuclease, Ape1, tumor suppressor protein, pp32 and transcriptional regulator and nucleoside diphosphate kinase, NM23-H1. The SET complex components participate also in other cellular complexes and functions (3). It may be naturally involved in regulating chromatin structure, integrity and gene expression and in oxidative stress repair response since it has been shown to translocate to the nucleus in response to oxidative stress (3, 4). This complex is most possibly important in preventing oncogenic transformation, since three of its members (SET, pp32, NM23-H1) are implicated in cancer (3).

Granzyme A dependent cell death

As stated above, Granzyme A is involved in alternative PCD. Cell death induced by Granzyme A and perforin is rapid. Within minutes the integrity of the membrane is disrupted and membrane blebbing appears. Within a few hours, chromatin condensation and nuclear fragmentation occur. However, there is neither caspase activation, nor the activation of caspase key substrates like Bid and DFF40. Although the mitochondrial transmembrane potential and function are disrupted and reactive oxygen species (ROS) accumulate, there is no release of cytochrome C or apoptotic mediators like apoptosis inducing factor (AIF), endonuclease G etc. (3).

When Granzyme A enters the cell it causes a rise in ROS that facilitates the translocation of the SET complex to the nucleus (4). There, Granzyme A has an effect on several components:

1. It cleaves the SET complex releasing NM23-H1 thus allowing it to nick DNA.
2. It cleaves Ape1 thus impairing its ability to repair DNA.
3. It attacks the nuclear envelope and the chromatin. It cleaves lamins, the key intermediate filaments that reside beneath the nuclear envelope and provide the anchor sites for chromatin and nuclear pore complex. This feature is common both to Granzyme A and to caspase dependent PCD.
4. It degrades the linker histone H1, which anchors the DNA around the core histones. This action enables most probably the accessibility of exogenous nucleases, such as Nm23-H1, DFF40 and Endonuclease G to DNA.

It is important to note that mitochondrial damage imposed by Granzyme A is caspase independent and is not affected by pan-caspase synthetic inhibitors or Bcl-2 overexpression (3).

Multi Drug Resistance

Multi drug resistance (MDR) is a phenomenon of cancer and other cells acquiring simultaneous resistance to several unrelated drugs that leads to poor prognosis in therapy (5).

The classical drug resistance arises as a result of expression of ATP dependent efflux pumps with broad drug specificity. They belong to a family of ATP binding cassette (ABC) and share sequence and structure homology. The resistance is a

result of increased drug efflux that significantly lowers the intracellular drug concentration. The drugs affected by this resistance include Vinca alkaloids, (vinblastine, vincristine), Anthracyclines (doxorubicin, daunorubicin), Podophyllotoxins (etoposide),Taxanes (taxol), Actinomycin D, Paclitaxel and more (5).

All ABC family pumps are also expressed on normal tissues. They protect the brain, testis, placenta, gastrointestinal tract and kidneys from cytotoxins.

P-glycoprotein (Pgp) pump is the main mediator of MDR. It is a 170 kDa membrane protein encoded by the MDR1 gene. It has two identical 6 transmembrane regions and two ATP binding sites. The transmembrane regions bind hydrophobic positively charged or neutral drugs. Pgp flips the drug from inner leaflet to the outer leaflet and into extracellular media. In order to transport one drug molecule, two ATP hydrolysis events are needed: one, to change the conformation in order to release the substrate and in the second, another ATP is utilized to re-set to the original conformation of the pump.

Pgp is highly expressed in breast, colon, kidney, adrenocortical and hepatocellular cancers. In acute myelogenous leukemia (AML), pumps' expression correlates with reduced complete remission rate and poor prognosis, which are supported by ex vivo data of Pgp reducing the intracellular accumulation of daunorubicin in leukemic cells.

Pgp can also confer resistance to apoptosis induced by several non-drug stimuli: Fas, TNF, UVB, serum starvation and γ irradiation. Apparently it inhibits caspase activation, specifically caspases 3 and 8, even when its effluxing ability is damaged. This may be due to Pgps' ability to elevate pH, which interferes with caspase activation occurring at acidic conditions. It is known that many chemotherapeutic drugs act through caspases. Hence, cell death that functions in the absence of caspase activation can kill Pgp positive cells.

IL-2 receptor (IL-2R)

IL-2 is a cytokine produced by activated T cells. It has an important role in both activation and maintenance of an immune response and in lymphocyte development. It acts by binding to high affinity IL-2 receptor (IL-2R).

IL-2R is composed of 3 subunits. The α subunit has a long extracellular domain responsible for the IL-2 binding. It is expressed on activated T and B lymphocytes.

Alone it represents a low affinity state. β and γ chains both have long intracellular domains. They are constitutively expressed on resting lymphocytes, monocytes and neutrophils and form together an intermediate affinity state receptor. β and γ chains are also part of other cytokine receptors, thus the α subunit is a key for receptor specificity. The three subunits together comprise a high affinity IL-2R.

Given its central role in the immune system, IL-2R was utilized to treat conditions like neoplasia, autoimmune diseases and organ allograft rejection. In cancer therapy, many IL-2 based proteins have been developed, the best known one being an FDA approved chimeric protein named Ontak. It is an IL-2 molecule fused to Diphtheria toxin that was produced by recombinant technology (6). Ontak is used to treat cutaneous T cell lymphoma (CTCL). Because medications like Ontak contain bacterial or plant toxins, which are not of human origin, it raises several complications and side effects. The body may develop neutralizing antibodies against the toxin, which accelerates the clearance of the drug from the body. Such chimeras may cause flu like syndrome, vascular leak syndrome and hypersensitivity reactions.

WO 97/46259 discloses chimeric toxins comprising cell targeting moieties and cell killing moieties for recognizing and for destroying neoplastic cells, wherein the cell targeting moieties consist of gonadotropin releasing hormone homologues and the cell killing moieties consist of Pseudomonas Exotoxin A.

WO 97/22364 discloses a chimeric protein for the therapy of allergic responses. The protein is comprised of a cell targeting moiety for FcεRI expressing cells and a cell killing moiety. The preferred killing moiety is the bacterial toxin Pseudomonas exotoxin (PE).

WO 99/45128 discloses chimeric proteins with cell-targeting specificity and apoptosis-inducing activities. In particular, the invention is illustrated by a recombinant chimeric protein between human interleukin-2 (IL2) and Bax. The chimeric protein specifically targets IL2 receptor (IL2R)-expressing cells and induces cell-specific apoptosis.

SUMMARY OF THE INVENTION

An object of the invention is to provide a chimeric protein which may be useful in the treatment of cancer cells in general, and of MDR positive cells in particular.

Thus, the present invention provides a chimeric protein comprising a cell targeting moiety and a cell killing moiety, wherein the cell killing moiety induces alternative programmed cell death.

In the present specification, *alternative programmed cell death* or *caspase independent cell death* is characterized by one or more, and preferably most or all, of the following characteristics: (1) no caspase activation, (2) not inhibited by Bcl-2, (3) no cytochrome C release and (4) single stranded DNA nicks.

Moreover, as this is a chimeric protein that targets the IL-2 receptor; it can be applied to kill any cells expressing the high affinity IL2 receptor. The chimeric protein of the invention may therefore be used to treat graft rejection, autoimmune diseases, infectious diseases, etc.

The cell killing moiety may be any molecule which induces alternative programmed cell death. In one embodiment, the cell killing moiety is of human origin. Examples of molecules which can serve as the cell killing moiety include, but are not limited to, perforin, and the granzyme family. Members of the granzyme family include ten granzymes (A, B, C, D, E, F, G, H, K and M) which have been identified in the mouse, and five which are presently known in humans (A, B, H, M and tryptase-2, which is also known as granzyme 3).

In one embodiment, the *cell targeting moiety* is selected from antibodies, cell membrane receptor ligands, cytokines, growth factors and hormones. In a further embodiment, the cell targeting moiety is selected from interleukin 2 (IL-2), gonadotropin releasing hormone (GnRH), myelin basic protein (MBP), the Fc fragment of an IgE antibody, epidermal growth factor (EGF) and insulin-like growth factor.

