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(54) **SENSIBILISATION DE CELLULES A DES COMPOSES UTILISANT UN APPORT DE GENE ET DE COMPOSE INDUIT PAR LIPIDES**

(54) **SENSITIZING CELLS TO COMPOUNDS USING LIPID-MEDIATED GENE AND COMPOUND DELIVERY**

(57) La présente invention a trait à des méthodes et des compositions de sensibilisation d'une cellule à un composé tel qu'un promédicament.

(57) The present invention relates to methods and compositions for sensitizing a cell to a compound such as a prodrug.

CORRECTED VERSION*

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(57) Abstract

The present invention relates to methods and compositions for sensitizing a cell to a compound such as a prodrug.

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SENSITIZING CELLS TO COMPOUNDS USING LIPID-MEDIATED GENE AND COMPOUND DELIVERY

FIELD OF THE INVENTION

This invention relates to methods and compositions for sensitizing a cell to a compound such as a prodrug.

BACKGROUND OF THE INVENTION

Working systems for *in vivo* human gene therapy are now established. Gene therapy vectors can be delivered to human cells *in vivo* by direct (local) injection or inhalation; by modified adenoviruses (reviewed in Englehardt, J.F. "Methods for Adenovirus-Mediated Gene Transfer to Airway Epithelium" Chapter 11 in Methods in Molecular Medicine, Gene Therapy Protocols, Ed. P. Robbins, 1997, Humana Press Inc., Totowa, NJ); by retroviruses (Olsen, J.C., *et al.*, "Methods for the Use of Retroviral Vectors for Transfer of the CFTR Gene to Airway Epithelium," Chapter 10, *Ibid.*); by cationic lipid-plasmid aggregates (Nabel, G.J., *et al.*, "Methods for Liposome-Mediated Gene Transfer to Tumor Cells in Vivo," Chapter 21, *Ibid.*; Son, K., *et al.*, "Cationic Liposome-Mediated Gene Transfer to Tumor Cells in Vitro and In Vivo, Chapter 23, *Ibid.*); or, simply, by delivery of naked DNA (*see*, U.S. Patent No. 5,589,466 to Felgner, *et al.*).

Systemic delivery for *in vivo* gene therapy, *i.e.*, delivery of the vector to a distal target cell via body systems such as the circulation, which is a less well explored avenue, has been achieved using lipid-plasmid particles such as those disclosed in published PCT Patent Application WO 96/40964, U.S. Patent No. 5,705,385, and U.S. Patent Applications Serial Nos. 08/485,458, 08/484,282, 08/660,025, 08/856,374, 60/063,473 and 09/169,573, filed October 9, 1998, all of which are assigned to the assignee of the instant invention and incorporated herein by reference. This latter format provides a fully encapsulated lipid-plasmid particle that protects the vector from nuclease degradation in serum, is non-immunogenic, is small in size and has a prolonged circulation half-life.

A variation of the basic gene therapy technique that is useful for therapeutic treatment is gene-delivered enzyme prodrug therapy ("GDEPT"). GDEPT is also known as the "suicide gene/prodrug" system and was first developed by Moolten, F.L., *Cancer Res.* 46:5276-5281 (1986). In addition, for a detailed review of GDEPT, *see*, Moolten, F.L., Chapter 11 (1995), The Internet Book Of Gene Therapy, Cancer Therapeutics, Eds. Sobol, R.E., Scanlon, K.J., Appelton & Lange. In this method, a heterologous gene, encoding an enzyme that promotes the metabolism of a first compound, to which the cell is less sensitive (*i.e.*, the "prodrug"), into a second compound to which is cell is more sensitive, is delivered to a cell. The cell takes up the gene and expresses it. Then the prodrug is delivered to the cell. The enzyme will process the prodrug into the second compound, and respond accordingly. A suitable system proposed by Moolten is the herpes simplex virus - thymidine kinase (HSV-TK) gene, and the prodrug ganciclovir. This method has recently been employed in work such as that of Zerrouqui, *et al., Cancer. Gen. Therapy* 3(6):385-392 (1996). Cationic lipid-nucleic aggregates were used for local delivery of the TK gene to mouse tumors in Sugaya, S., *et al., Hum. Gen. Ther.* 7:223-230 (1996). Human clinical trials using a GDEPT system employing viral vectors have been proposed (*see*, *Hum. Gene Ther.* 8:597-613 (1997) and *Hum. Gen. Ther.* 7:255-267 (1996)).

Patent applications relating to the GDEPT method have been published under the following numbers: WO 97/19180; WO 97/07118; WO 96/22277; WO 97/19183; WO 96/16179; WO 96/03515; WO 96/03515; WO 96/03151; EP 690129; EP 657541; EP 657539; WO 95/05835 and EP 415731.

Prior art methods suffer from many deficiencies. Firstly, the vector systems employed to date in GDEPT are designed for local delivery of the vector only. These systems are also highly immunogenic and hinder repeat dosing. This limits the range of applications for GDEPT. Secondly, non-specific toxicity of the prodrug can prohibit delivery of a satisfactory amount of the prodrug for effecting the transformed cell. In addition, prior art prodrug formulations are rapidly cleared from the blood, thereby requiring less desirable treatment modalities such as repeat injections or intravenous drip.

Another deficiency with the prior art methods is that even though researchers struggle to improve gene delivery systems, virtually no work has explored

improvements in the prodrug delivery system, or the possible advantages of syncopating methods of delivering prodrugs and the vector. If the gene vector element is delivered in a format different from the prodrug element (*i.e.*, adenovirus delivered vector versus free drug), the biodistribution of the elements is different, thus complicating dosage requirements and associated toxicities.

Clearly, there remains a need in the art for a method that solves the problem of targeting both elements of the suicide gene/prodrug system to the same organ, tumor or disease site of interest. It would be advantageous if this method could also reduce non-specific toxicities of the suicide gene/prodrug elements and extend their half-life in blood. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The present invention provides, *inter alia*, methods and compositions for sensitizing a cell to a compound, such as a prodrug. In one embodiment, the present invention provides a method of sensitizing a cell to a compound, the method comprising: (a) transfecting a cell with a nucleic acid (*e.g.*, a vector) comprising a nucleotide sequence that produces a gene-product (*e.g.*, a polypeptide or RNA) that promotes the processing, *i.e.*, conversion, of a first compound (*e.g.*, a prodrug) into a second compound; and (b) delivering to the cell the first compound in a lipid formulation; wherein the cell is more sensitive to the second compound than to the first compound.

In the above method, both the nucleic acid and the first compound can be delivered in lipid formulations which can be the same or different. The lipid formulations, whether used to deliver the nucleic acid or first compound (*e.g.*, prodrug), can be prepared from a variety of lipids, lipid conjugates and additional compatible components known in the art. The lipid formulations can be prepared, for example, from sphingomyelin and cholesterol. Moreover, the lipid formulations can contain additional components which improve the properties or characteristics of the formulations, such as leakiness, longevity in circulation, reduced toxicity, encapsulation efficiency, *etc.* Such components include, for example, cationic lipids, ATTA-lipid conjugates, PEG-lipid conjugates, targeting agents, *etc.* Once prepared, the lipid formulations can be administered or delivered to the cell using a variety of techniques known to those of skill

in the art. For instance, the lipid formulations can be delivered systemically, regionally or locally.

In a preferred embodiment, the nucleic acid is also delivered in a lipid formulation, such as a lipid-encapsulated formulation that is prepared for *in vivo* administration. In another preferred embodiment, the nucleic acid is a cationic lipid-nucleic acid aggregate or particle. The nucleic acid can be, for example, a modified adenovirus, modified retrovirus or naked DNA. The gene-product can be any product which promotes the processing, *i.e.*, conversion, of a first compound (*e.g.*, a prodrug) into a second compound to which the cell of interest is sensitive or receptive. Examples of suitable gene-products include, but are not limited to, herpes simplex virus thymidine kinase, cytosine deaminase, xanthine-guaninephosphoribosyl transferase, purine nucleoside phosphorylase, cytochrome P450 2B1 and their analogs. Other gene products suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

In a preferred embodiment, the first compound is a prodrug, *i.e.*, a compound to which the cell of interest in not initially sensitive to, but which the gene-product converts into a compound to which the cell of interest is sensitive. Examples of suitable prodrugs include, but are not limited to, ganciclovir, acyclovir, bromovinyldeoxyuridine, 5-fluorocytosine, 6-thioxanthine, MeP-dr and cyclophosphamide. Other prodrugs suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

In another embodiment, the present invention provides a method of sensitizing a cell to a compound, the method comprising: a) delivering to a cell an enzyme which promotes the processing of a first compound into a second compound; and b) delivering to the cell the first compound in a lipid formulation; wherein the cell is more sensitive to the second compound than the first compound. In a presently preferred embodiment, both the enzyme and the first compound are delivered in lipid formulations.

In yet another embodiment, the present invention provides a composition for treating a human condition (*e.g.*, a human medical disorder or disease state), the composition comprising a prodrug in a lipid formulation and a pharmaceutically acceptable carrier. In a presently preferred embodiment, the composition further comprises a nucleic acid or an enzyme in a lipid formulation.

In still another embodiment, the present invention provides a kit for the treatment of a human medical disorder, the kit comprising: a) a nucleic acid in a lipid formulation; and b) a prodrug in a lipid formulation.

In addition, the present invention provides methods for preparing lipid formulated prodrugs, nucleic acids and enzymes which can be used in carrying out the methods of the present invention.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the relationship of citrate concentration in the dialysis buffer and the DODAC mol% in the lipid for the preparation of lipid-plasmid particles. The solid dots represent good quality formulations having high association efficiencies (>40%), small size (<100 nm) and low values of size polydispersity (chi-square less than 10, preferably less than 3) on a NICOMP particle sizer. The stars represent formulations containing aggregates or having large polydispersity values, and the open circles represent formulations having low association efficiencies (<40%). Proper tuning of the citrate buffer concentration to the cationic lipid charge appears to improve the formulation. Alternative anionic buffers may also be used if the counterions can prevent the cationic lipid from aggregating during the detergent removal step.

Figure 2 illustrates the biodistribution in various organs (*i.e.*, blood, spleen and liver) of 303i in C57-Lewis Lung mice.

Figure 3 illustrates the accumulation of 303i at the tumor site in C57 mice.

Figure 4 illustrates a time course of gene product activity at distal (metastatic) tumor sites.

Figure 5 illustrates gene expression in LS180 tumors (dose response of 303i after 48 hrs).

Figure 6 illustrates the pattern of HSV-TK gene expression within peritoneal tumors.

Figure 7(A) illustrates in vivo efficacy studies using a tumor model.

Figure 7(B) illustrates a 16-day treatment regimen on test mice after tumor inoculation.

Figure 8(A) illustrates an assessment of the tumor growth, with the empty formulation showing the largest tumor volume.

Figure 8(B) illustrates the efficacy of the suicide gene SPLP of this invention.

Figure 9 illustrates serum clearance of liposomal GCV. Plasma was recovered at the indicated time points and assayed for ^{3}H-ganciclovir and ^{14}C-CHE lipid. Results are +/- SEM with n = 4.

Figure 10 illustrates *in vivo* kinetics of GCV release. Plasma was recovered at the indicated time points and assayed for ^{3}H-ganciclovir and ^{14}C-CHE lipid. Retention of ganciclovir in liposomal GCV was evaluated by monitoring the ratio of ganciclovir to lipid.

Figures 11A, B, C and **D** illustrate accumulation of liposomal GCV in distal tissue. Organs were recovered at the indicated time points and assayed for ^{3}H-ganciclovir and ^{14}C-CHE lipid. Results are +/- SEM with n = 4. Figure 11A, liver; Figure 11B, spleen; Figure 11C, lung; and Figure 11D, kidney.

Figure 12 illustrates tumor accumulation of liposomal GCV. Tumors were recovered at the indicated time points and assayed for ^{3}H-ganciclovir and ^{14}C-CHE lipid. Results are +/- SEM with n = 4.

Figure 13 illustrates efficacy of liposomal ganciclovir in the B16TK tumor model. Female C57BL/6 mice were seeded subcutaneously in the hind flank with 150,000 B16 murine melanoma cells stably transfected with HSV-TK. Five days later, tumor measurement began. Starting on day five, mice were treated with liposomal or free GCV (25 mg/kg ganciclovir) or HBS (Hepes Buffered Saline) every other day for a total of six injections. Measurements at day 18 indicate that tumors in the control mice are 5-fold larger than those of the treated groups. Tumor volume was determined by measuring tumor in three dimensions with calipers.

Figure 14A illustrates pINEX L018 plasmid contruct description and map.

Figure 14B illustrates pINEX TK10 plasmid construct.

DEFINITIONS

"Sensitizing" refers to the ability to increase the sensitivity of a designated system, such as a cell. This meaning includes changing a cell to make it more responsive

to a compound to which it previously was not sensitive or was less sensitive. Sensitizing and "more sensitive" also include increasing the sensitivity of a cell such that exposure to a previously non-killing substance results in cell death.

"Nucleic acid vector" or "vector" refer to a composition comprising a nucleic acid sequence encoding a gene product. This is usually a plasmid or viral genome, but can also include other compositions such as linear nucleic acids, protein/nucleic acid conjugates, *etc*. Depending on usage, vector can also refer to a nucleic acid delivered in a virus encapsulated or protein coated format, wherein the entire composition is known as a vector.

"Lipid formulation" refers to any lipid composition that can be used to deliver a compound including, but not limited to, liposomes, wherein an aqueous volume is encapsulated by an amphipathic lipid bilayer; lipid particles, wherein the lipids coat an interior comprising a large molecular component, such as a plasmid, with a reduced aqueous interior; or lipid aggregates or micelles, wherein the encapsulated component is contained within a relatively disordered lipid mixture. As used herein, a "lipid encapsulated" can refer to a lipid formulation which provides a compound with full encapsulation, partial encapsulation, or both.

"Therapeutically effective amount," as used herein, refers to an amount that is sufficient or necessary to give rise to a desired therapeutic effect. The therapeutic effect can be obtained directly or indirectly. For instance, the therapeutic agent can lead to activation of other therapeutic agents or can act in combination with additional therapeutic agents.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript. The RNA transcript can be therapeutic of its own accord as in the case of antisense or ribozyme transcription plasmids, or the RNA transcript can be translated into a polypeptide that is also a gene product.

"Serum-stable" in relation to lipid/therapeutic nucleic acid particles means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Suitable assays include, for example, a standard serum assay or a DNAse assay such as those described in the Example section.

"Systemic delivery," as used herein, refers to delivery that leads to a broad biodistribution of a compound within an organism. Some techniques of administration

can lead to the systemic delivery of certain compounds, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of a compound is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the compound is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid/therapeutic nucleic acid particles is preferably obtained by intravenous delivery.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention provides a qualitative improvement to the GDEPT system by employing lipid formulations for one or both of the nucleic acid encoding the gene and the prodrug. In a preferred embodiment of this invention, both the nucleic acid nucleic acid and prodrug are delivered separately in lipid formulations. In this embodiment, the invention preferably uses lipid-nucleic acid particles, wherein the nucleic acid is fully encapsulated and protected from nuclease degradation, and wherein the particles have a small diameter (50-200 nm) and have other attributes suitable for systemic delivery. The composition of the lipids in the formulation can be the same or different depending on the desired efficacy. Alternatively, the nucleic acid and the prodrug can be delivered together in the same lipid formulation at the same time. The use of a lipid formulation for the prodrug, with or without a lipid formulation for the nucleic acid, confers an exquisite sensitivity to GDEPT not previously known.