In one exemplary embodiment of the invention, the chimeric protein is composed of the targeting moiety, IL-2 and the killing moiety, Granzyme A. This chimeric protein is referred to as IL-2-Granzyme A (IGA). IL-2 binds to cells expressing the high affinity IL-2 receptor. Use of a chimeric protein composed of human proteins, such as the proposed IGA, is not expected to provoke an immune response, thus improving its future application for human clinical use.

In one embodiment, the IL-2-Granzyme A molecule of the invention comprises the following amino acid sequence (SEQ. ID. NO:1):

MADPTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMP
KKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELK
GSETTFMCEYADETATIVEFLNRWITFCQSIISTIPEGEAFIIGGNEVTPHSR
PYMVLLSLDRKTICAGALIAKDWVLTAAHCNLNKRSQVILGAHSITREE
PTKQIMLVKKEFPYPCYDPATREGDLKLLQLTEKAKINKYVTILHLPKK
GDDVKPGTMCQVAGWGRTHNSASWSDTLREVNITIIDRKVCNDRNHY
NFNPVIGMNMVCAGSLRGGRDSCNGDSGSPLLCEGVFRGVTSFGLENK
CGDPRGPGVYILLSKKHLNWIIMTIKGAVZ

The invention also provides nucleotide sequences coding for the chimeric protein of the invention, particularly that of the IL-2-Granzyme A. In view of the degenerate nature of the genetic code, there are many different nucleotide sequences which encode a given protein such as the IL-2-Granzyme A. All such sequences are included within the scope of the invention. The following is one embodiment of a DNA sequence coding for the IL-2-Granzyme A (SEQ. ID NO:2):

ATGGCAGATCCTACTTCAAGTTCTACAAAGAAAACACAGCTACAACT
GGAGCATTTACTGCTGGATTTACAGATGATTTTGAATGGAATTAATA
ATTACAAGAATCCCAAACTCACCAGGATGCTCACATTTAAGTTTTAC
ATGCCCAAGAAGGCCACAGAACTGAAACATCTTCAGTGTCTAGAAG
AAGAACTCAAACCTCTGGAGGAAGTGCTAAATTTAGCTCAAAGCAA
AAACTTTCACTTAAGACCCAGGGACTTAATCAGCAATATCAACGTAA
TAGTTCTGGAACTAAAGGGATCTGAAACAACATTCATGTGTGAATAT
GCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTAC
CTTTTGTCAAAGCATCATCTCAACAATCCCCGAGGGCGAAGCTTTCA
TTATTGGAGGAAATGAAGTAACTCCTCATTCAAGACCCTACATGGTC
CTACTTAGTCTTGACAGAAAAACCATCTGTGCTGGGGCTTTGATTGC
AAAAGACTGGGTGTTGACTGCAGCTCACTGTAACTTGAACAAAAGGT
CCCAGGTCATTCTTGGGGCTCACTCAATAACCAGGGAAGAGCCAACA
AAACAGATAATGCTTGTTAAGAAAGAGTTTCCCTATCCATGCTATGA
CCCAGCCACACGCGAAGGTGACCTTAAACTTTTACAGCTGACGGAAA
AAGCAAAAATTAACAAATATGTGACTATCCTTCATCTACCTAAAAAG
GGGGATGATGTGAAACCAGGAACCATGTGCCAAGTTGCAGGGTGGG

GCAGGACTCACAATAGTGCATCTTGGTCCGATACTCTGAGAGAAGTC
AATATCACCATCATAGACAGAAAAGTCTGCAATGATCGAAATCACTA
TAATTTTAACCCTGTGATTGGAATGAATATGGTTTGTGCTGGAAGCCT
CCGAGGTGGAAGAGACTCGTGCAATGGAGATTCTGGAAGCCCTTTGT
TGTGCGAGGGTGTTTTCCGAGGGGTCACTTCCTTTGGCCTTGAAAATA
AATGCGGAGACCCTCGTGGGCCTGGTGTCTATATTCTTCTCTCAAAG
AAACACCTCAACTGGATAATTATGACTATCAAGGGAGCAGTTTAA

Also included within the scope of the invention, as aforesaid, are nucleotide sequences that differ from SEQ. ID. NO:1 in view of the degenerate nature of the genetic code.

Also included in the invention is a polypeptide which consists of an amino acid sequence of SEQ. ID. NO: 1 in which one or more amino acid residues is added, deleted or replaced, without significantly affecting the biological characteristics of the modified molecule as compared to the unmodified molecule. According to certain embodiments of the invention, the amino acid sequences may have at least 90%, preferably at least 92%, at times at least 94%, particularly at least 95%, and particularly preferably at least 95%, 96%, 97%, 98% and at times even at least 99% identity to SEQ. ID. NO:1. Also included within the scope of the invention are nucleic acid sequences encoding for any of the amino acid sequences of said certain embodiments. Particular examples of such nucleic acid sequences are such having at least 90%, preferably at least 92%, at times at least 94%, particularly at least 95%, and particularly preferably at least 95%, 96%, 97%, 98% and at times even at least 99% identity to SEQ. ID. NO:2.

The term *"biological characteristics"*, with respect to the polypeptide, refers to the polypeptide's ability to induce alternative programmed cell death in the specific cells recognized by the cell targeting moiety, including but not limited to the biological activities reported below in the Examples.

In another aspect of the invention, there is provided a pharmaceutical composition comprising the chimeric protein of the invention and a pharmaceutically acceptable excipient.

In another aspect of the invention, there is provided a method for treating a subject suffering from a disease involving cells which are multi-drug resistant (MDR)

positive comprising administrating to the subject a pharmaceutical composition comprising the chimeric protein of the invention.

The chimeric protein of the invention may be used to treat a variety of diseases involving cells which are MDR positive such as cells having an over-expression of the P-gp pump. In a preferred embodiment, the disease which may be treated is cancer. In one embodiment, the cancer is selected from Adrenocortical cancer; Malignant melanoma; Non-melanoma skin cancer; Cutaneous T-cell Lymphoma; Kaposi's Sarcoma; Bladder cancer; Colon cancer; Colorectal cancer; Rectal cancer; Neuroectodermal and Pineal cancer; Childhood Brain Stem Glioma; Childhood Cerebellar Astrocytoma; Childhood Cerebral Astrocytoma; Childhood medulloblastoma; Childhood visual pathway Glioma; Meningioma; Mixed Glioma; Oligodendroglioma; Astrocytoma; Ependymoma; Pituitary adenoma; Metastasic Adenocarcinoma; Acoustic neuroma; Paravertebral Malignant teratoma; Breast cancer; Ductal carcinoma; Mammary gland neoplasia; Ovarian cancer; Carcinoid tumour; Cervical cancer; Uterus cancer; Endometrial cancer; Vaginal cancer vulva cancer Gestational Trophoblastic cancer; Fallopain cancer; Uterine sarcoma; Leukemia; Lymphoma (Hodgkin's disease and Non Hodgkin's disease); Neuroblastom; Retinoblastoma; Soft tissue Sarcomas; Wilm's tumour; Fanconi Anaemia; Langerhan's Cells Histiocytosis; Malignant Rhabdoid Tumour of Kidney; Liver cancer; Neuroblastoma; Retinoblastoma; Choriocarcinoma; Endocrine cancers; Endometrial cancer; Esophageal cancer; Ewing's Sarcoma; Eye cancer; Gastric cancer; Gastrointestinal cancers; Genitourinary cancers; Glioma; Gynaecological cancers; Head and neck cancer; Hepatocellular cancer; Hypopharynx cancer; Islet call cancer; Kidney cancer; Laryngeal cancer; Lung cancer; Lymphoma; Male breast cancer; Melanoma; Mesothelioma; Myeloma, multiple; Nasopharyngeal cancer; Non-melanoma Skin cancer; Oesophageal cancer; Osteosarcoma; Ovarian cancer; Pancreas cancer; Pituitary cancer; Prostate cancer; Renal cell carcinoma; Retinoblastoma; Rhabdomyosarcoma; Sarcoma; Skin cancer; Squamous cell carcinoma; Stomach cancer; Testicular cancerthymus cancer; Thyroid cancer; Transitional cells cancer; Trophoblastic cancer; Uterus cancer; Acute Lymphatic leukemia; Acute myeloid leukemia; Adenocystic carcinoma; Anal cancer; Bone cancer; Bowel cancer; Ductal carcinoma; Liposarcoma; Neuroblastoma; Nephroblastoma; Osteosarcoma. However, in the last few years more and more data has accumulated showing that autoimmune diseases also involve over-

expression of the P-gp – see for example "*P-glycoprotein in autoimmune diseases*", Autoimmunity Reviews 3, 188-192, 2004. Neurodegenerative diseases such as Alzhiemer may also be treated using the chimeric protein of the invention.