In general, patient therapy can be achieved using the methods of the present invention as follows. In the first step, a gene product must be expressed in cells at the disease site or target site to increase the sensitivity of such cell(s). The first step therefore requires delivery of the nucleic acid to the disease site. Any delivery method known in the art can be employed in the methods of the present invention. Local (regional) delivery can be achieved, for example, by direct injection at the disease site or by inhalation of the nucleic acids, such as naked DNA, modified viruses, cationic lipid-plasmid aggregates, and by other means known to those of skill in the art. For systemic delivery, such as intravenous administration, fully encapsulated lipid-nucleic acid particles are preferred. Such particles offer the advantage of greater biodistribution and

prolonged circulation half-lives, and allow delivery of the plasmid to microscopic or otherwise un-injectable disease sites.

Several approaches for introducing nucleic acids into cells *in vivo* have been used. These include liposome based gene delivery by systemic administration (*e.g.*, parenteral, including intravenous and intraperitoneal delivery), intratracheal instillation, aerosolized gene delivery and the like. For example, Debs and Zhu WO 93/12240, Debs WO 92/1108 and Debs U.S. Patent No. 5,641,662 all describe aerosolized gene delivery of lipid DNA complexes to mammals. Similarly, Stribling, *et al.*, *PNAS 89*:11277-11281 (1992), describe lipid delivery to mice. McLachlan, *et al.*, *Gene Therapy 2*:614-622 (1995), describe DOTAP-mediated lipid delivery of hCFTR to mice. Canonico, *et al.*, *AM. J. Respir. Cell Mol. Biol. 10*:24-29 (1994), and Canonico, *et al.*, *The American Physiological Society* 415-419 (1994), describe lipofectin-mediated gene delivery of hr1AT to rabbits by aerosolized gene delivery. Alton, *et al.*, *Nature Genetics 5*:135-142 (1993), describe DC-chol:DOPE/ DOTAP-mediated delivery of hCFTR and t-gal by aerosol or tracheal instillation to mice. Capelen, *et al.*, *Nature Medicine*, *1(1)*:39 (1995), describe delivery of CFTR to the nasal epithelia of humans using a DC-Chol/DOPE mediated procedure, as does McLachlan, *et al.*, *Gene Ther. 3(12)*:1113-1123 (1996). A variety of reports of administration of lipid-DNA complexes by parenteral administration have also been made, including Brigham WO 91/06309 and U.S. Patent No. 5,676,954; and Debs and Zhu WO 93/24640. Accordingly, a variety of procedures for transducing cells *in vivo* using lipid-mediated techniques are known. Details of preferred formulations are given below.

The local or systemic delivery that is employed to deliver the nucleic acid to the cells must ensure that the nucleic acid is taken up and expressed sufficiently to sensitize the cell to the prodrug. Those skilled in the art know the variety of nucleic acid enhancements that can be used to improve expression of the suicide-gene product. Such enhancements include, but are not limited to, compounds or sequences that assist with intra-cellular localization of the nucleic acid, promoters that improve transcription or translation of the DNA sequence, features that improve the intracellular localization of the gene product, and the like. The full variety of functioning nucleic acid possibilities fall within the orbit of this invention.

In the second step, the prodrug in a lipid formulation is delivered to the cells. The use of lipid formulations has many surprising and previously undiscovered advantages over the delivery of free drug in the GDEPT system including, but not limited to, improved targeting to the disease site transfected by the nucleic acid, prolonged circulation half-life, increased drug loading, reduced toxicity towards non-target tissues, improved treatment modalities, such as a single bolus injection as opposed to IV drip, and the like. These advantages surmount the clear limitations of the prior art GDEPT systems. Further, the liposomal formulation of the prodrug will preferably provide similar biodistribution to a lipid nucleic acid formulation, thereby concentrating both the nucleic acid and the prodrug at the disease site.

Usually, the nucleic acid will be delivered to the target cell in advance of the prodrug, in order to allow synthesis of the suicide gene product prior to the arrival of the prodrug. Temporal separation can be obtained either by separate administration of nucleic acid and prodrug, or by providing the formulations simultaneously, wherein the nucleic acid formulation rapidly accumulates at the target site and delivers the nucleic acid, and the prodrug formulation accumulates or delivers its payload more slowly. Using the compositions and the methods of the invention, therefore, the nucleic acid is delivered to the cell to direct synthesis of the suicide gene product, the cell is thereby sensitized, the prodrug is delivered to the cell, and patient therapy, *i.e.*, reduction of tumor size, inflammation or infectious load and the like, is achieved.

A. Formulating the Nucleic Acid

The nucleic acid formulation can be achieved using any prior art method. The preferred methods for systemic (*i.e.*, intravenous or other parenteral) delivery result in a high-efficiency encapsulation, wherein little of the nucleic acid is exposed to free solution or adsorbed to the outer surface of the lipid particle. Such methods are disclosed in published PCT Patent Application WO 96/40964, U.S. Patent No. 5,705,385, and U.S. Patent Application Serial Nos. 08/485,458, 08/484,282, 08/660,025, 08/856,374, 60/063,473, 08/996,783, 06/082,665 and 60/086,917, all of which are assigned to the assignee of the instant invention and incorporated herein by reference. Specific embodiments of preferred formulations are set forth in the Examples below. Generally, high efficiency encapsulation provides low immunogenicity and improved tolerance

when injected for systemic delivery. Further, these lipid-plasmid particles are relatively easy to characterize and define compared to cationic lipid-plasmid aggregates used in local delivery methods.

Preferred encapsulation methods are set out in the Example section. The lipid-therapeutic nucleic acid particles obtained by these methods have identifiable characteristics which make them suitable for use in the invention. For instance, they are small particles typically having a mean particle size of about 50 to about 200 nm and, preferably, of about 60 to about 130 nm. Most preferably, particles are of a relatively uniform size and have a χ^2 (chi-squared) value of less than about 3, more preferably of less than about 1 and, more preferably, of less than about 0.5.

Moreover, the lipid-therapeutic nucleic acid particles of the present invention are serum-stable and, thus, not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Suitable assays for measuring serum stability include a standard serum assay or a DNase assay (which are described in the Example section). Nuclease resistance/serum stability is a measure of the ability of the formulation to protect the therapeutic nucleic acid from nuclease digestion either in an *in vitro* assay or in circulation. The encapsulated particles of the present invention have greater nuclease resistance and serum stability than lipid-plasmid aggregates (also known as cationic complexes), such as DOTMA/DOPE (LIPOFECTIN™) formulations.

In addition, the lipid-therapeutic nucleic acid particles of the present invention have a nucleic acid to lipid ratio that can be formulated at various levels. For use in the methods of this invention, the particles have a drug to lipid ratio of at least about 3 mg of nucleic acid per mmol of lipid, more preferably, at least about 14 mg of nucleic acid per mmol of lipid and, even more preferably, greater than about 25 mg of nucleic acid per mmol of lipid. The preferred particles, when prepared to an administration ready formulation, are about 60 – 80 mg nucleic acid per mmol lipid (*i.e.*, they are "high ratio" formulations). The method used for making high ratio formulations can also be employed using reduced amounts of DNA to obtain lower ratio formulations. As used herein, "drug to lipid ratio" refers to the amount of therapeutic nucleic acid (*i.e.*, the amount of nucleic acid that is encapsulated and that will not be rapidly degraded upon exposure to the blood) in a defined volume of preparation divided by the amount of lipid

in the same volume. This may be determined on a mole per mole basis, on a weight per weight basis, or on a weight per mole basis. For final administration ready formulations, the drug to lipid ratio is calculated after dialysis, chromatography and/or nuclease digestion have been employed to remove as much of the externally associated therapeutic agent as possible. Drug to lipid ratio is a measure of potency of the formulation, although the highest possible drug to lipid ratio is not always the most potent formulation.

An alternative description of the lipid-nucleic acid particles of the present invention is "high efficiency" formulations that emphasizes the active loading process involved and contrasts with low efficiency or passive encapsulation. Passive encapsulation of nucleic acid in lipid particles, which is known in the art, achieves less than 15% encapsulation of therapeutic agent, and results in low ratio particles having less than 3 mg of nucleic acid per mmol of lipid. The preferred lipid/therapeutic nucleic acid particles of the present invention have an encapsulation efficiency of greater than about 30%. As used herein, "encapsulation efficiency" refers to absolute efficiency, *i.e.*, the total amount of DNA added to the starting mixture that ends up in the administration competent formulation. Sometimes the relative efficiency is calculated, wherein the drug to lipid ratio of the starting mixture is divided by the drug to lipid ratio of the final, administration competent formulation. The amount of lipid lost during the formulation process may be calculated. Efficiency is a measure of the wastage and expense of the formulation.

Other beneficial features that flow from the use of the preferred particles of the present invention, such as low nonspecific toxicity, improved biodistribution, therapeutic efficacy and ease of manufacturing, will be apparent to those of skill in the art. It is possible to develop particles as described above by alternative methods of encapsulation. These methods may employ standard techniques for loading of liposomes that are well known for use with conventional drugs. These methods include freeze-thaw extrusion, dehydration/rehydration, reverse phase evaporation, and the like, some of which are disclosed in Monnard, *et al.*, "Entrapment of nucleic acids in liposomes, " *Biochim. Biophys. Acta.*, 1329:39-50 (1997). These methods are not high encapsulation efficiency formulations, nor high ratio formulations, but the instant disclosure suggests the utility of such particles in the use of gene therapy against distal tumor sites.

B. Formulating the Prodrug

The lipid-prodrug formulation can be achieved by any prior art method. The preferred methods result in a desirable drug:lipid ratio of about 0.1 to 0.25 (mol/mol). The lipid-prodrug formulation can be synthesized using standard freeze-thaw and extrusion techniques disclosed in Hope, *et al., Biochim. Biophys. Acta* 812:55-65 (1985). Other drug loading and encapsulation techniques that can be used are disclosed in U.S. Patent Application Serial Nos. 08/399692, 08/607614, 08/588542, 08/741622, the teachings of which are incorporated herein by reference. Sizing of the lipid formulation can be achieved using extruders, pressure cells, and other tools known to those of skill in the art.

In addition to the lipids disclosed herein, there are a tremendous number of additional lipid and nonlipid components which can be used to enhance delivery or targeting of the liposomal formulations disclosed herein. Additional lipid components include, but are not limited to, lipids with neutral, anionic, cationic or zwitterionic headgroups, and the like. These standard components are set out in the art and in the patent applications referred to above which are incorporated herein by reference. Charged lipids that are particularly preferred with the invention are N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), the subject of recently issued U.S. Patent No. 5,753,613, incorporated herein by reference and assigned to the assignee of the instant invention; and 1,2-Dioleoyl-3-dimethylammonium-propane (DODAP), the subject of U.S. Patent Application Serial No. 08/856,374, the teachings of which are incorporated herein by reference.

Both the nucleic acid and prodrug formulations can include additional components selected from a wide variety of lipids, lipid conjugates and compatible additional components known in the art. For instance, cholesterol and its derivatives can be used in the nucleic acid and prodrug formulations of the present invention. Still other formulations can employ polycationic compounds that can condense DNA into small sizes before lipid encapsulation. Polylysine and polyethyleneimine, among other compounds, have been used by those of skill in the art in this capacity. Condensed particles can also be used in the methods of this invention.

In addition, cloaking agents can be used to reduce elimination by the host immune system. Such cloaking agents include, for example, polyamide oligomer-lipid

conjugates, such as ATTA-lipids, disclosed in U.S. Patent Application Serial No. 08/996,783, filed February 2, 1998 (TTC Attorney Docket No. 16303-005800) and PEG-lipid conjugates disclosed in U.S. Patent Application Serial Nos. 08/486,214, 08/316,407 and 08/485,608, the teachings of which are incorporated herein by reference. These components can also be targeting agents that encourage the lipid formulations to accumulate at the area of the disease or target site. In addition, these components can be compounds that improve features of the formulation, such as leakiness, longevity in circulation, reduction in toxicity, encapsulation efficiency, *etc.* Examples of these components and others that can usefully be included in the formulations of the invention are known to and used by those skilled in the art.

With respect to both the nucleic acid formulation and the prodrug formulation, it is sometimes preferable to employ a programmable fusogenic lipid formulation. This refers to a formulation which has little tendency to fuse with cell membranes and deliver its payload until a given signal event occurs. This allows the lipid formulation to distribute more evenly after injection into or delivery to an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or simply time. In this last event, the fusion delaying or "cloaking" component, such as the ATTA-lipid conjugate or PEG-lipid conjugate, can simply exchange out of the liposome membrane over time. By the time the formulation is suitably distributed in the body, it is calculated to have lost sufficient cloaking agent so as to be fusogenic. With other signal events, it may be desirable to choose a signal event which is associated with the disease site or target cell, such as increased temperature at a site of inflammation.

The possible lipid components of the prodrug formulations of the invention are all those components typically used in the art including, but not limited to, sphingosomes disclosed in U.S. Patent No. 5,543,152, and U.S. Patent Application Serial Nos. 08/536584, 08/316399, 08/485608, 08/442267.

C. ***Nucleic acid/Prodrug Combinations***

Any suicide gene/prodrug combination can be used in the fashion disclosed herein. Several suicide gene/prodrug combinations suitable for use in the methods of the present invention are cited in Sikora in OECD Documents, Gene Delivery

Systems at pp.59-71 (1996), incorporated herein by reference, and include, but are not limited to, the following:

Suicide Gene Product	Less Active ProDrug	Activated Drug
Herpes simplex virus type 1 thymidine kinase (HSV-TK)	ganciclovir(GCV), acyclovir, bromovinyl-deoxyuridine, or other substrates	phosphorylated dGTP analogs
Cytosine Deaminase (CD)	5-fluorocytosine	5-fluorouracil
Xanthine-guanine-phosphoribosyl transferase (XGPRT)	6-thioxanthine (6TX)	6-thioguano-sinemonophosphate
Purine nucleoside phosphorylase	MeP-dr	6-methylpurine
Cytochrome P450 2B1	cyclophosphamide	[cytotoxic metabolites]
Linamarase	amygdalin	cyanide
Nitroreductase	CB 1954	nitrobenzamidine
Beta-lactamase	PD	PD mustard
Beta-glucuronidase	adria-glu	adriamycin
Carboxypeptidase	MTX-alanine	MTX
Glucose oxidase	glucose	peroxide
Penicillin amidase	adria-PA	adriamycin
Superoxide dismutase	XRT	DNA damaging agent
Ribonuclease	RNA	cleavage products

Any prodrug can be used if it is metabolized by the heterologous gene product into a compound to which the cell is more sensitive. Preferably, cells are at least 10-fold less sensitive to the prodrug than its metabolite.

Modifications of the GDEPT system that may be useful with the invention include, for example, the use of a modified TK enzyme construct, wherein the TK gene

has been mutated to cause more rapid conversion of prodrug to drug (*see*, for example, Black, *et al.*, *PNAS (USA)*, 93:3525-3529 (1996)). Alternatively, the TK gene can be delivered in a bicistronic construct with another gene that enhances its effect. For example, to enhance the "bystander effect" also known as the "neighbor effect" (wherein cells in the vicinity of the transfected cell are also killed), the TK gene can be delivered with a gene for a gap junction protein, such as connexin 43. The connexin protein allows diffusion of toxic products of the TK enzyme from one cell into another. The TK/Connexin 43 construct has a CMV promoter operably linked to a TK gene by an internal ribosome entry sequence and a Connexin 43-encoding nucleic acid.