In a further aspect of the invention, there is provided a use of the chimeric protein of the invention in the preparation of a pharmaceutical composition for use in treating a subject suffering from a disease involving cells which are MDR positive such as cancer or autoimmune disease.

In a still further aspect of the invention, there is provided a use of the chimeric protein of the invention in treating a subject suffering from a disease involving cells which are MDR positive such as cancer or autoimmune disease.

The chimeric protein of the invention may be prepared in the following manner, using IGA as a non-limiting example.

1. Construction of the chimeric protein. Insertion and fusion of two moieties (targeting and killing) encoding the sequences into an expression plasmid using molecular biology techniques.

2. Expression and characterization of the protein. The protein may be expressed using an E. coli expression system, partially purified and characterized by SDS-PAGE and Western blots. Its function may be tested by an enzymatic in vitro cell free assay, for example using a synthetic Granzyme A substrate, such as Z-Gly-Pro-Arg-AFC-TFA.

3. Internalization. The ability of IGA to internalize into specific target cells may be examined by Western blots and confocal microscopy.

4. Activity of the chimera.

A. IGAs' ability to kill high affinity IL-2R overexpressing cells may be tested on sensitive and MDR lymphocyte cell lines as well as on primary cultures from healthy donors and patients which are MDR positive or negative.

B. In addition, cell lines expressing different levels of the high affinity IL-2R and different sub-units of the receptor may be used to test proteins' activity. The efficiency of the chimera may be investigated in the treatment of MDR cells as compared to that of chemotherapy using drugs such as Doxorubicin or Vinblastine.

5. Specificity of the chimera. The protein's specificity may be examined by its activity on negative cell lines and by introducing anti-IL-2R antibodies and recombinant IL-2, blocking the chimeras' ability to bind to its receptor (competition assay) on target

cells. Positive and negative control proteins may include IL-2-PE, Granzyme A without the targeting IL-2 moiety, respectively.

6. Characterization of cell death caused by IGA. Death caused by IGA may be investigated and characterized. This may be done by testing activity or lack of activity of various proteins known to be targets of Granzyme A by using specific antibodies. For example, IGAs' influence on translocation of the SET complex to the nucleus; the activation or lack of activation of proteins, such as caspase 3, BID, PARP, involved in caspase dependent apoptosis; type of DNA fragmentation (double stranded fragmentation as opposed to single stranded fragmentation) caused by IGA dependent cell death and more, may be tested.

7. The *in vivo* model. The ability of IGA chimera to control tumor progress in MDR transgenic mice, overexpressing MDR1 gene and nude mice may be tested and side effects may be monitored.

DETAILED DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1 is a schematic representation of the IGA chimeric protein;

Figs. 2A and **2B** show an SDS-PAGE gel and Western blot illustrating IGA expression. IGA was expressed in the E.coli strains, HMS and BL21 codon+, and the whole cell extract (w.c.e), soluble (sol.) and insoluble refolded (ref.) fractions were run on a SDS-PAGE (2A) and tested by Western blotting using anti-Granzyme A antibodies (**2B**). It may be seen that the chimeric protein is enriched in the insoluble refolded fraction;

Fig. 3 shows a Western blot illustrating an internalization assay. Lm-1 cells were incubated with the chimeric protein for the indicated time periods. Cell lysates were prepared and analyzed by Western blotting using anti Granzyme A antibodies. Tubulin was used as a marker for the total amount of protein loaded;

Figs. 4A and **4B** show microphotographs by a confocal microscope illustrating internalization of IGA into target cells. Confocal microscopy was used to study the internalization of the chimeric protein. The parental, Lm-1 (4A) and the resistant, Lm-1-

MDR (4B) cell lines were incubated with the chimeric protein or PBS for 24 hours. The slides were exposed to anti IL-2 antibodies (zoom x 400);

Figs. 5A and **5B** show bar graphs illustrating the effect of IGA and Doxorubicin on target and non-target cells. Lm-1, Lm-1-MDR, L1210 and HMC cells were incubated with the chimeric protein (5A) or with the chemotherapeutic drug, Doxorubicin (5B) for 72 hours, and % death of the cells was determined;

Fig. 6A shows a Western blot with anti-Caspase3 or anti-activated Caspase3, illustrating the amount of caspase 3 and its activated subunit (17KDa subunit) present in sensitive and resistant cell lines, Lm-1 (1) and Lm-1-MDR (2), respectively, after being incubated with IGA for 6, 24 and 48 hours, or with doxorubicin for 48 hours. Tubulin was measured as a control for loading;

Fig. 6B shows Caspase3 enzymatic activity measured in Lm-1 and Lm-1-MDR cells treated with IGA or doxorubicin for 48 hr. Enzymatic activity was measured using a commercial kit (Apo-ONE, Caspase3/7 assay; Promega);

Fig. 7 shows % death of Lm-1 and Lm-1-MDR cells after 48 hours incubation with IGA or doxorubicin. Treatment of the cells was with 1.2 μg IGA or 0.2μM doxorubicin in both assays (Figs. 6B & 7);

Fig. 8A is a graph showing the release of fluorescence from the granzyme A substrate Z-GPR-AMC as a function of IGA concentration;

Fig. 8B is a bar graph which compares the release of fluorescence by IGA using a granzyme A substrate Z-GPR-AMC to the release of fluorescence by a control protein (RFB-cas3);

Fig. 9 is a Western Blot analysis of LM-1 cells treated with IGA showing that treatment of target cells with IGA causes the traslocation of the DNAse NM23H1 from the cytoplasm to the nucleus;

Figs. 10A and **10B** are photographs of a polyacrylamide denaturing gel showing the results of the Klenow incorporation assay carried out on LM-1 (10A) and LM-1-MDR (10B) cells treated with IGA; and

Figs. 11A and **11B** show photographs of the same results as in Fig. 10 with LM-1 (11A) and LM-1-MDR (11B) cells on a standard acrylamide DNA gel.

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

EXAMPLE I

Materials and methods

1. cDNA for human Granzyme A was obtained by RT-PCR using RNA isolated from fresh human PHA activated lymphocytes. Total RNA was isolated and the reverse transcribed into first strand cDNA using the reverse transcription system. The Granzyme A fragment was generated by PCR using the above cDNA and a specific pair of primers, covering the coding sequence of the active enzyme.

IL-2 was cut from pAY1 vector with specific restriction enzymes. Granzyme A and IL-2 were ligated to the vector fragment resulting in pET28 vector encoding IL-2 sequence 5' to the Granzyme A sequence.

2. The chimeric protein was expressed in Escherichia coli strains HMS and BL21 Codon +.

A pellet of expressing cells was lysed, sonicated and centrifuged. It was denatured in buffer containing 6M guanidium and diluted 1:50 in refolding buffer. The enriched protein fractions were dialyzed against PBS to remove guanidium and kept in aliquots at –20°C.

3. Protein expression was analyzed by SDS-PAGE and Western blot analysis.

4. Cell lines: Mouse lymphocytic leukemia cell line, L1210 and mouse lymphoma cell line, Lm-1 were grown in RPMI and DMEM medium, respectively supplemented with 10% HIFCS, 10 units/ml Penicillin, 100 μg/ml streptomycin and 2mM L-Glutamine. The resistant Lm-1-MDR cell line transduced with a retroviral vector containing the human MDR-1 cDNA was grown in the Lm-1 medium containing 750ng/ml colchicine (5). Negative cells used were HMC cells, human mast cells.

5. Confocal microscopy. The cells were allowed to adhere to poly (L-lysine) covered coverslips. The slides were incubated with the chimeric protein for various time periods. After fixation, permabilization and incubation with first and secondary antibodies, the slides were examined using a Zeiss LSM 410 confocal laser scanning system.

6. Testing the effect of IGA on target and non-target cells. Cell viability was preformed by seeding cells on 96 well plates. Different concentrations of the chimeric protein or equal volumes of PBS were added and after 72 hour incubation, cell viability assay was preformed using a cell titer blue kit (Promega).

Results

1. Cloning of the plasmid encoding the IGA. Human fresh lymphocytes, from a healthy donor after three days activation were used as a source to extract total RNA. The RNA was reversed transcribed into cDNA. The primers for Granzyme A were designed so that only an active Granzyme A sequence was included (without the signal peptide and the activation sequence). The RT-PCR fragment thus obtained was inserted into the pET28 vector. The IL-2 fragment was cut by restriction enzymes from the IL-2-Bax pAY1vector. Both fragments were fused together with IL-2 being 5' to the Granzyme A sequence (Fig. 1). The sequence was confirmed by restriction and sequencing analyses.

2. Expression of the chimeric protein. The protein was expressed in several E. coli bacterial strains and was found to be highly expressed in both BL21 codon + and HMS strains. SDS –PAGE and Western blot analyses demonstrated that most of the chimera is located in the insoluble fraction. It was expressed at the expected size of 42kDa (Fig. 2). The insoluble fraction expressing cells, after denaturation and refolding was used as a source of the chimeric protein.

3. Internalization assays. A. The assay was preformed by incubating target sensitive Lm-1 cells with the chimeric protein for the indicated time periods. Samples containing equal amounts of total protein were analyzed by Western blots using anti-Granzyme A specific antibodies and revealed internalization of the chimera into the cells 30 minutes after the beginning of the incubation (Fig. 3). The protein was detected in the cells also following 4 hours of incubation.

B. Confocal microscopy was yet another method to demonstrate the internalization of IGA into target cells expressing the high affinity IL-2R. The sensitive and the resistant cell lines, Lm-1 and Lm-1-MDR were incubated with the chimeric protein for 24 hours. As can be seen in Fig. 4, the intensity of IL-2 staining is greater in the treated parental Lm-1 and resistant Lm-1-MDR cell lines as compared to control non-treated cells. The control non-treated cells are also stained because these are T-cells, expressing most probably endogenous IL-2.

4. Activity of IGA on cultured cells. The ability of the chimera to kill target cells was assayed by carefully following the viability of cells exposed to the chimeric protein. Experiments were preformed with Lm-1, Lm-1-MDR (Lm1-750), L1210 cells

expressing the high affinity IL-2R and the negative HMC cell line, lacking IL-2R expression. The cells were incubated with the chimera and with a chemotherapeutic drug, doxorubicin, for 72 hours. The results showed a marked dose dependent decrease in the viability of IL-2R positive cells while the negative cell lines weren't affected (Fig. 5A). It is evident that the chemotherapeutic drug, Doxorubicin, affects only the sensitive Lm-1 cell line while its effect on the Lm-1-MDR is very limited (Fig. 5B). In contrast, the chimeric protein affects both sensitive and MDR cell lines to a similar degree (Fig. 5A).

Thus, it seems that the IGA chimera is a potent protein for treating cancer sensitive and MDR cells expressing the high affinity IL-2R.

EXAMPLE II

This experiment is intended to show that while death induced by the chemotherapeutic drug Doxorubicin induces activation of Caspase3, death induced by IGA does not involve activation of Caspase3 (as expected by death induced via granzyme A).

$3*10^6$ Lm-1 and Lm-1-MDR cells were seeded per 5 ml medium. The cells were incubated at 37°C with 0.08 ug/ul IGA chimeric protein for 0, 6, 24 and 48 hours. The cells were then harvested, protein was extracted and loaded on an SDS gel. Protein concentration was determined by measuring O.D using the Bradford reagent. Western blot analysis was carried out using anti-tubulin (control), anti-Caspase3 and anti-active caspase3 antibodies.

The results are shown in Fig. 6A. It may be seen that IGA does not cause activation of caspase3 in either of the cell lines, as opposed to doxorubicin which activates caspase3 in the sensitive cell line, but not in the resistant cell line. In other words, IGA causes alternative programmed cell death. This was also confirmed by measuring caspase3 enzymatic activity as illustrated in Fig. 6B.

In parallel, the treatment efficacy on cell death was determined. Fig. 7 shows that while IGA causes death of both the sensitive and resistant target cells, doxorubicin kills only the non-MDR cells. Thus, doxorubicin causes cell death that is caspase3 dependent, while cell death is found to be independent of caspase3 activation in the case of IGA.

EXAMPLE III

It would be advantageous to have an *in vitro* cell free assay to quickly and effectively check the enzymatic activity of different batches of chimeric proteins according to the invention. In the case of IGA, advantage may be taken of existing synthetic substrates of Granzyme A, such as Z-Gly-Pro-Arg-AFC-TFA (Z-GPR-AMC).

A stock solution (10mM) of the substrate Z-GPR-AMC (BIOMOL Ltd.) was prepared in DMSO and diluted to 0.5mM in 20mM Tris-HCl pH 8, 150 mM NaCl. 20ul of different amounts of IGA were added to 80ul of the substrate solution and incubated at 37°C for 1 Hr and then at room temperature overnight. An identical control experiment was carried out using the non-relevant chimeric protein RFB-cas3. Fluorescence intensity was measured using a fluorometer at excitation and emission wavelengths of 390 and 460nm, respectively. The results are shown in Figs. 8A and 8B.

The IGA chimeric protein showed in vitro enzymatic activity on the Granzyme A substrate Z-GPR-AMC in a dose dependent manner (Fig. 8A and Table 1). This activity was found to be specific as the control protein RFB-cas3 did not exhibit this enzymatic activity (Fig. 8B and Table 2).

Table 1: Fluorescence as a function of IGA concentration

(FI=Fluorescence Intensity)

IGA [units]	**FI Units**
3	1198
2.25	646
1.5	600
1.125	232
0.75	344

Table 2: Specific enzymatic activity of IGA

Protein	**FI units**
IGA	2121
RFB-cas3	173

EXAMPLE IV

As stated above, granzyme A cleaves the SET complex releasing the transcriptional regulator and nucleoside diphosphate kinase NM23-H1, thus allowing it to nick DNA. As a result of granzyme A activity, NM23-H1 is translocated from the cytoplasm to the nucleus. In this experiment, the effect of IGA treatment on NM23-H1 in target cells was investigated.

LM-1 cells were incubated with IGA (20ug/ul) for 30 hrs. The cells were harvested and subjected to sub-fractionation separating the cytoplasm and the nucleus. Sub cellular fractions were run on SDS-gels and Western Blotted using Anti-tubulin antibodies (Serotec, Ltd.; dilution-1:10,000) and Anti-NM23H1 antibodies (Santa-Cruz, Ltd.; dilution-1:5000). The results are shown in Fig. 9.

It may be seen that treatment of target cells with IGA caused the translocation of the NM23H1 from the cytoplasm to the nucleus, similar to the effect seen with natural Granzyme A.

EXAMPLE V

In order to verify that IGA treatment not only causes translocation of NM23H1 into the nucleus of target cells, but also causes single-stranded DNA nicks, LM-1 and LM1-MDR cells were incubated with IGA (12ug/ul) for various time periods. DNA was extracted by proteinase K treatment and Phenol-Chloroform precipitation. Klenow fragment of DNA polymerase was used to label DNA breaks. DNA was incubated with 1 U Klenow (Takara Ltd.) and 2 uCi ^{32}P-dCTP (Amersham Ltd.) for 40 minutes at 37°C. Samples were then loaded on columns to remove excess radioactivity. The samples were analyzed by a denaturing alkaline polyacrylamide gel (Sambrook et al., 1989).

Fig. 10 shows the results of the Klenow incorporation assay on a polyacrylamide denaturing gel. It may be seen that treatment of the target cells leads to the activation of the DNAse NM23H1 that causes single-stranded DNA nicks. The nicks are not seen on a standard acrylamide DNA gel (Figs. 11A and 11B).

EXAMPLE VI

1. Further analysis of the chimera may be by competition assays. Cells are incubated with the chimera in absence and in presence of specific anti IL-2 receptor antibodies and recombinant IL-2. Activity of the IGA may be assessed by comparing it

to the negative and the positive controls. As a positive control protein, the IL-2-PE chimeric protein, known to be a very potent killer of cells bearing the high affinity IL-2R, may be used. Alternatively, a TAT-Granzyme A construct may be a useful tool. TAT is a transcription trans activation protein of HIV-1 able to cross cell membranes in the form of fusion protein (7). This construct may serve as a positive control and may allow the seeking of other cell lines as potential target, not necessarily connected to the immune system or cancer. As a negative control protein the Granzyme A protein, lacking the targeting IL-2 moiety, may be used as well as chimeric protein composed of an irrelevant targeting sequence. All control proteins may be prepared similarly to that of IGA.