In addition to the GDEPT systems, there exists a very wide variety of therapeutic nucleic acids that can be employed in the instant invention. The nucleic acids can be human, nonhuman (*i.e.*, from any other plant, animal or microorganism) or entirely synthetic (*i.e.*, non-naturally occurring). The nucleic acids can be endogenous to the cells of the patient, or can be exogenous, meaning that the nucleic acid is not normally found in cells of the patients. Since treatment of neoplasia does not necessarily require long term or stable expression of the delivered nucleic acid, genes effective in transient expression systems, such as toxins or immune stimulatory proteins, are also useful in the methods of the present invention.

When the therapeutic nucleic acid is one that is endogenous to the patient, a modified sequence, an increased copy number, or a construct that has increased transcriptional activity relative to the native gene can be delivered. The gene product can be directly toxic, indirectly toxic or it can induce apoptosis or cell differentiation. In the most preferred system, the gene product of the therapeutic gene will demonstrate low toxicity to nontarget tissues, and high toxicity to the disease site. For example, when delivered in the preferred lipid-nucleic acid particles of the invention, the gene product preferably has greater toxicity to tumor cells than liver or spleen cells, where a large portion of particles are normally cleared. Disease site specificity can also be enhanced by employing tissue/disease specific promoters for gene transcription or translation. Tissue specific promoters, and methods of associating them with therapeutic nucleic acids are known to those skilled in the art.

Preferred endogenous genes suitable for use in the methods of this invention include, but are not limited to, pro-apoptotic genes; poreifirin; tumor suppressor

genes (p53 and the like); cytokines (IL-2, IL-12, IL-15, GM-CSF, *etc.*); heat shock proteins; immunodominant Ag (or tumor-specific protein genes); genes activated in embryos only; TIMP-2 (tissue inhibitor of metallo proteinase-2) and other metastasis inhibiting proteins; replacement genes, such as CFTR, DMD; LDL-R and the like; and anti-angiogenic genes, such as endostatin or angiostatin (*see*, WO 97/15666; WO 95/29242; Boehm, *et al.*, *Nature*, 309:404-407 (1997); and Kerbel, *et al.*, *Nature*, 309:335 (1997)). IL-12 is a preferred endogenous gene that can be employed as a therapeutic nucleic acid in the instant invention (*see*, Tahara, H. and Lotze, M.T., *Gene Ther.*, 2:96-106 (1995)). A suitable IL-12 plasmid construct for delivery is pNGVL3-mIL12 provided by the National Gene Therapy Vector Laboratory at the University of Michigan (Ann Arbor, Michigan).

Exogenous genes which are not naturally found in the cells of the patients, can be advantageous because their gene products can also serve to induce an immune response. For example, genes used in a suicide gene/pro-drug system can have this effect.

Preferred exogenous genes include, but are not limited to, genes used in GDEPT combinations (treatment in conjunction with pro-drugs); ribozymes or transcription plasmids encoding ribozymes or antisense transcripts; toxin genes, such as saporin, ricin, diphtheria toxin and cholera toxin (or any other plant, bacterial or fungal gene); viral protein genes, such as E1A; mutated E6; SV40 Tag, *etc.* Other exogenous genes suitable for use in the methods of the present invention will be readily apparent to those of skill in the art.

Methods of constructing plasmids or other vectors that carry the therapeutic nucleic acids disclosed herein are well known to those skilled in the art (*see*, *e.g.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.* (eds.) 1995, the teachings of which are incorporated herein by reference). Therapeutic activity can be enhanced by the addition of transcription or translation promoters and other nucleic acid elements, again, all of which are known to those skilled in the art.

A preferred pro-drug is the lipophilic elaidic acid ester analogue of ganciclovir ("E-GCV"). E-GCV has a lipophilic moiety which may enhance encapsulation and delivery of the pro-drug by the pro-drug/liposome formulations of the invention. This enhanced delivery using liposomes may enhance the benefits set out in

the research which describes the E-GCV compound and methods for its synthesis: Balzarini, *et al.*, *Gene Therapy, 5*:419-426 (1998).

D. ***Disease Site Targeting by Systemic Delivery***

One of the great advantages of the invention is its versatility in targeting a broad range of disease sites. In particular, lipid encapsulated formulations are usefully employed in targeting and killing tumor cells and other neoplasia, or other cell types that can usefully be sensitized to perform some other function. Other cell types include, but are not limited to, sites of inflammation, sites where genes are aberrantly expressed, sites of infection, *etc.*

In a preferred embodiment, both the nucleic acid and the prodrug are delivered in a lipid encapsulated formulation by intravenous administration. This method takes advantage of the known tendency of lipid encapsulated formulations to accumulate at tumors and neoplasia, even without specific targeting aspects. This ability is thought to be the result of "leaky" vasculature at sites of neoplasia that is easily invaded by small sized lipid particles (*see*, R.K. Jain, *Sci. Am. 271*:58-65 (1994)).

Where specific cell type targeting is preferred, the lipid formulation can contain, *e.g.*, on the outer surface, antigens or markers which are recognized by, or which recognize, receptors on the target cell. Examples of such targeting methods can be found in, for example, Forum: Liposome Targeting in Animal Models (Ed. L. Huang), *Journal of Liposome Research 7(4)*: 315-534 (1997), the teachings of which are incorporated herein by reference.

Generally, when administered intravenously, the nucleic acid and/or the prodrug formulation will be formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers can be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used and include, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These

compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. Carriers may also be employed when delivering the nucleic acid or prodrug formulations by other parenteral methods known in the art, such as subcutaneous, intratumoral or intramuscular injection, inhalation, and the like.

The instant invention also demonstrates that encapsulated prodrug will also improve the related system of Antibody Directed Enzyme Prodrug Therapy ("ADEPT") (Bagshawe, K., *et al.*, *B.J. Cancer 58*:700-703 (1988)). In this system, targeted antibodies are used to deliver enzyme directly to the tumor or disease cell. Often these enzymes function in the extracellular matrix near such cells. At the target site, the prodrug is converted to the toxic metabolite by the enzyme. Clearly, delivery of prodrug in a lipid formulation would have similar advantages to those shown in the GDEPT system, such as syncopation with the gene/enzyme delivery, reduced toxicity, improved targeting, prolonged circulation, and the like.

E. Dosages of Nucleic Acid and Prodrug

The precise dosage to be administered to a patient, whether as part of the GDEPT system or as part of combination therapy, will ultimately be dependent upon the discretion and professional judgment of the attendant physician and will be in part dependent on such factors as the age, weight and the particular neoplasia of the patient. The amounts and precise regime will of course depend on other factors including the severity of the condition to be treated.

In other systems, the exact dosage regime will need to be determined by individual clinicians which will be controlled by the exact nature of the nucleic acid to be

delivered and the condition to be treated, but some general guidance can be given. In general, dosage can easily range from about 0.1 μg to 1 g or more of nucleic acid. More preferably, the dose of nucleic acid will range from about 0.1 μg to about 5 mg per kilogram for a typical 70 kilogram patient, and doses of nucleic acids, which include a viral particle, are calculated to yield an equivalent amount of therapeutic nucleic acid.

In GDEPT systems, a suitable dose of the nucleic-acid lipid particle will be the amount of nucleic acid which will produce about 500 to about 200,000 enzyme units/m^2 (*e.g.*, 20,000 enzyme units/m^2). The dose of the prodrug will advantageously be in the range of about 0.1 to 250 mg per kilogram of body weight of recipient per day, preferably about 0.1 to 100 mg per kilogram bodyweight.

Typically, the nucleic acid will be administered to the patient and then the uptake and transfection into cells will be monitored, for example by recovery and analysis of a biopsy sample of the targeted neoplastic tissue. This can be determined by clinical trials which involve administering a range of trial dosages to a patient and measuring the degree of transfection in a target cell or tumor. In the methods of the current invention, the prodrug will usually be administered following administration of the nucleic acid encoding a gene product.

The invention will be described in greater detail by way of specific examples carried out in accordance with *Canadian Council on Animal Care*, Vol. 2nd Ed., "Guide to the care and use of experimental animals," Eds. Olfert, E., Cross, B. and McWilliam, A. (1993). The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

EXAMPLES

A. EXAMPLE 1

This examples illustrates the synthesis of lipid-plasmid particles for systemic delivery.

Materials: Plasmids are preferably supercoiled, 4000 to 15000 bp in length, encoding genes and enhancer elements, *etc.* as desired. The plasmids employed in these examples are:

pINEX L018 - an expression vector in which the *Photinus pyralis* luciferase gene (Promega, Madison, WI) is under the control of the CMV promoter. Other genes and sequences are set out in **Figure 14A**.

pINEX-TK10 - an expression vector comprising a pBR322 derived plasmid with CMV promoter linked to a "hyper" HSV-TK gene (*see*, Black, *et al.*, *PNAS USA*, 93:3525-3529 (1996). Other genes and their orientation are set out in **Figure 14B**.

pINEX-IL-12 - is similar to pINEX-TK10 except that the therapeutic gene comprises the IL-12 gene linked to the CMV promoter.

Cationic lipid, N,N-dioleyl-N,N-dimethyl ammonium chloride ("DODAC") and monomethoxy polyethylene2000 glycol succinate-(C8:0-ceramide) ("PEG-Cer-C8") were synthesized at Inex Pharmaceuticals Corp. Dioleyl-phosphatidylethanolamine (DOPE) was supplied by Northern Lipids, Vancouver. Standard dialysis membranes: Spectro/Por 5 regenerated Cellulose (12-14,000 MWCO) was purchased from VWR (Manufactured by Spectrum Medical Industries Inc.). Sodium Citrate was purchased from BDH. Sodium Chloride, Triton X-100 and Octyl-beta-D-glucopyranoside ("OGP") were obtained from VWR Scientific, Fisher Scientific or Sigma Chemical Company.

<u>INEX 303 (or, Alternatively, Formulation 1.1)</u>

Plasmid (50-400 μg) is incubated with DODAC in 500 μL of the prep solution containing 0.2 M OGP in 150 mM NaCl; 5 mM HEPES pH 7.4, for 30 min at room temperature. This mixture is added to a mixture of DOPE and PEG-Cer-C14 or PEG-Cer-C20 or PEG-Cer-C8 in 500 μL of the same prep solution. The total lipid concentration was either 5 or 10 mg/ml, with the molar ratio of DOPE:DODAC:PEG-Cer being 84:6:10. The mixture was dialyzed against 150 mM NaCl; 5 mM HEPES (pH 7.4) for 36-48 h with two buffer changes.

Nonencapsulated DNA was removed by anion exchange chromatography on DEAE-Sepharose column (1 X 4 cm). Empty liposomes were removed by pooling lipid/DNA samples that co-eluted on the DEAE column on top of a sucrose density gradient in 12.5 ml ultracentrifuge tubes. The gradient was formed with 3 ml each of 10% sucrose, 2.5% sucrose and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 36,000 rpm (160,000 X g) for 2 h at 20°C in a

Beckman Optima XL-100K ultracentrifuge using an SW-28 rotor. Separated bands were removed from top to bottom. Fractions were assayed for ^{3}H-plasmid and ^{14}C-CHE by dual-label scintillation counting using a Beckman LS6500 scintillation counter. The lipid encapsulated plasmid DNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. The formulation can be concentrated in 12-14,000 MWCO dialysis tubing against 500,000 MW PEG (Aquacide II). When the desired volume is reached, the formulation was transferred into a new dialysis bag and dialyzed overnight against HBS to adjust the NaCl concentration to 150 mM.

INEX 351

Lipid Concentration: 5.0 mg/ml (or 5.3 mM)

Plasmid Concentration: 200 μg

Initial Volume: 1.0 ml

Lipid Stock Solutions: (in 95:5 benzene:methanol, 2:1 chloroform:methanol or ethanol)

By mg, 10-20 mg/ml suitable (10 mg/ml preferred, in the above solvents, absolute ethanol is suitable at these lower concentrations).

Calculated by molarity (dissolved in 95:5 benzene:methanol or 2:1 chloroform:methanol).*

DOPE (744 g/mol): 40 mM

DODAC (582 g/mol): 40 mM

PEG-C8 (2515 g/mol): 20 mM

Formulation for 351: 42.5:42.5:15 (mole %) DOPE:DODAC:PEG-C8

	DOPE	DODAC	PEG-C8
mg	1.68	1.315	2.005
mole %	42.5	42.5	15
μmol	2.25	2.25	0.8
μl	56.2	56.2	40

Formulation Procedure (1 ml scale):

Aliquot lipid stock solutions into a clean, dry test tube and dry to a lipid film using a stream of N_2 gas and then dry under vacuum for at least 2 hrs. Add 50 µL 2M OGP and add 500 µL of 2X strength dialysis buffer, add 200 µg of plasmid and mix by vortexing to dissolve the lipid film. Make up to 1.0 mL with sterile deionized H_2O, mix and allow to incubate approximately 30 min at room temperature. Place the solution into a dialysis bag and dialyze for 40-48 hrs against 2 L of dialysis buffer with 1-2 changes of buffer after approximately 24 hrs, and determine the volume of the sample by weighing in a tarred tube (assume density of 1.0). These steps may be followed by DEAE cleaning and/or sucrose density gradient centrifugation, as described above.

After DEAE cleaning and sucrose density centrifugation, as described above, the final INEX 351 formulation has a concentration of about 200 µg/ml plasmid and 5 mg/ml total lipid.

NOTES for INEX 351:

Note 1: Appropriate dialysis buffer concentrations:

p53 : 150 mM $NaPO_4$ + 150 mM NaCl (try 140 - 160 mM NaCl), pH 7.4

pLuc: + 175 mM NaCl (about 150 - 170 mM NaCl), pH 7.4

Note 2: 150 mM $NaPO_4$ buffer, pH 7.4:

35.77 g dibasic sodium phosphate (Na_2HPO_4)

6.62 g monobasic sodium phosphate (NaH_2PO_4)

add appropriate quantity of NaCl dissolve in 2 L (final volume) of deionized water with stirring. The final pH may vary between a pH of about 7.3 and about 7.4; this has not normally been adjusted and has not affected the performance of the formulation.

Note 3: Use 0.2 µm filtered buffer with the lipid/plasmid/detergent solution

Note 4: As an alternative to adding 2X dialysis buffer, the plasmid may be pre-dialyzed against dialysis buffer and the formulation may be diluted to its final volume normal strength dialysis buffer. While this means that there will be a slight difference in the buffer concentration, this does not affect the encapsulation efficiency or resulting particle size.

Note 5: If the volume of the formulation is increased (*i.e.*, above 5 mL), add another dialysis change.

Note 6: DEAE-Sepharose columns are often pre-treated by eluting 50 µL of a 10 mg/ml extruded or sonicated 1:1 phosphatidylcholine:cholesterol vesicle formulation (diluted in 2 mL) to block any nonspecific lipid binding to the column.

To reduce the cationic surface charge of INEX 351 formulations, it may be desirable to reduce the amount of cationic lipid (*i.e.*, DODAC) employed. If the amount of DODAC is changed, the amount of DOPE is changed to maintain the same total amount of lipid. Formulations below 30% DODAC are preferably made in 10 mg total lipid. Dialysis buffer may be changed as in Table 1, below:

Table 1. Characterization of representative large scale formulations.

Conc.	Starting volume	Buffer	Encapsulation efficiency	Nicomp particle size (nm)[a]
42.5 %	30 ml	150 mM $NaPO_4$, 130 mM NaCl	49 %	131
30%	12 ml	150 mM $NaPO_4$	56.8 %	109
24%	30 ml	130 mM $NaPO_4$	50.7 %	250
20%	15 ml	105 mM $NaPO_4$	63 %	178

[a]Nicomp analysis of mean particle size, gaussian dist., volume weighting, before DEAE cleaning and isolation.

INEX 321

Lipid-plasmid particles with 10-30% DODAC are also useful in the present invention. These may be formulated, as described above, or as follows.

Lipid stock solutions: Individual stock solutions of each lipid were dissolved in chloroform/methanol (2:1 v/v) to a final concentration of 2 or 20 mg/ml.

OGP solution: 1.0 M OGP solution was prepared in MilliQ grade water.

Citrate buffer: Sodium citrate buffer was used for dialysis to remove detergent from the formulation. The citrate concentrations were varied according to the

amount of DODAC. Buffer also contains 150 mM NaCl and 5 mM HEPES at pH 7.4, unless indicated otherwise. In general, a 10X solution was prepared and diluted 1:10 in MilliQ Plus water for dialysis using a graduated cylinder.

Preparation of lipid/DNA/OGP mixture: A typical formulation contained 10 mg of lipid of DODAC/DOPE/PEG-Cer-C8 and 200 μg DNA. Appropriate amounts of stock solutions containing DODAC, DOPE and PEG-Cer-C8 (normally 15 mol % in this formulation) were mixed in a glass test tube. If the amount of DODAC is changed, the amount of DOPE is changed to maintain a total of 10 mg lipid. The solvent was first removed under a stream of N_2 gas followed by incubation under vacuum for 3-5 h. To the lipid, 0.2 mL of 1 M OGP was added. The suspension was vortexed until the lipid was totally dissolved and the solution became clear. Then a 0.2 mL DNA (1 mg/ml) solution containing 200 μg DNA and 0.6 mL HBS (HEPES buffered saline) or citrate buffer (concentrations designated in **Figure 1**) were added to a final total volume of 1 mL. If the solution did not become clear, a small amount of OGP (50 μL) was added. The solution was incubated at room temperature for 1 hr to allow the components to equilibrate.

Dialysis: Dialysis tubes were soaked in 60% ethanol (or in distilled water if sterilization was not required) for 30 min. The mixture of DNA/lipid/OGP solution was then transferred to the dialysis tube. The sample was dialyzed for 2 days in 2-4 L citrate buffer (concentration as described in **Figure 1**) with two changes of buffer daily.

After preparation, empty liposomes can be removed by DEAE cleaning and sucrose density centrifugation, as described above. Having been taught the various lipid-plasmid particle formulations suitable for systemic delivery in this example, it would be obvious to one skilled in the art to modify them, for example, for improved plasmid delivery and/or intracellular expression using one or more possible variations. Variations of the following type are suggested: percentage of PEG-lipid; size of PEG; length of hydrophobic (anchor) chain; pH sensitive PEG-lipids; replacement of PEG by ATTA (disclosed in U.S. Patent Application Serial Nos. 08/996,783, filed December 23, 1997, and 06/073,852, filed February 2, 1998, all of which are assigned to the assignee of the instant invention; addition of membrane modifying lipids, such as cholesterol or DOPE; use of alternative cationic lipids, such as DMRIE, DOTAP, DOTMA, DODMA,

AL-1, *etc.*; use of fusogenic components, such as pH sensitive lipids, peptides (EALA) or polymers (PEAA); use of targeting agents; use of DNA condensing peptides (*i.e.*, polylysine or spermine) or polymers (*i.e.*, PEI); use of negatively charged lipids, such as phosphatidylserine; or use of alternative PEG-lipid linkers, such as SPDP or PDPH (disclosed in U.S. Patent Application Serial Number 08/536,584, which is assigned to assignee of the instant invention).

Formulation 1.4

Formulation 1.4 contains DOPE:DODAC:PEG-Cer-C20 (83:7:10) – mol %. The synthesis protocol is as follows: Aliquot the lipid stock solutions (in ethanol) into an autoclaved, clean, dry round bottom flask. The solution is dried to a lipid film using a rotavap in a 65°C water bath and vacuumed overnight. Add HBS with octylglucopyranoside (OGP) to a final OGP concentration of 200 mM. Swirl the mixture to dissolve the lipid film and, if necessary, heat to 37°C to ensure the lipid is fully dissolved. Plasmid DNA is then added (400 μg / 10 mg lipid) to the dissolved lipid films. After incubation at room temperature for 30 min, place the resulting solution in a dialysis bag that has been pre-soaked in filter sterilized distilled H_2O and autoclaved. Dialyze overnight against 20 L of dialysis buffer (5 mM HEPES, 150 mM NaCl, pH 7.4, filter sterilized through a 0.2 micron sterile filter) with two buffer changes.

Nonencapsulated DNA was removed by anion exchange chromatography on a DEAE-Sepharose CL-6B column. Collect the particle suspension as it appears in the eluate, and concentrate using the Amicon difiltration system (YM 30 membrane). Next, empty liposomes were removed using a sucrose density gradient. The gradient was formed by layering 10% sucrose, 5.0% sucrose, and 2.5% sucrose in HBS, pH 7.4. The sample is loaded by floating it on top of the 2.5% sucrose layer and centrifuged at 28,000 rpm for 18 h at 20°C using a Beckman Optima XL-100K ultracentrifuge and an SW-28 rotor. After centrifugation, remove the lower band with a syringe and needle and pool the samples. The sucrose is removed and the sample is concentrated simultaneously using the Amicon system. Filter sterilize the final volume through a 0.2 micron filter. DNA concentration is analyzed by Picogreen assays, lipid concentration by HPLC and particle size by Nicomp analysis.

Formulation 1.5

This method, set out in PCT patent publication WO 96/40964, which is incorporated herein by reference, is an alternative high-efficiency formulation of the lipid/nucleic acid particle. It is, in essence, a preparation of lipid therapeutic nucleic acid particles in organic solvent. The following stock solutions of lipid are prepared in 100% ethanol: DSPC – 20 mg / mL (20 mol%) = 128.4 µL; Chol – 20 mg / mL (25 mol%) = 113.1 µL; DODAP – 40 mg/mL (45 mol%) = 44.5 µL; PEG-Cer-C20 (or C14) – 50 mg/mL (10 mol%) = 67.6 µL.

The lipids are mixed together and the volume is increased to a total volume of 0.400 mL with 100% ethanol. An appropriate volume of 300 mM citrate buffer (pH 3.3) is added to the DNA to a final volume of 600 µL and pH 3.8. Warm the two solutions to 65°C for 2 min. While vortexing the DNA tube, use a Pasteur pipette to add lipid (in ethanol) in a dropwise manner to the DNA solution. The resulting solution will get cloudy and can bubble, but no aggregates should be present. Place the solution in presoaked dialysis tubing (12-14,000 MWCO) and dialyze for 1 h against 300 mM citrate buffer (pH 3.7-4.0). Transfer the dialysis tubing to HBS (pH 7.5) and dialyze for 12 h. Nonencapsulated DNA was removed by anion exchange chromatography using a DEAE-sepharose column equilibrated in HBS. If necessary, the final preparation can be concentrated using the Amicon system (YM 30 membrane). DNA concentration is analyzed by Picogreen assays and the lipid concentration by HPLC.

All of the above lipid-therapeutic nucleic acid formulations have beneficial characteristics that make them suitable for use in the methods of the present invention. Such characteristics include, but are not limited to, the following: First, they are small particles with mean sizes of about 50 about 200 nm and, more preferably, of about 60 to about 130 nm. Most preferably, particles are of a relatively uniform size and have a î2 value of less than 3, more preferably, of less than 1 and, even more preferably, of less than 0.5. Second, they are serum-stable and, thus, are not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA. Third, they have a nucleic acid to lipid ratio that can be formulated at various levels. For use in the methods of this invention, particles are preferably at least about 3 mg nucleic acid per mmol lipid, more preferably at least about 14 mg per mmol lipid and, most preferably,

greater than about 25 mg per mmol. The lipid-nucleic acid particles of the present invention have other advantageous features, such as low nonspecific toxicity, improved biodistribution, therapeutic efficacy and ease of manufacturing.

Assays for Serum Stability

Lipid/therapeutic nucleic acid particles formulated according to the above noted techniques can be assayed for serum stability by a variety of methods.

For instance, in a typical DNase 1 digestion, 1 μg of DNA encapsulated in the particle of interest is incubated in a total volume of 100 μL of 5 mM HEPES, 150 mM NaCl, 10.0 mM MgCl2 pH 7.4. DNase treated samples are treated with either 100 or 10 U of DNase I (Gibco – BRL). 1.0 % Triton X-100 can be added in control experiments to ensure that lipid formulations are not directly inactivating the enzyme. Samples are incubated at 37°C for 30 min after which time the DNA is isolated by addition of 500 μL of DNAZOL followed by 1.0 mL of ethanol. The samples are centrifuged for 30 min at 15,000 rpm in a tabletop microfuge. The supernatant is decanted and the resulting DNA pellet is washed twice with 80% ethanol and dried. This DNA is resuspended in 30 μL of TE buffer. 20 μL of this sample is loaded on a 1.0% agarose gel and subjected to electrophoresis in TAE buffer.

In a typical serum assay, 50 μg of DNA in free, encapsulated, or encapsulated + 0.5% Triton X100 was aliquoted into 1.5 mL Eppendorf tubes. To the tubes were added 45 μl normal murine or human serum, dH_2O (to make final volume 50 μL). The tubes were sealed with parafilm and incubated at 37°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an Eppendorf tube and stored at -20°C. Aliquots were taken at various time points, added to GDP buffer containing proteinase K (133 μg/mL) and immediately frozen in liquid nitrogen to stop the reaction. Once all of the time points were collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to denature any remaining exonuclease. Proteinase K digested samples were applied to polyacrylamide gels to assess levels of exonuclease degradation.

Particles disclosed above demonstrate serum stability by showing less than 5% and preferably undetectable amounts of DNA degradation (partial or total) as a result of such treatment, even in the presence of 100 U DNase 1. This compares favorably to free DNA, which is completely degraded, and plasmid/lipid complexes (such as DOTMA or DODAC:DOPE complexes), wherein DNA is substantially (*i.e.*, greater than 20%, often 80%) degraded after such treatment.

B. EXAMPLE 2

This example illustrates the measurement of the therapeutic effect of lipid formulated ganciclovir on subcutaneous tumors transfected with lipid encapsulated HSV-TK.

Group	Tumor	Plasmid	Prodrug	Route	Assay	Mice per Group
A	B16	L018	PBS	IV	Tumor Volume	6 C57
B	B16	L018	GCV	IV	Tumor Volume	6 C57
C	B16	pTK10	PBS	IV	Tumor Volume	6 C57
D	B16	pTK10	GCV	IV	Tumor Volume	6 C57

The pINEX-TK10 construct consists of a pBR322 derived plasmid containing a CMV promoter linked to a "hyper" HSV-TK gene, bovine growth hormone polyadenylation sequence and kanamycin resistance gene. "hyper" HSV-TK is a more active form of the HSV-TK gene as disclosed by Black, *et al.*, *PNAS (USA)*, **93**:3525-3529 (1996). The plasmid construct employed is set forth in **Figure 14B**. The plasmid L018 has a luciferase reporter gene linked to the CMV promoter.

On day zero, 24 female C57 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded sub-cutaneously with 100,000 B16 mice melanoma cells (NCI catalog B16BL-6) in a total volume of 50 μL (groups A, B, C, D). Tumor volume is determined daily by measuring the length, width and height of the tumor with skin calipers as soon as possible and every day thereafter. Groups A to D are treated with 200 μg plasmid of the appropriate lipid-formulated plasmid, formulated according to Example 1, once daily beginning at 9:00 a.m. on day five and on every day following. The plasmid formulation is injected IV in the tail vein in a total volume of 200 μL PBS. Groups B and D are treated

with lipid formulated ganciclovir, prepared according to Example 3, once daily beginning at 3:00 p.m. on day five and on every day following. 0.5 mg ganciclovir (~25 mg/kg) are injected IV in the tail vein in a total volume of 200 µL PBS. On day 21, mice are sacrificed. Tumors are collected and weighed.

The results obtained demonstrate that the mice of group D either did not develop tumors, or else developed tumors significantly more slowly than mice of control group C.

C. *EXAMPLE 2A*

This example illustrates the measurement of therapeutic effect of systemic delivery of lipid formulated ganciclovir on SCID mice having human (SKOV-3) intraperitoneal (IP) tumors.

Group	Tumor	Plasmid	Prodrug	Route	Assay	Mice per Group
A	SKOV-3	L018	PBS	IV	Tumor Volume	6 C57
B	SKOV-3	L018	GCV	IV	Tumor Volume	6 C57
C	SKOV-3	pTK10	PBS	IV	Tumor Volume	6 C57
D	SKOV-3	pTK10	GCV	IV	Tumor Volume	6 C57

On day zero, 24 female C57 mice are seeded intraperitoneally with 5,000,000 SK-OV-3 human ovary adenocarcinoma cells (ATCC HTB-77)) in a total volume of 500 μL (groups A, B, C, D). Groups A to D are treated with 200 μg plasmid of the appropriate lipid-formulated plasmid, formulated according to Example 1, once daily beginning at 9:00 AM on day five and on every day following. The plasmid formulation is injected IV in the tail vein in a total volume of 200 μL PBS. Treatment will continue for two weeks.

Groups B and D are treated with lipid formulated ganciclovir, prepared according to Example 3, once daily beginning at 3:00 p.m. on day five and on every day following. 0.5 mg ganciclovir (~25 mg/kg) are injected IV in the tailvein in a total volume of 200 μL PBS.

Mice are monitored for survival. If tumors develop, mice are sacrificed and the tumors are collected and weighed.

The results obtained demonstrate that the mice of group D either did not develop tumors, or else developed tumors significantly more slowly than mice of control group C.

D. EXAMPLE 3

This example illustrates the protocol for the preparation of lipid formulated ganciclovir in a sphingomyelin/cholesterol lipid formulation.

1. Weigh out lipids slightly in excess of what is needed. For a 1 mL preparation, use 100 mg (180 μmole) of lipid. 55 mol % is sphingomyelin (99 μmoles,) and 45 mol % (81 μmoles) is cholesterol (Northern Lipids, Vancouver, BC). Dissolve

each lipid in 1 mL of chloroform. Aliquot the required amounts of each lipid into one tube to obtain a 55/45 SM/Chol mixture.

2. Add 4500 dpm/μmole of lipid of ^{14}C-CHE (^{14}C-cholesteryl hexadecyl ether).
3. Dry the lipid to near dryness under nitrogen.
4. Apply to the lyophilizer overnight.
5. Make up a 30/70% solution of ethanol/HBS (HBS is 20 mM Hepes, 145 mM NaCl, pH 7.45)
6. Dissolve 100 mg ganciclovir (109 mg ganciclovir-Na, Hoffman LaRoche Ltd.) in 1 mL of 30/70% ethanol/HBS. Vortex well. Add 42000 dpm/ mole ^{3}H-GCV (7.5 μL of a 1 μCi/mL stock)
7. Add ganciclovir solution to the lipid film and vortex well. Vortex until the solution appears homogeneous.
8. Freeze-thaw for 5 cycles (liquid nitrogen and 65°C). Warm the cryovial up slightly before putting in the water bath.
9. Take 2-10 μL pre-extrusion samples and assay for radioactivity using the dual label program. Take note of the final volume and use this to determine specific activity for both the lipid and GCV.
10. Extrude the sample 2 x thru 3 x1 00 nm filters at 65°C at 350psi. At this point the sample becomes quite viscous. Add 1 mL HBS to the samples and mix by pipetting up and down.
11. Continue extrusion for a total of 10 passes.
12. Take 2-10 μL post-extrusion samples and assay for radioactivity.
13. Hydrate some dialysis tubing (MW cutoff 12,000-14,000) in dH_20 for 15min.
14. Put the extruded sample in the tubing and dialyze for 1 hr against 2 L HBS
15. Change to fresh buffer and dialyze overnight.
16. Take 2-10 μL samples and assay for radioactivity
17. Determine the percentage loading by comparing the pre-extrusion and post-dialysis ratios of ^{3}H/^{14}C. For example: ^{3}H/^{14}C pre-extrusion = 12.0; ^{3}H/^{14}C post-dialysis = 1.2; 1.2/12.0 x 100% = 10% encapsulation.