2. Purification of IL-2-Granzyme A. Highly purified and concentrated IGA is obtained by chromatographic techniques.

CLAIMS:

1. A chimeric protein comprising a cell targeting moiety and a cell killing moiety, wherein the cell killing moiety induces alternative programmed cell death.

2. The chimeric protein of claim 1 wherein the cell killing moiety is of human origin.

3. The chimeric protein of claim 1 wherein the cell killing moiety is a member of the granzyme family.

4. The chimeric protein of claim 3 wherein the cell killing moiety is selected from the granzymes A, B, C, D, E, F, G, H, K and M.

5. The chimeric protein of claim 4 wherein the cell killing moiety is Granzyme A.

6. The chimeric protein of any of claims 1-5 wherein the cell targeting moiety is selected from antibodies, cell membrane receptor ligands, cytokines, growth factors and hormones.

7. The chimeric protein of claim 6 wherein the cell targeting moiety is selected from interleukin 2 (IL-2), gonadotropin releasing hormone (GnRH), myelin basic protein (MBP), the Fc fragment of an IgE antibody, epidermal growth factor (EGF) and insulin-like growth factor.

8. The chimeric protein of claim 7 wherein the cell targeting moiety is IL-2.

9. The chimeric protein of claim 8 comprising IL-2 and Granzyme A.

10. A method for treating a subject suffering from a disease involving cells which are multi-drug resistant (MDR) comprising administrating to the subject a pharmaceutical composition comprising the chimeric protein of any of claims 1-9.

11. The method of claim 10 wherein the cells overexpress the P-glycoprotein (Pgp) pump.

12. The method of either of claims 10 or 11 wherein the cells are cancer cells.

13. The method of claim 10 wherein the cancer is selected from Adrenocortical cancer; Malignant melanoma; Non-melanoma skin cancer; Cutaneous T-cell Lymphoma; Kaposi's Sarcoma; Bladder cancer; Colon cancer; Colorectal cancer; Rectal cancer; Neuroectodermal and Pineal cancer; Childhood Brain Stem Glioma; Childhood Cerebellar Astrocytoma; Childhood Cerebral Astrocytoma; Childhood medulloblastoma; Childhood visual pathway Glioma; Meningioma; Mixed Glioma; Oligodendroglioma; Astrocytoma; Ependymoma; Pituitary adenoma; Metastasic

Adenocarcinoma; Acoustic neuroma; Paravertebral Malignant teratoma; Breast cancer; Ductal carcinoma; Mammary gland neoplasia; Ovarian cancer; Carcinoid tumour; Cervical cancer; Uterus cancer; Endometrial cancer; Vaginal cancer vulva cancer Gestational Trophoblastic cancer; Fallopain cancer; Uterine sarcoma; Leukemia; Lymphoma (Hodgkin's disease and Non Hodgkin's disease); Neuroblastom; Retinoblastoma; Soft tissue Sarcomas; Wilm's tumour; Fanconi Anaemia; Langerhan's Cells Histiocytosis; Malignant Rhabdoid Tumour of Kidney; Liver cancer; Neuroblastoma; Retinoblastoma; Choriocarcinoma; Endocrine cancers; Endometrial cancer; Esophageal cancer; Ewing's Sarcoma; Eye cancer; Gastric cancer; Gastrointestinal cancers; Genitourinary cancers; Glioma; Gynaecological cancers; Head and neck cancer; Hepatocellular cancer; Hypopharynx cancer; Islet call cancer; Kidney cancer; Laryngeal cancer; Lung cancer; Lymphoma; Male breast cancer; Melanoma; Mesothelioma; Myeloma, multiple; Nasopharyngeal cancer; Non-melanoma Skin cancer; Oesophageal cancer; Osteosarcoma; Ovarian cancer; Pancreas cancer; Pituitary cancer; Prostate cancer; Renal cell carcinoma; Retinoblastoma; Rhabdomyosarcoma; Sarcoma; Skin cancer; Squamous cell carcinoma; Stomach cancer; Testicular cancerthymus cancer; Thyroid cancer; Transitional cells cancer; Trophoblastic cancer; Uterus cancer; Acute Lymphatic leukemia; Acute myeloid leukemia; Adenocystic carcinoma; Anal cancer; Bone cancer; Bowel cancer; Ductal carcinoma; Liposarcoma; Neuroblastoma; Nephroblastoma; Osteosarcoma.

14. A pharmaceutical composition comprising the chimeric protein of any of claims 1-9 and a pharmaceutically acceptable excipient.

15. A pharmaceutical composition according to Claim 14, for use in treatment of cancer and autoimmune disease.

16. Use of the chimeric protein of any of claims 1-9 in the preparation of a pharmaceutical composition for use in treating a subject suffering from a disease involving cells which are MDR positive.

17. Use of the chimeric protein of any of claims 1-9 in treating a subject suffering from a disease involving cells which are MDR positive.

18. A method for treating a subject suffering from a disease involving cells which express IL-2 receptor comprising administrating to the subject a pharmaceutical composition comprising the chimeric protein of either of claims 8 or 9.

19. Use of the chimeric protein of either of claims 8 or 9 in the preparation of a pharmaceutical composition for use in treating a subject suffering from a disease involving cells which express IL-2 receptor.

20. Use of the chimeric protein of either of claims 8 or 9 in treating a subject suffering from a disease involving cells which express IL-2 receptor.