E. EXAMPLE 3A

Stable transfection of B16 tumor cells with HSV-TK, for use in Examples 4 and 4A, is achieved as described in *Short Protocols in Molecular Biology*, Third Edition, page 9-13 to 9-15, or as described therein, with the following modifications. According to the method, the following materials were used:

Plasmids: pCMVTKIRESneo includes a CMV promoter, HSV-TK gene, internal ribosome entry site and neomycin resistance gene. L018 is the same, but with a luciferase gene in place of HSV-TK.

1. Plate B16 murine melanoma cells in a tissue culture flask (T-75) at 5 x 10^5 cells/flask in 10 ml MEM media with addition of 10% FBS and Glutamine and grow overnight in CO_2 incubator at 37°C to 70% confluency.
2. Aspirate media and feed cells with 3.8 ml fresh media per flask 2 hrs prior to transfection.
3. Prepare plasmid/lipid Lipofectin (GIBCO BRL) aggregate in polystyrene tube according to manufacturer's instructions as follows:
   - dilute plasmid to 20 µg/ml in sterile distilled water.
   - use Luciferase L018 plasmid as a control for selection in Geneticin (G418), use Thymidine Kinase (neomycin) 20A for TKneo stable cells.
   - dilute lipid to 1 mM in sterile distilled water.
   - dilute lipid to charge ratio 1 in sterile distilled water (1.2 ml lipid/5 ml water).
   - add volume of DNA (20 mg/ml) to equivalent volume of lipid (CR1) dropwise while vortexing.
   - incubate DNA/lipid complex for 30 min at room temperature.
4. Slowly add 1.2 ml DNA/lipid complex/T75 flask, mix gently and incubate 24 hr in CO_2 incubator at 37°C (complex is diluted 1:4 in media).
5. Aspirate media, wash with PBS buffer and split each T75 flask into 2-100 x 20 mm tissue culture dishes
6. 24 hr after plating into dishes, add the selective agent, Geneticin (G418), at the appropriate concentration to kill nontransfected cells, yet allow cells with transfected TKneo to stay alive. The Luciferase control cells should die.
7. Every 2-3 days, change the media to remove dead cell debris.

8. Within 10 days, clones are visible on bottom of 100 mm dish which are neomycin resistant and TK positive.

9. Scrape clones into 1 ml media in 24-well plate and expand up into T-75 flask.

10. Cells that stably express TK may then be used for local, regional or systemic injection into mice.

11. To screen identified clones for TK expression:

- plate 2000 cells/well in 96 well plate in 150 μL volume and grow 48 hr in CO_2 incubator at 37°C.
- add the specific prodrug for TK, ganciclovir, in a dilution series across the plate made up at 2.5x concentrated and add 100 μL/well (add to 150 μL volume)
- incubate 3 days in CO_2 incubator at 37°C.
- aspirate media from wells and add Alamar Blue as per manufacturer's instructions (Biosource International) (1:10 dilution in media).
- 100 μl/well and incubate for 1,2,4 hr and read plate at time intervals on fluorescent plate reader (550,595 nm; 750V; 70 offset; 100 ms integration time).

F. EXAMPLE 4

This example illustrates the effects of systemically delivered lipid-formulated ganciclovir on tumor growth in mice having B16 intradermal tumors stably transfected with HSV-TK.

Group	Tumor	Prodrug	Route	Assay	Timepoint (assay)	Mice per Group
A	B16	PBS	IV	Tumor Volume	DAILY	8 C57
B	B16 TK	PBS	IV	Tumor Volume	DAILY	8 C57
C	B16	LIPO-GCV	IV	Tumor Volume	DAILY	8 C57
D	B16 TK	LIPO-GCV	IV	Tumor Volume	DAILY	8 C57

32 female C57 mice (Harlan Sprague Dawley, Inc., Indianapolis) were seeded intradermally with B16 tumor cells stably transfected and expressing HSV-TK (B16 TK) (prepared as in previous example) at a dose of 150,000 cells in a total volume of 50 μL phosphate buffered saline on day zero. Intradermal tumor volume was determined daily by measuring the length, width and height of the tumor with skin calipers as soon as possible and every day thereafter.

The mice were treated with the ganciclovir prodrug, lipid formulated as in Example 3, once every two days beginning on day four and on every second day following. The ganciclovir dosage of 0.5 mg (~25 mg/kg) was injected IV in a total volume of 200 μL PBS (phosphate buffered saline). Mice received a total of nine treatments. On day 21, mice were sacrificed. Tumors were collected and weighed prior to fixation for sectioning.

Intradermal tumors stably transfected with HSV-TK showed no measurable growth when treated systemically with lipid formulated ganciclovir. Untreated B16 tumors, and treated B16 tumors without TK, were not affected by the drug.

G. EXAMPLE 4A

This study was to determine the effect of lipid formulated ganciclovir on TK gene expression in B16 tumor cells stably transfected with HSV-TK and implanted intravenously.

Group	Tumor	Prodrug	Route	Assay	Timepoint (assay)	Mice per Group
A	B16 TK	PBS	IV	Tumor Volume	DAILY	8 C57
B	B16 TK	LIPO-GCV	IV	Tumor Volume	DAILY	8 C57

16 female C57 mice were seeded with B16 tumor cells stably expressing HSV-TK by tail vein injection at a dose of 150,000 cells in a total volume of 200 μL phosphate buffered saline on day zero. The mice were treated with the ganciclovir prodrug, lipid formulated as in Example 3, once every day beginning on day two and on the two days following. The ganciclovir dosage of 0.5 mg (~25 mg/kg) was injected IV in a total volume of 200 μL PBS (phosphate buffered saline). Mice received a total of three treatments. On day 21, mice were sacrificed and tumors were scored. Livers, lungs, spleen and pancreas were photographed.

The metastatic tumor nodules of the mouse treated with the lipid-ganciclovir formulation were significantly smaller than those of the untreated mice.

H. EXAMPLE 5

This example illustrates gene expression in distal metastatic tumors using INEX 303 lipid plasmid particles.

On day zero, C57BL/6 mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded with 300,000 LL/2 (Mouse Lewis Lung Carcinoma) cells (ATCC CRL-1642) by intravenous/tail vein injection with total volume 200 μL. On day 10, the mouse is intravenously injected with INEX 303 plasmid-lipid particles, formulated according to Example 1. The particles carry plasmid L018, which is a standard construct containing the luciferase gene linked to the CMV promoter. At various time points after plasmid injection, mice are sacrificed, and organs and tumors are quickly frozen in liquid nitrogen, then stored at -70°C. Organs/tumors are assayed for the luciferase gene to demonstrate delivery to the organ/tumor site. Biodistribution results for organs are shown in **Figure 2**. Accumulation at the tumor site is illustrated in **Figure 3**. Southern blot data shows presence of intact plasmid at the tumor site increasing to at least 96 h. Cell protein from organs/tumors is also prepared and assayed for luciferase according to standard

techniques. A time course of gene product activity at distal (metastatic) tumor sites is demonstrated in **Figure 4**.

I. EXAMPLE 6

This example illustrates the systemic vector delivery and gene expression in an *in vivo* human tumor.

SCID mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) are seeded with 1 x 10^6 LS180 human colon adenocarcinoma cells (ATCC CL-187) by subcutaneous injection on day zero. On day 11, mice in groups A, B and C are injected intravenously with indicated doses of L018 plasmid in lipid formulation INEX 303, which is formulated according to Example 1, in 200 μL total volume. On day 17, mice in group D and E are injected intravenously with L018 plasmid in lipid formulation INEX 320, using C8 or C20 PEG-Cer according to Example 1, in 200 μL total volume. At the times indicated after plasmid injection, mice are sacrificed and organs (liver, spleen and lungs) and tumors are harvested. Expression of the enzyme luciferase is assayed according to standard techniques on all samples.

The data obtained demonstrates the excellent transfection and expression of the reporter luciferase achieved in an *in vivo* human tumor using the lipid-nucleic acid particles of the present invention (*see*, **Figure 5**).

A	303 (75 μg)	Luciferase	48 hr	5
B	303 (100 μg)	Luciferase	48 hr	5
C	303 (125 μg)	Luciferase	48 hr	5
D	320 (100 μg)	Luciferase	24 hr	4
E	320 (100 μg)	Luciferase	48 hr	4
F	PBS	Luciferase	48 hr	1

J. EXAMPLE 7

This example demonstrates systemic delivery and expression at an *in vivo* tumor site of a vector containing the HSV-TK gene, using a lipid-nucleic acid particle prepared according to Example 1.

C57 mice are intraperitoneally seeded with 100,000 B16 tumor cells in a total volume of 200 µL PBS on day zero. On day 14, test mice are injected with INEX 351 plasmid formulation (100 µg DNA in 500 µL PBS) prepared according to Example 1. The plasmid vector used is pCMVTKIRESneo as described earlier. 24 h later, mice are sacrificed, and tumors are isolated, fixed within 5 minutes, and prepared in paraffin sections using standard techniques. The expression of the HSV-TK gene at the distal tumor site is assayed by *in situ* RNA/RNA hybridization using techniques standard in the art. One such technique is summarized below.

The pattern of HSV-TK gene expression within peritoneal tumors is demonstrated in **Figure 6**. In all cases of gene expression, positive signal is observed as a cellular content of B16 cells or endothelial cells. Positive stained cells are localized in proliferative zone associated with blood vessels or peripheral area.

<u>Protocol for RNA/RNA *in situ* hybridization of *in vivo* tumors transfected by systemically delivered plasmid.</u>

Tumors are prepared for *in situ* investigation by paraffin embedding and staining. Specifically, peritoneal tumors are collected into 4% paraformaldehyde/PBS fixative (Sigma Chemical Co.) and fixed overnight at 4°C. Serial 5 µm sections are prepared after the samples have been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in paraffin wax (Paraplast Plus, Fisher).

When ready to be used, prepared samples are treated with two changes of xylene for 10 min., each followed by rehydration in graded ethanol solutions to 50% ethanol. Samples are prehybridized by standard rinsing, incubation with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride, followed by treatment at 56°C for at least 60 minutes in hybridization buffer containing: 40% deionized formamide, 10% dextran sulfate, 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10 mg/ml RNase- free bovine serum albumin), 4 x SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, and 1 mg/ml denatured and sheared salmon sperm DNA.

Labelling of RNA probe by *in vitro* transcription of DNA was done as follows. The fragment of 599bp (532 - 1131) from HSV-TK (pTK10) was cloned into KpnI and BamHI sites of the vector pGEM-7Zf(+) (pTK11). The plasmid is cloned by standard techniques and prepared using Qiagene 500 (Qiagen, Inc.). For the anti-sense probe, this plasmid is linearized by cutting it with KpnI at the original 5' end of the cDNA HSV-TK and purified. The same logic is used for sense (control) probe (*i.e.*, cut at the side of the 3-end of insert by BssHII or BamH or SacI). The plasmid is purified by ethanol precipitation. The following are then mixed in a 1.5 mL sterile RNase free microcentrifuge tube on ice: 4 μL (4 μg) purified, linearized plasmid DNA, 5 μL of 10 x concentrated DIG RNA Labeling Mix (supplied by manufacturer), 10 μL 5 x concentrated Transcription Buffer (400 mM Tris-HCl (pH 8.0, 20°C), 60 mM $MgCl_2$, 100 mM Dithiothreitol (DTT), 20 mM spermidine, 2 μL RNasin, 3 μL RNA polymerase (SP6 for antisense or T7 for sense), and sterile, redistilled water to make a total reaction volume of 50 μL.

The components are mixed and centrifuged briefly, and then incubated for 2 hrs at 37°C (for T7 RNA polymerase) or at 40°C (for SP6 polymerase). **Note:** *Longer incubations do not increase the yield of labeled RNA. To produce larger amounts of RNA, scale up the reaction components.* After incubation, add 3 μL DNase I, RNase free and 1 μL RNasin to the tube and incubate for 15 min at 37°C. Then add 2.5 μL 0.5M EDTA (pH 8.0) to the tube to stop the polymerization reaction.

The labeled RNA transcript is precipitated by performing the following steps. Add to the reaction tube 6.25 μL 4 M LiCl and 180 μL prechilled (-20°C) 100% ethanol incubate overnight at -70°C. Centrifuge the tube (at 13,000 x g) for 15 min at 4°C. Discard the supernatant. Wash the pellet with 50 μL ice-cold 70% (v/v) ethanol. Centrifuge the tube (at 13,000 x g) for 5 min at 4°C. Discard the supernatant and dry the pellet at room temperature. Dissolve the RNA pellet for 30 min at 37°C or (R.T.) in 20 μL DEPC (diethylpyro-carbonate)-treated, sterile, redistilled water, and add 20 μL deionized formamide and 1 μL RNasin. Keep transcript at -20°C or -70°C.

An accurate quantification of DIG-labeled RNA obtained in the labeling reaction is most important for optimal and reproducible results in various membrane or *in*

situ hybridization techniques. Too high of a probe concentration in the hybridization mix usually causes background, while too low of a concentration leads to weak signals. The estimation of yield can be performed in a side by side comparison of the DIG-labeled sample nucleic acid with a DIG-labeled control that is provided in the labeling kits. Dilution series of both are prepared and spotted on a piece of membrane. Subsequently, the membrane is colorimetrically detected. Direct comparison of the intensities of sample and control allows the estimation of labeling yield.

The hybridization reaction is then performed. Drain pre-hybridization buffer from the pre-hybridized slides and overlay each section with 200 µL of hybridization buffer containing 0.2-1 ng of digoxigenin-labeled RNA probe (0.2 ng/ L). Cover samples with a 24 x 30 mm hydrophobic plastic coverslip. Incubate sections at 56°C overnight in a humid chamber. Washes may include an RNAse step which reduces the background, but decreases the signal as well. It is important to keep the tissue sections moist at all times during washing.

Wash the slides in 2 x SSC at 55°C for 30 min.

Wash in 50% formamide, 2 x SSC at 65°C for 30 min.

Wash in 2 x SSC 3 times at 37°C for 5 min. each.

Wash in RNase 10 µg/ml washing solution at 37°C for 30 min.

Wash in 50% formamide, 2 x SSC at 65°C for 30 min.

Wash in 2 x SSC at 37°C for 15 min.

Wash in 0.2 x SSC 5 times at 37°C for 5 min. each.

After hybridization, cells are incubated DIG-specific antibody. Wash the slides in TBS at RT for 30 min. Incubate sections with blocking solution (TBS and 2% goat serum) at RT for 1 h. Decant blocking solution and incubate sections with goat anti-DIG-alkaline phosphatase (Fab fragment) dilution 1:500 at RT for 1 h. Wash the slides in TBS at RT for 30 min.

Wash the slides in substrate buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$) at RT for 30 min.

Prepare a color solution containing: 10 ml substrate buffer, 50 µL NBT (nitroblue tetrazolium) and 37 µL BCIP. Slides are immersed in color solution at room

temperature for 1-2 hr or at 4°C for 2-3 days. Slides are washed with water and observed by light microscopy. Results are shown in **Figure 6**.

K. EXAMPLE 8

This example describes the treatment of tumors using the method of the present invention. In particular, this example demonstrates the effect of pINEX-TK10 in Formulation 1.1 in inhibiting the growth of MCA-207 tumors following treatment with ganciclovir. The general method is set out in **Figures 7A** and **7B**.

Group	Formulation	GCV	Route*	Assay	Timepoint	# of Mice
A	PBS	PBS	IP	Volume/CTL	---	6 C57
B	Empty 1.1	PBS	IP	Volume/CTL	---	6 C57
C	1.1 TK	PBS	IP	Volume/CTL	---	6 C57
D	1.1 TK	GCV	IP	Volume/CTL	---	6 C57

* It is noted that the "Route" refers to the delivery of the prodrug, *i.e.*, gangciclovir.

24 female C57 mice were seeded with 100,000 MCA-207 fibrosarcoma tumor cells (provided by S. Rosenberg, National Cancer Institute, Frederick/Bethesda, MD) by intra-dermal injection on day zero. The tumor cells had been cultivated and prepared according to standard techniques using RPMI media with 10% Fetal Bovine Serum (*see, for example, Current Protocols in Molecular Biology*). Beginning on day 5, all animals were treated with the lipid/therapeutic nucleic acid formulation listed in the chart, *supra*. The formulation was delivered intravenously through the tail vein. 80 μg of pINEX-TK10 DNA were injected in a total volume of 200 μL. Treatments were administered on days 5, 7, 9, 11 and 13.

Beginning on day 5, all animals were treated with ganciclovir twice daily. 1 mg (~50 mg/kg) were injected intra-peritoneally in a total volume of 200 μL PBS. Treatments continued twice daily for 12 days (*see*, **Figures 7A** and **7B**). Mice were monitored for tumor growth.

Figure 8A sets out in more quantitative terms the effect of the treatments. Mice treated with HSV-TK in Formulation 1.1 have greatly reduced tumors compared to control treated mice. Not shown is data of control mice which demonstrates that treatment with empty liposomes and ganciclovir has no effect on tumor reduction.

Figure 8B demonstrates the effect of the treatment on test mice in comparison with control mice at day 16 after tumor inoculation.

L. EXAMPLE 9

1. The Materials

Sphingomyelin and cholesterol were obtained from Northern Lipids (Vancouver, BC). ^{14}C-cholesteryl hexadecyl ether (^{14}C-CHE) was purchased from Amersham (Mississauga, ON). Ethanol, chloroform, methanol, N-(2-hydroxyethyl) piperazine N'-(2-ethanesulphonic acid) (HEPES), hydrogen peroxide and sodium chloride (NaCl) were from VWR Scientific (Mississauga, ON). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma (St. Louis, MO). Solvable® and Picofluor® scintillation fluids were provided by Canberra Packard Canada (Mississauga, ON). Ganciclovir (GCV) was acquired from Hoffman LaRoche Ltd. (Mississauga, ON). ^{3}H-GCV was purchased from Moravek Biochemicals (Brea, CA). Female C57BL/6 mice were from Harland Sprague Dawley (Indianapolis, IN). The B16 cell line was acquired from the NCI (Frederick, Maryland). B16TK cells were produced at Inex according to standard methodology (*see*, previous Examples).

2. The Preparation of GCV-TCS

Large unilamellar vesicles were prepared according to the method of Hope, *et al., Biochim. Biophys. Acta* 812:55-65 (1985). 55 mole% sphingomyelin and 45 mole% of cholesterol were dissolved in chloroform with a drop of methanol and approximately 0.035 μCi of ^{14}C-CHE. Lipids were dried down under a stream of nitrogen gas and the resulting lipid film was then placed under high vacuum for at least 3 hrs. A solution of ganciclovir containing ^{3}H-GCV was made up in 30% ethanol, 70% HBS (20 mM Hepes, 145 mM NaCl pH 7.45) to 50 or 100 mg/mL depending on the sample size. The lipid to GCV weight ratio was initially 1 to 1. The GCV solution was added to the lipid film. The lipid-GCV solution was heated to 65°C for 5 min and

vortexed well in order to obtain a homogenous solution. The resulting solution was subjected to five freeze-thaw cycles before extrusion through 100 nm filters using an extrusion apparatus (Lipex Biomembranes) heated to 65°C. An extra 1 mL of buffer was added to the solution after the third pass of extrusion. Once extruded, the formulation was dialyzed against HBS overnight with one buffer exchange after one hr of dialysis. The formulation was sized using a quasielastic light scattering (QELS) particle sizer (Nicomp 370). The lipid concentration was determined by scintillation counting of ^{14}C-CHE label. The GCV concentration was determined by counting ^{3}H-GCV and a Bligh Dyer assay.

3. Methods

Groups of four female C57BL/6 mice per time point were injected subcutaneously with 150,000 B16 tumor cells. The tumors were grown for 14 days at which time a single iv dose of free GCV or liposomal GCV was given via the lateral tail vein. This dose, given in a total of 200 µL, contained approximately 5.6 mg (10 µmoles) lipid and 0.5 mg (1.96 µmoles) GCV resulting in lipid and GCV doses of 300 mg/kg and 25 mg/kg, respectively. Circulating levels of lipid and GCV were determined at 15 min, 1, 8, 24 and 48 hrs for the liposomal GCV groups. Levels of GCV were determined at 15 min and 1 hr for the free GCV groups. At each time point, animals were anesthetized with ketamine/xylazine and blood was collected via cardiac puncture. Blood was put into EDTA coated microtainers and spun at 500 g for 10 min in order to pellet blood cells and obtain plasma. Lipid and GCV levels in the plasma were determined. Lipid and GCV were measured by counting ^{14}C-CHE and ^{3}H-GCV. One hundred or 200 µL aliquots of plasma were measured on a Beckman LS3801 scintillation counter.

4. Results

a. Pharmacokinetics of GCV-TCS in B16 tumored mice

This example demonstrates plasma clearance rates of free ganciclovir and liposomal ganciclovir. Levels of lipid and GCV were monitored in the plasma by tracing ^{14}C-CHE and ^{3}H-GCV labels. A direct comparison of the pharmacokinetic properties of free and liposomal GCV was made at 15 min and 1 hr. **Figure 9** shows that liposomal

encapsulation confers enhanced pharmacokinetics of GCV resulting in an increased circulation lifetime compared to free drug, with a hundred-fold increase in serum drug levels 1 hr after administration. After 1 hr, 41% of the injected dose of liposomal GCV remains in circulation compared with only 0.4% of the free GCV. Animals treated with liposomal GCV have significant amounts of drug (1.5% of the injected dose) in the circulation even after 48 hrs.

b. In vivo kinetics of GCV release

The bioavailability of lipid-formulated GCV after intravenous administration was determined by comparing lipid and ganciclovir levels in plasma at various times after tail vein injection. The resulting plasma drug-to-lipid ratio was determined. A decrease in the *in vivo* drug-to-lipid ratio indicates that a portion of the GCV is no longer associated with the liposomal formulation. This free GCV is rapidly cleared from the circulation. The decrease in the relative percent recovery shown in **Figure 10** is suggestive of gradual *in vivo* drug release.

M. EXAMPLE 10

This example illustrates biodistribution studies.

1. Methods

Biodistribution data was collected from those mice that were in the pharmacokinetic studies. Following heart puncture, mice were terminated by cervical dislocation. Tissues were harvested from each animal and weighed. Saline was added to each organ prior to homogenization by either a Polytron homogenizer (livers) or a Fast Prep® machine. Tissues were solubilized in Solvable for 3 to 16 hr at 50°C. The samples were then cooled to room temperature before defoaming and decolourizing with EDTA and hydrogen peroxide respectively. Picofluorscintillation fluid was added to the samples prior to counting on the scintillation counter.

2. Results

a. Biodistribution of GCV-TCS in B16 tumored mice

Tumor bearing mice were treated with free or liposomal GCV. Liver, lungs, spleen, and kidneys were analyzed for lipid and ganciclovir content. The major route of elimination for many drugs is the kidney. **Figures 11A, B, C** and **D** show that free ganciclovir was essentially absent from all of the organs assayed, except for a small amount, 3.5 +/- 1.8% of the total injected dose, which was found in the kidneys at the 15 minute time point. This finding is consistent with previous studies in humans that found that intravenously administered ganciclovir is rapidly eliminated in the urine. *See*, Paul, *et al.*, *J. Am. Med. Sci.*, **304**:272-277 (1992), and Markham, *et al.*, *Drugs*, **48(3)**:455-484 (1994). For this reason, free GCV biodistribution data were not collected beyond 1 hr. A similar amount of free GCV, 3.1 +/- 0.1% of the initial injected dose, was found in the liver 15 minutes after administration, but this represents a much lower relative dose because of the large size of this organ.

The biodistribution of liposomal GCV was considerably different from that of the free drug. Liposomal formulations are typically cleared from circulation by the organs of the reticuloendothelial system (RES), the spleen and liver. *See*, Lim, *et al.*, *J. Pharmacol. Exp. Ther.*, **281(1)**:566-573 (1997), Gregoridis, *et al.*, *Drugs*, **45(1)**:15-28 (1993) and Cullis, *et al.*, "Liposomes as Pharmaceuticals," *Liposomes* (Ostro, M. (ed.), Marcel Dekker: New York, pp. 39-72 (1987)). **Figure 11A** illustrates a significant increase in liposomal GCV accumulation in the liver over time, with a maximum accumulation of 13.6 +/- 3.1% of the total injected dose 48 hrs after administration. The liver also accumulates a similar amount of lipid at early time points. However, at 24 and 48 hrs, a significantly greater percentage of the lipid dose accumulates in the liver. This may represent an accumulation of empty liposomal particles or a remodeling of lipid components with concomitant uptake in the form of lipoproteins.

The kinetics of liposomal GCV uptake in the spleen differ significantly from the situation in liver. **Figure 11B** illustrates that the spleen rapidly accumulates as much as 8.5 +/- 4.8% of the total injected liposomal GCV dose with a peak at 1 hr. At this time, only 0.04 +/- 0.02% of the free drug remains in the spleen. This difference in kinetics suggests a mechanistic difference, possibly involving uptake and redirection of liposomes by homing macrophages. Intravenous administration of liposomal GCV resulted in a minimal accumulation of GCV in the kidney (*see*, **Figure 11D**) and lung (*see*, **Figure 11C**) persisting from 1 to 48 hr. This maintenance of GCV in the kidney

may be a reflection of the rapid clearance of GCV from the blood as it becomes bioavailable. Minimal accumulation of free GCV or liposomal GCV over the entire time course (less than 0.35% of the total injected dose) was observed in the lung. Liposomes of this size (100 nm) typically do not accumulate in the lung.

In summary, the free GCV accumulated to the largest extent in the kidney, whereas the liposomal GCV accumulated in the spleen and liver.

b. Tumor Accumulation of GCV-TCS

Tumor bearing mice were treated intravenously with 25 mg/kg of free or liposomal GCV. Lipid and GCV were quantified using ^{14}C-CHE and ^{3}H-GCV labels. **Figure 12** illustrates that the accumulations of both free and encapsulated GCV in the tumor are comparable at early time points, but that encapsulated GCV has extended bioavailability at the disease site. Free ganciclovir is rapidly cleared from the tumor, with only 1.1 +/- 0.1% of the total injected dose per gram of tumor detected at 1 hr. Liposomal formulation results in maintenance of GCV in the tumor over the course of the experiment. Forty-eight hrs after treatment, there is still 3.9 +/- 0.5% of the total injected dose per gram of tumor tissue. The high drug-to-lipid ratio at the tumor suggests that some of the GCV that is no longer associated with lipid is accumulating there. The mechanism of action of GCV involves inhibition of DNA polymerase and termination of DNA synthesis via chain termination (*see*, Paul, *et al.*, *J. Am. Med. Sci.*, **304**:272-277 (1992), Markham, *et al.*, *Drugs*, **48(3)**:455-484 (1994) and Oja, *et al.*, *BBA*, **1281**:31-37 (1996)). Since not all tumor cells can be expected to undergo DNA synthesis during the short time period in which free GCV is accumulating, and because the intracellular stability of GCV, GCV-MP and GCV-TP is not well understood, it is believed that there are significant benefits associated with long-term, continuous GCV exposure. Quite importantly, the liposomal formulations described herein have extended bioavailability at the disease site compared with the free drug.

N. EXAMPLE 11

This example illustrates efficacy studies.

1. Methods

Groups of 4 or 5 female C57BL/6 mice were injected intradermally with B16 cells stably transfected with HSV-TK. Once tumors were measurable (day 5), the mice were injected intravenously with liposomal GCV (25 mg/kg GCV). Injections were continued every other day for 6 injections. Tumor measurements were typically taken every other day.

2. Results

a. In vivo efficacy of GCV-TCS in the B16TK model

To test whether the lipid-formulated ganciclovir retains its biological activity, an *in vivo* efficacy experiment was performed. Mice were seeded with B16 melanoma cells stably transfected with HSV-TK. **Figure 13** illustrates that the treatment of mice with liposomal GCV significantly inhibits the growth of B16-TK melanoma. Measurements on day 18 indicated that tumors in the control HBS-treated mice were fivefold larger than those in the treated groups. These results confirm that the formulation process did not compromise the structural integrity of GCV, and provide additional evidence of the bioavailability of the drug.

It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications and publications, are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A method of sensitizing a cell to a compound, said method comprising:

a) transfecting a cell with a nucleic acid that comprises a nucleotide sequence that produces a gene-product that promotes the processing of a first compound into a second compound; and

b) delivering to said cell said first compound in a lipid formulation;

wherein said cell is more sensitive to said second compound than said first compound.

2. The method of claim 1 wherein said nucleic acid is in a lipid formulation.

3. The method of claim 2 wherein said nucleic acid is in a lipid encapsulated formulation.

4. The method of claim 3 wherein said nucleic acid is fully encapsulated in said lipid encapsulated formulation.

5. The method of claim 1 wherein said nucleic acid is a cationic lipid-nucleic acid aggregate.

6. The method of claim 1 wherein said nucleic acid comprises a vector.

7. The method of claim 1 wherein said nucleic acid comprises a modified adenovirus or modified retrovirus.

8. The method of claim 2 wherein said nucleic acid is naked DNA.

9. The method of claim 2 wherein said lipid formulation comprises a cationic lipid, and a compound which delays clearance of said lipid formulation from the circulation.

10. The method of claim 8 wherein said lipid formulation comprises DODAC, and a polyamide oligomer linked lipid.

11. The method of claim 1 wherein said lipid formulation comprises sphingomyelin and cholesterol.

12. The method of claim 1 wherein said lipid formulation comprises a cationic lipid and said nucleic acid is in a lipid formulation comprising a cationic lipid.

13. The method of claim 1 wherein the cell is in an organism and the nucleic acid is delivered systemically.

14. The method of claim 1 wherein the cell is in an organism and the nucleic acid is delivered regionally or locally.

15. The method of claim 13 or 14 wherein the organism is a mammal.

16. The method of claim 1 wherein the gene-product is a polypeptide.

17. The method of claim 1 wherein said gene-product is a member selected from the group consisting of herpes simplex virus thymidine kinase, cytosine deaminase, xanthine-guaninephosphoribosyl transferase, purine nucleoside phosphorylase, cytochrome P450 2B1 and their analogs.