Figure 1

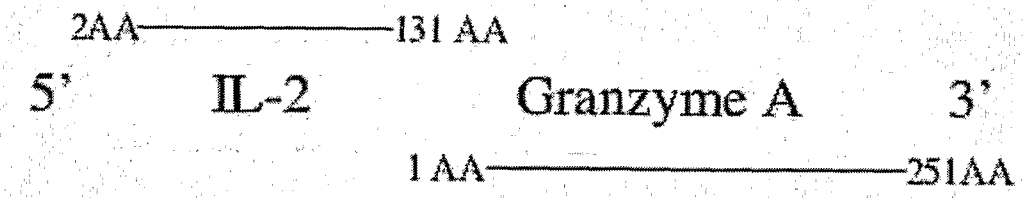


Figure 2A

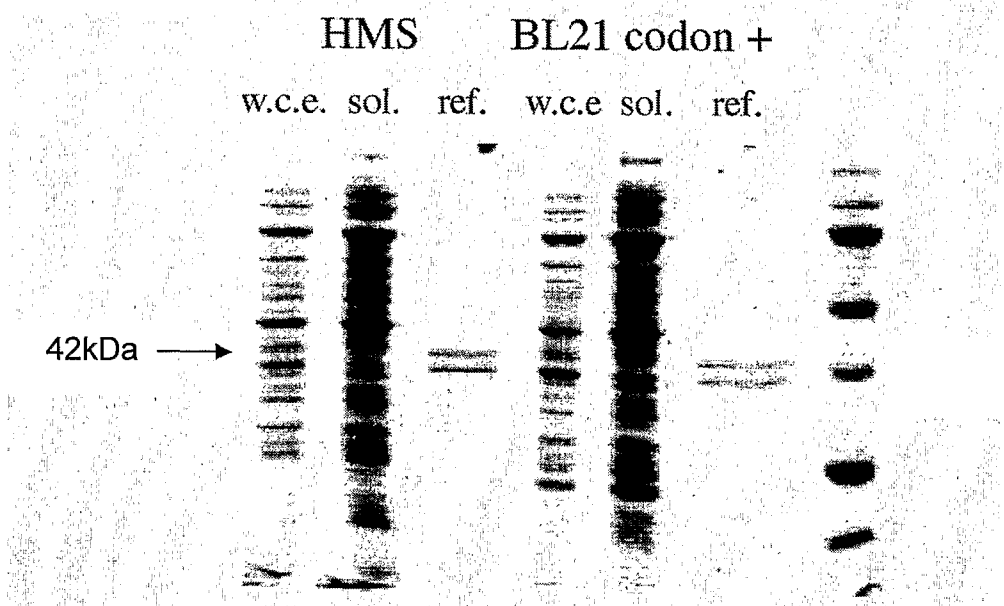


Figure 2B

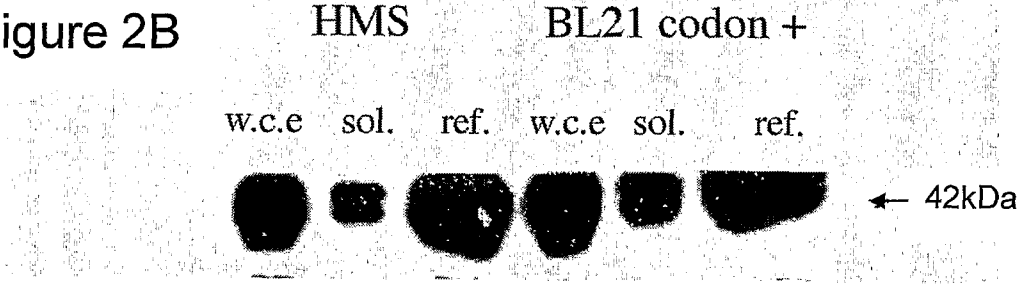


Figure 3

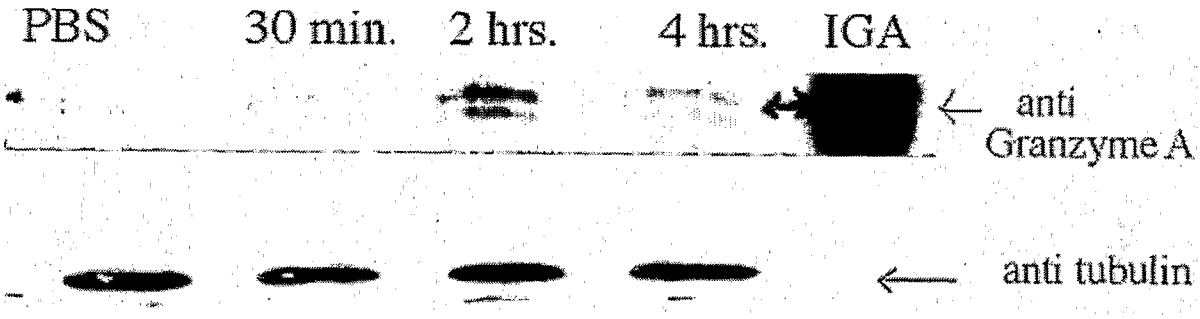


Figure 4A

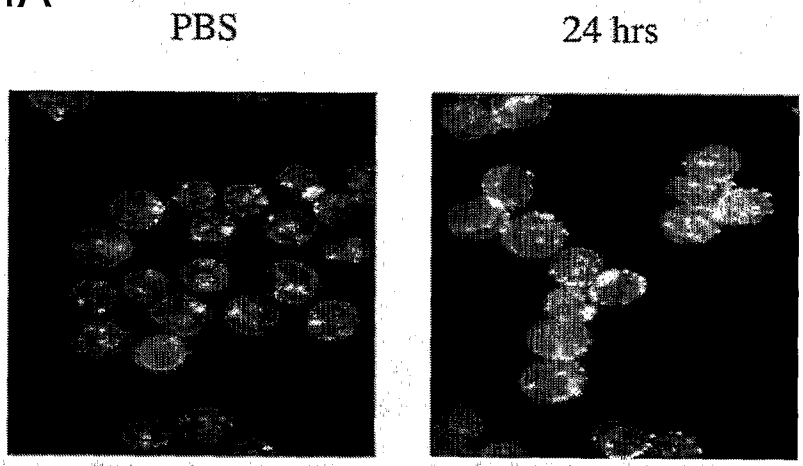
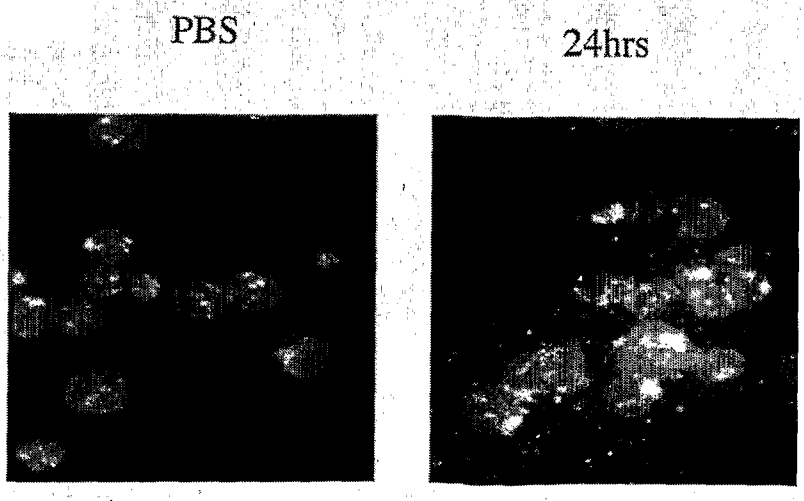