18. The method of claim 1 wherein said first compound is a prodrug selected from the group consisting of ganciclovir, acyclovir, bromovinyldeoxyuridine, 5-fluorocytosine, 6-thioxanthine, MeP-dr and cyclophosphamide.

19. The method of claim 1 wherein said prodrug is ganciclovir and said lipid formulation comprises sphingomyelin.

20. A method of sensitizing a cell to a compound, said method comprising:

a) delivering to a cell an enzyme that promotes the processing of a first compound into a second compound; and

b) delivering to the cell the first compound in a lipid formulation;

wherein said cell is more sensitive to said second compound than said first compound.

21. A kit for the treatment of a human medical disorder, said kit comprising:

a) a nucleic acid in a lipid formulation; and

b) a prodrug in a lipid formulation.

22. A composition, said composition comprising a ganciclovir in a lipid formulation and a pharmaceutically acceptable carrier.

23. The composition of claim 22 wherein said composition further comprises a nucleic acid.

24. The composition of claim 23 wherein said nucleic acid comprises a vector.

25. The composition of claim 23 wherein said nucleic acid is in a lipid formulation.

26. The composition of claim 22 wherein said composition further comprises an enzyme.

27. The composition of claim 26 wherein said enzyme is in a lipid formulation.

28. The use of a lipid formulation to deliver to a cell a first compound wherein said cell is more sensitive to a second compound than said first compound and wherein said cell is transfected with a nucleic acid comprising a nucleotide sequence that produces a gene-product that promotes the processing of said first compound into said second compound.

29. The use of a lipid formulation according to claim 28 wherein said nucleic acid is in a lipid formulation.

30. The use of a lipid formulation according to claim 29 wherein said nucleic acid is in a lipid encapsulated formulation.

31. The use of a lipid formulation according to claim 30 wherein said nucleic acid is fully encapsulated in said lipid encapsulated formulation.

32. The use of a lipid formulation according to claim 28 wherein said nucleic acid is a cationic lipid-nucleic acid aggregate.

33. The use of a lipid formulation according to claim 28 wherein said nucleic acid comprises a vector.

34. The use of a lipid formulation according to claim 28 wherein said nucleic acid comprises a modified adenovirus or modified retrovirus.

35. The use of a lipid formulation according to claim 29 wherein said nucleic acid is naked DNA.

36. The use of a lipid formulation according to claim 29 wherein said lipid formulation comprises a cationic lipid, and a compound which delays clearance of said lipid formulation from the circulation.

37. The use of a lipid formulation according to claim 35 wherein said lipid formulation comprises DODAC, and a polyamide oligomer linked lipid.

38. The use of a lipid formulation according to claim 28 wherein said lipid formulation comprises sphingomyelin and cholesterol.

39. The use of a lipid formulation according to claim 28 wherein said lipid formulation comprises a cationic lipid and said nucleic acid is in a lipid formulation comprising a cationic lipid.

40. The use of a lipid formulation according to claim 28 wherein the cell is in an organism and the nucleic acid is delivered systemically.

41. The use of a lipid formulation according to claim 28 wherein the cell is in an organism and the nucleic acid is delivered regionally or locally.

42. The use of a lipid formulation according to claim 40 or 41 wherein the organism is a mammal.

43. The use of a lipid formulation according to claim 28 wherein the gene-product is a polypeptide.

44. The use of a lipid formulation according to claim 28 wherein said gene-product is a member selected from the group consisting of herpes simplex virus thymidine kinase, cytosine deaminase, xanthine-guaninephosphoribosyl transferase, purine nucleoside phosphorylase, cytochrome P450 2B1 and their analogs.

45. The use of a lipid formulation according to claim 28 wherein said first compound is a prodrug selected from the group consisting of ganciclovir, acyclovir, bromovinyldeoxyuridine, 5-fluorocytosine, 6-thioxanthine, MeP-dr and cyclophosphamide.

46. The use of a lipid formulation according to claim 28 wherein said prodrug is ganciclovir and said lipid formulation comprises sphingomyelin.

47. The use of a lipid formulation to deliver to a cell a first compound wherein said cell is more sensitive to a second compound than said first compound and wherein an enzyme that promotes the processing of said first compound into said second compound is delivered to said cell.

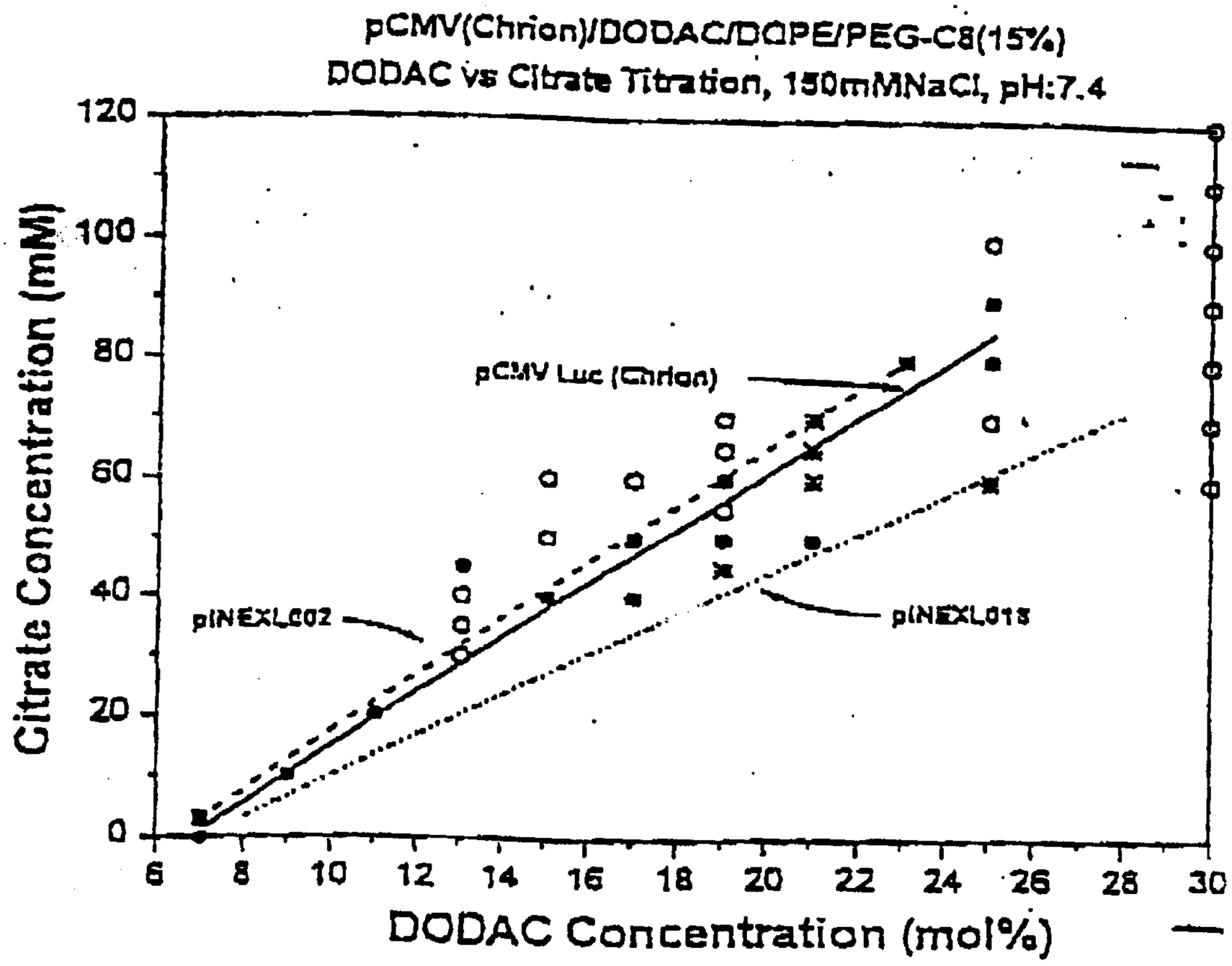
pCMV(Chrion)/DODAC/DOPE/PEG-C8(15%)
DODAC vs Citrate Titration, 150mMNaCl, pH:7.4
Citrate Concentration (mM)
DODAC Concentration (mol%)
pCMV Luc (Chrion)
pINEXL002
pINEXL013
120
100
80
60
40
20
0
6
8
10
12
14
16
18
20
22
24
26
28
30

Figure 1

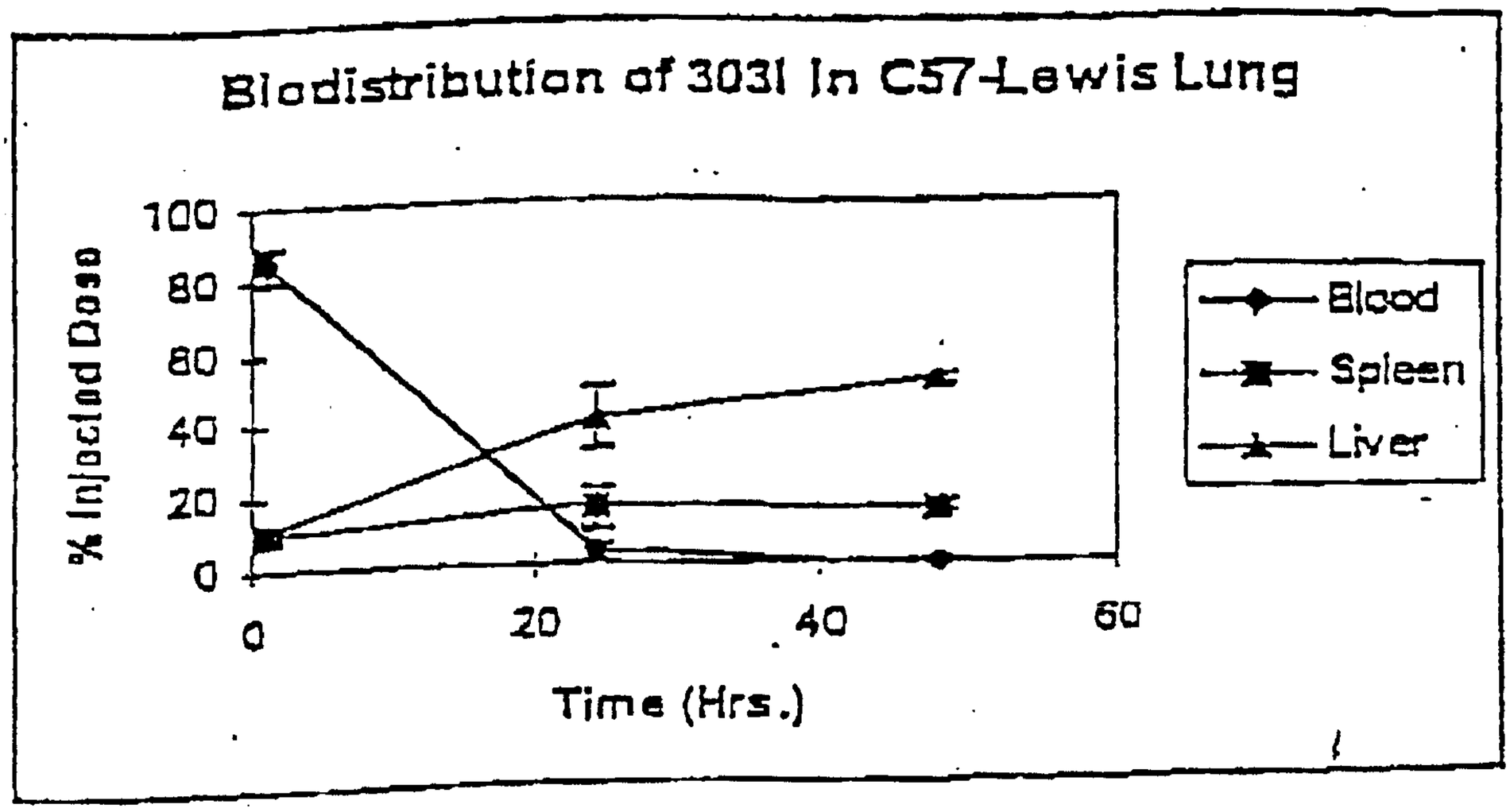
Biodistribution of 3031 In C57-Lewis Lung
% Injected Dose
100
80
60
40
20
0
0
20
40
60
Time (Hrs.)
Blood
Spleen
Liver

Figure 2

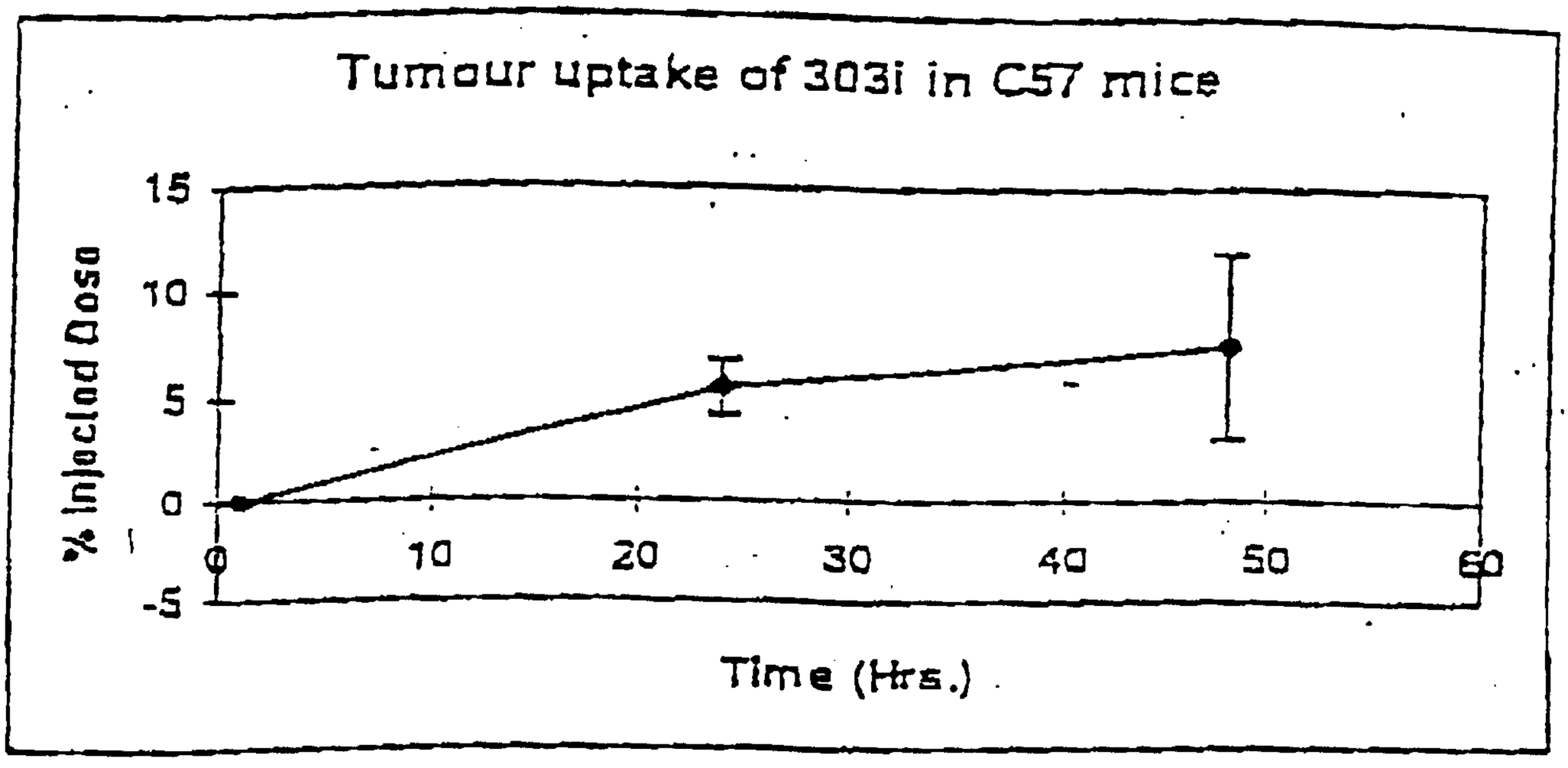
Tumour uptake of 303i in C57 mice
% Injected Dose
15
10
5
0
-5
0
10
20
30
40
50
60
Time (Hrs.)