Figure 4B



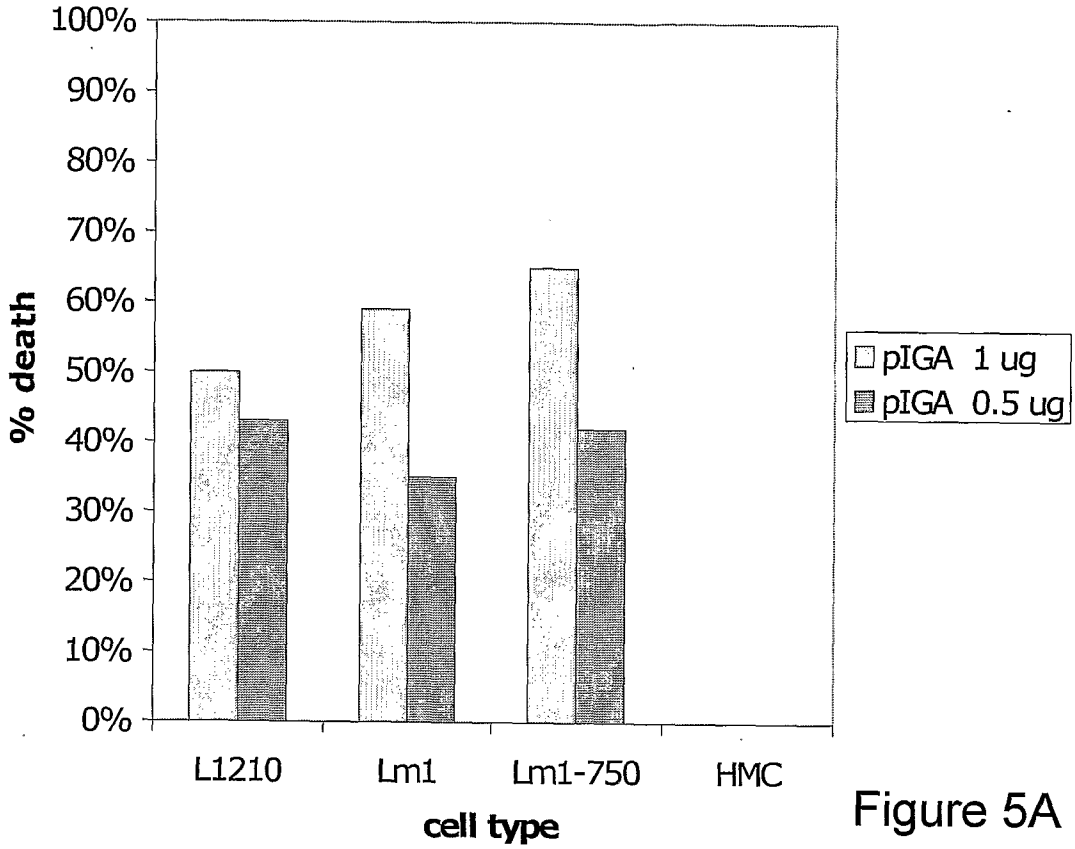


Figure 5A

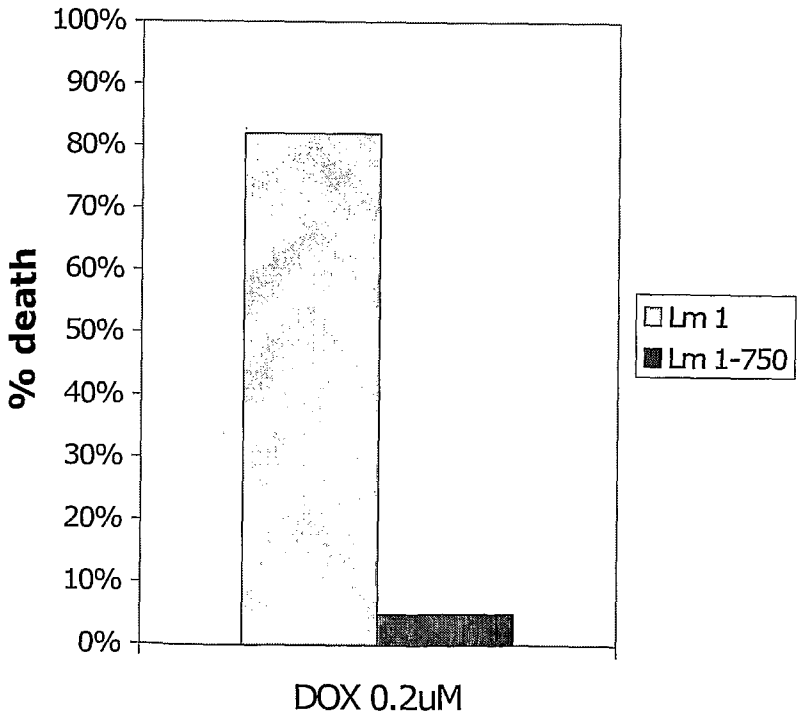


Figure 5B

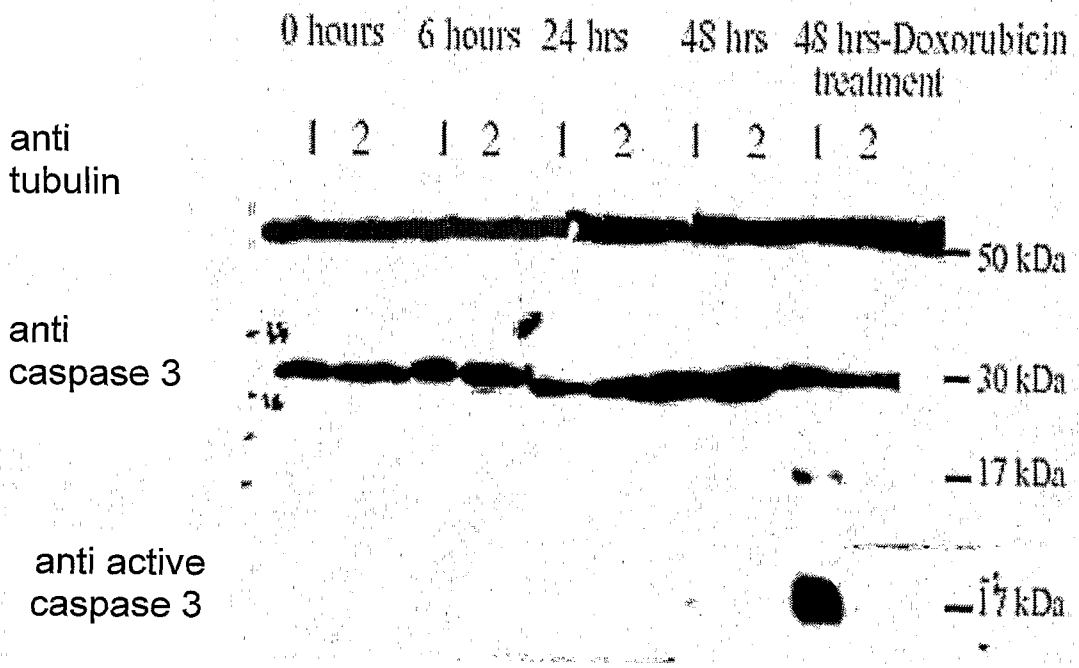


Figure 6A

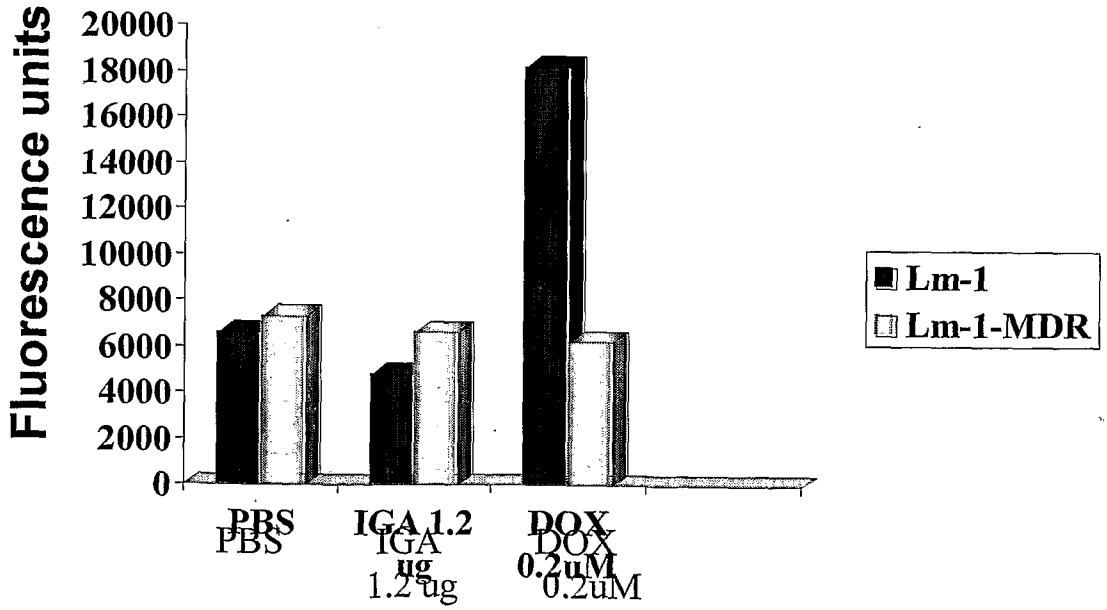


Figure 6B

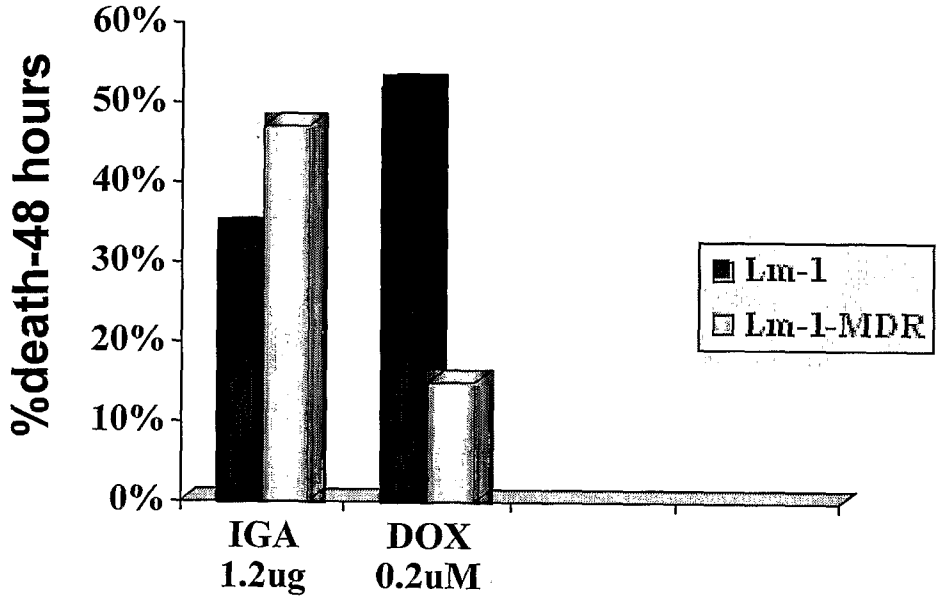


Figure 7

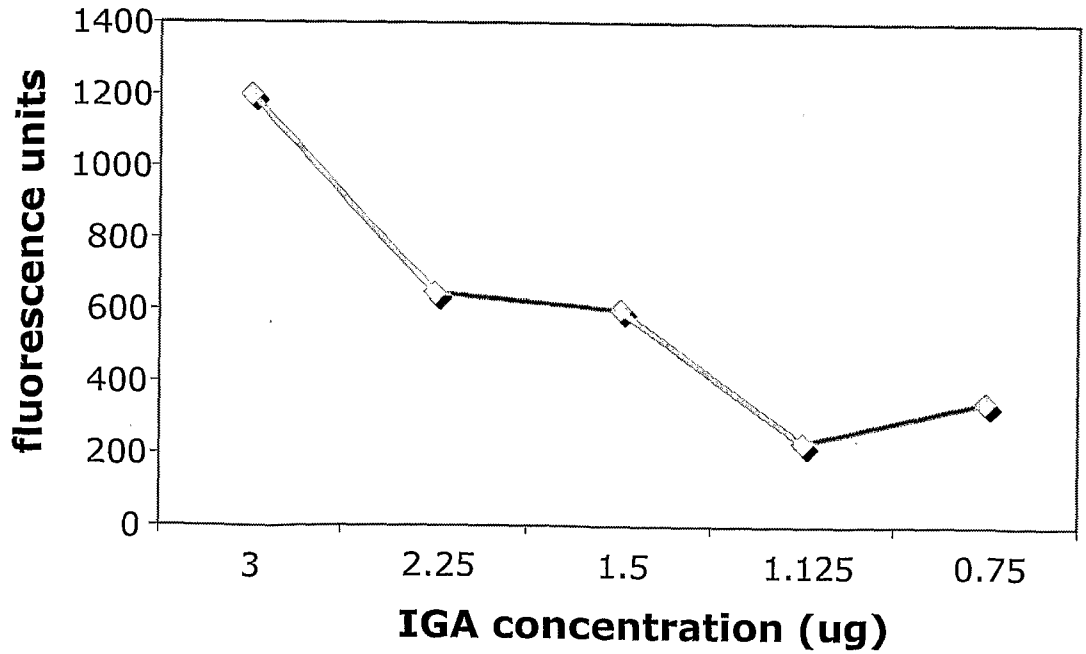


Figure 8A

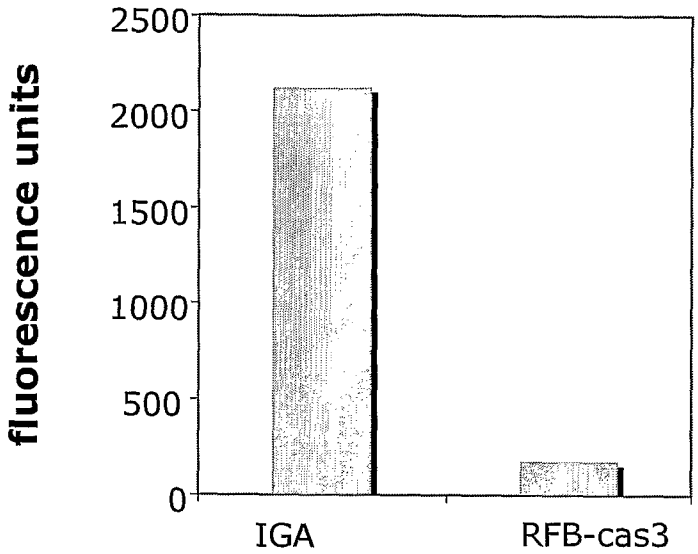


Figure 8B

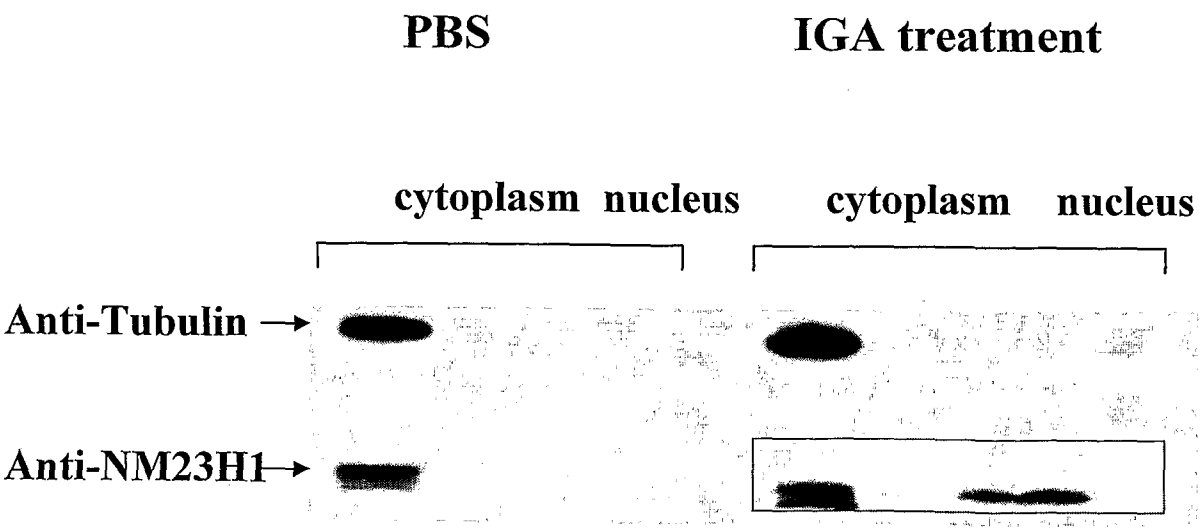


Figure 9

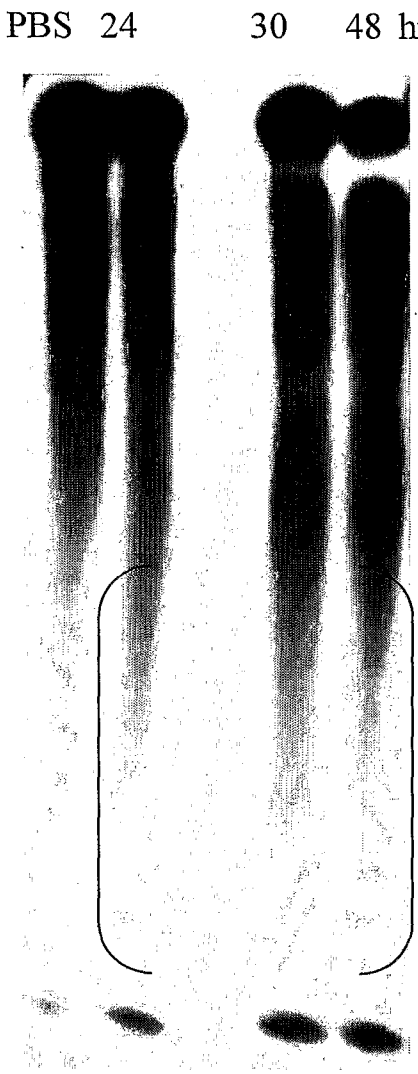


Figure 10A

Figure 10B

PBS 24 30 48 hrs

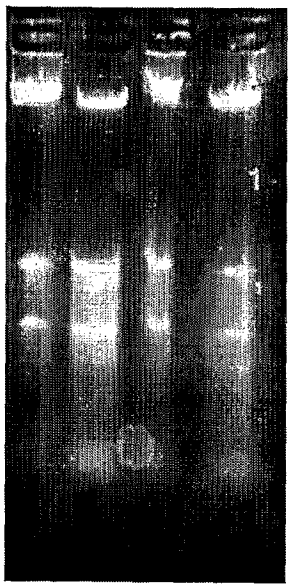


Figure 11A

PBS 24 30 48 hrs

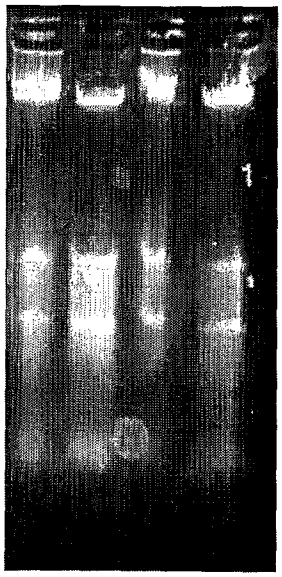


Figure 11B