Figure 3

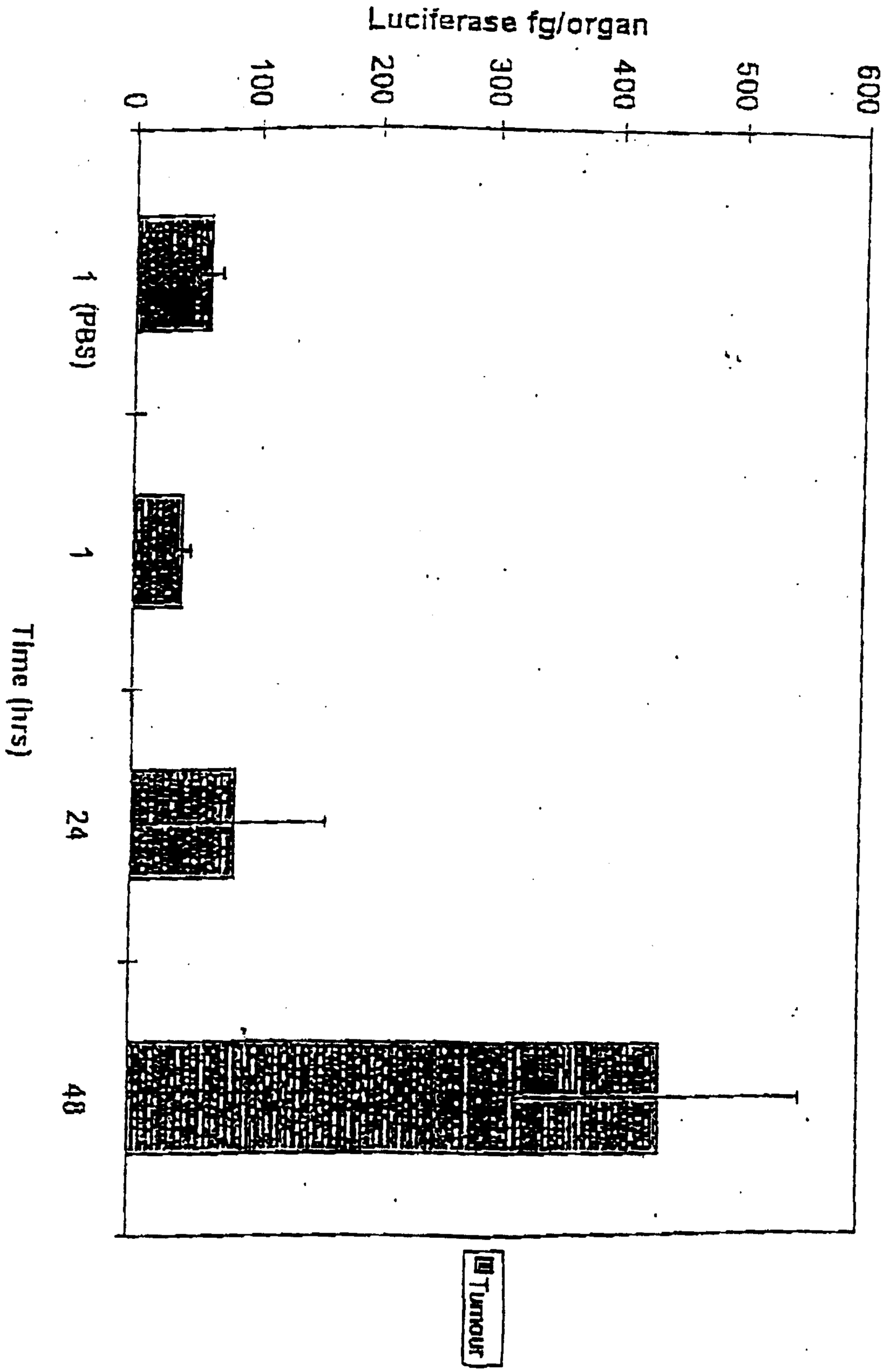
Luciferase fg/organ
0
100
200
300
400
500
600
1 (PBS)
1
24
48
Time (hrs)
Tumour

Figure 4

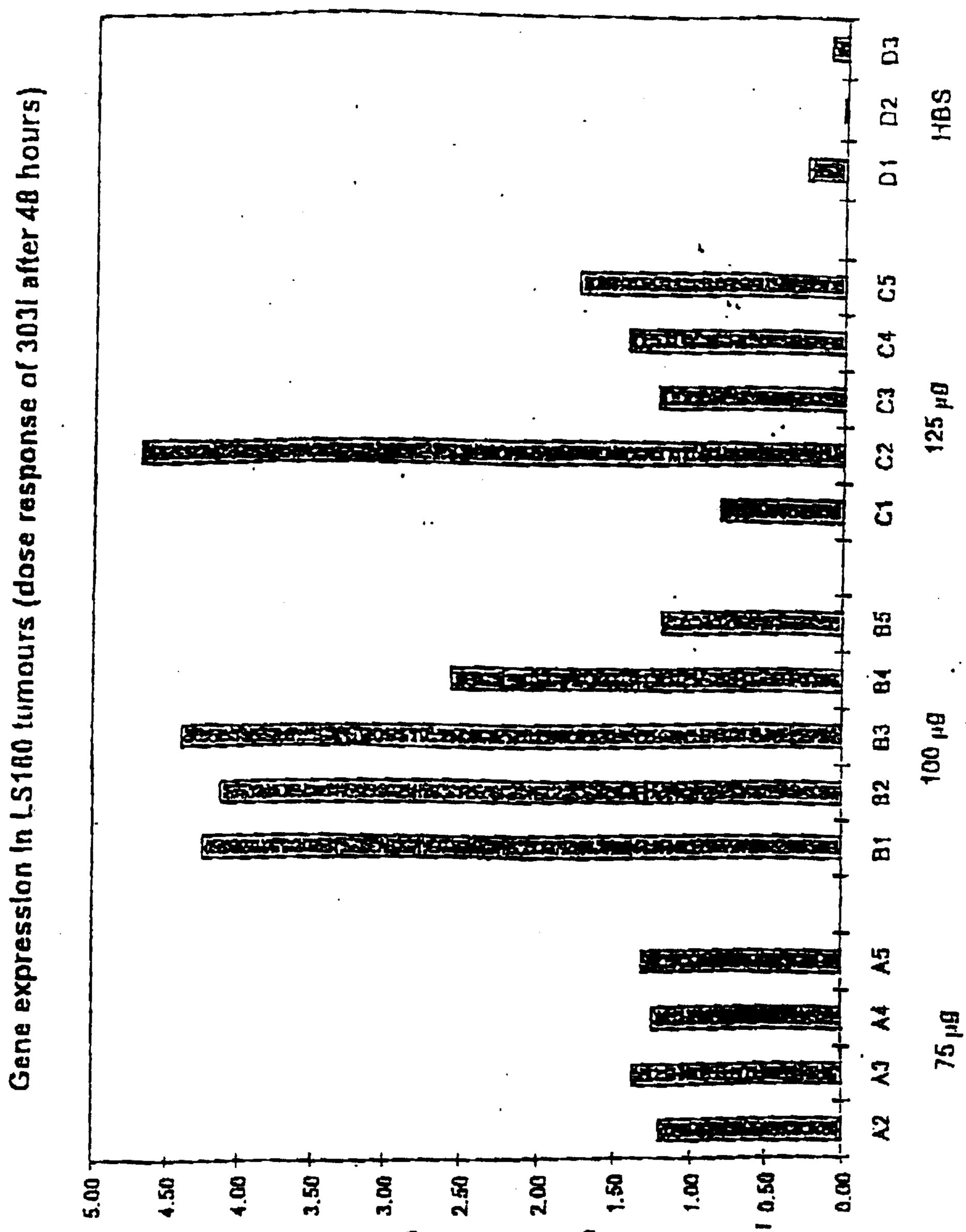
Gene expression in LS180 tumours (dose response of 303l after 48 hours)
fg luciferase/mg tumour
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
A2 A3 A4 A5 B1 B2 B3 B4 B5 C1 C2 C3 C4 C5 D1 D2 D3 HBS
75 µg
100 µg
125 µg

Figure 5

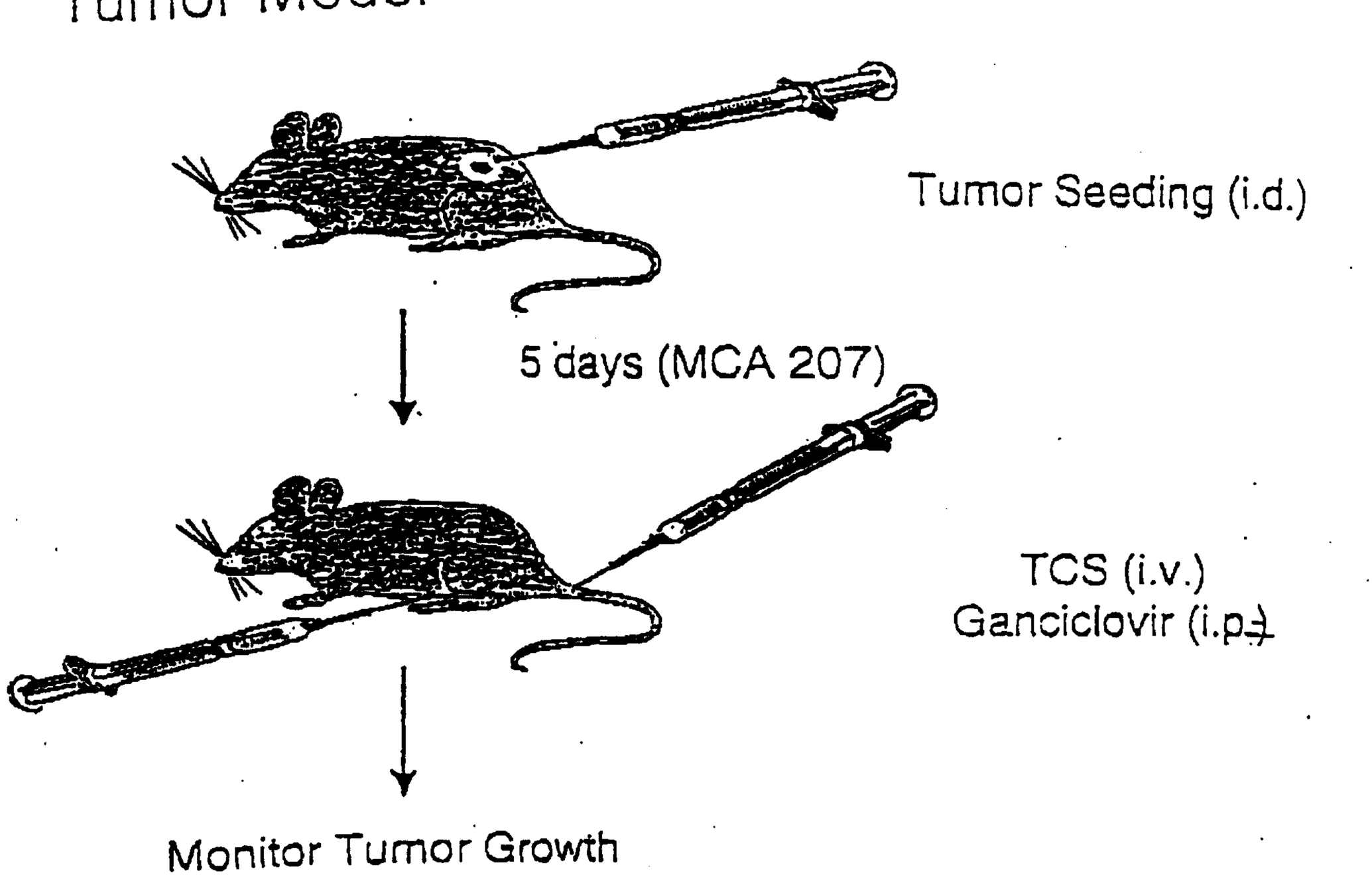

FIGURE 7A

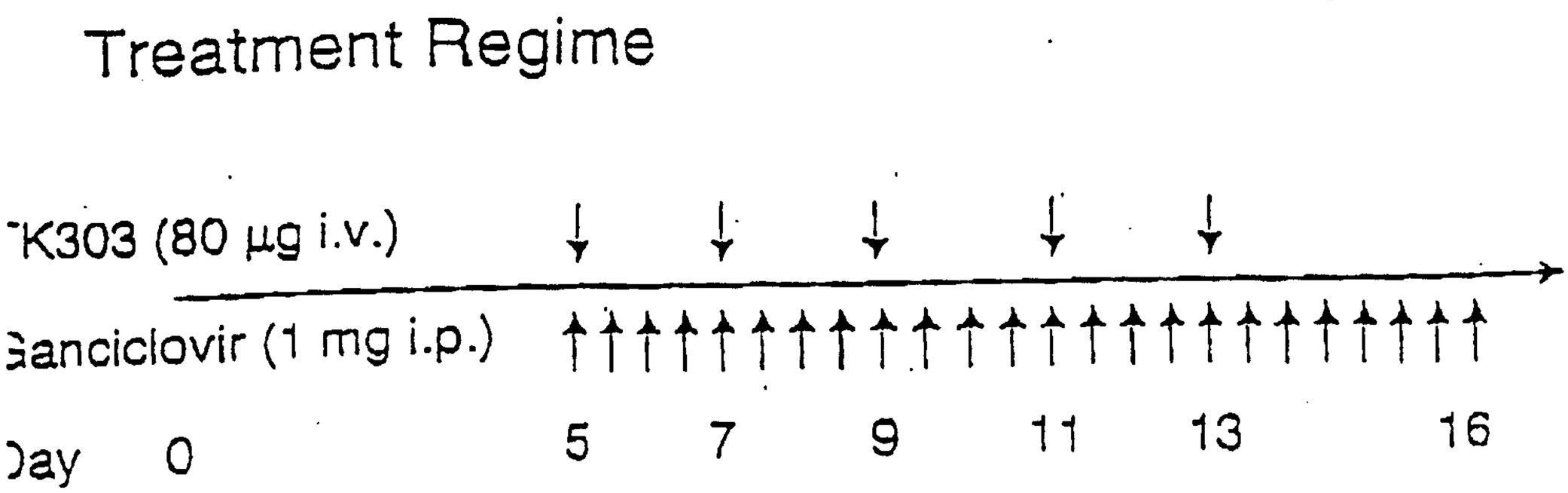

FIGURE 7B

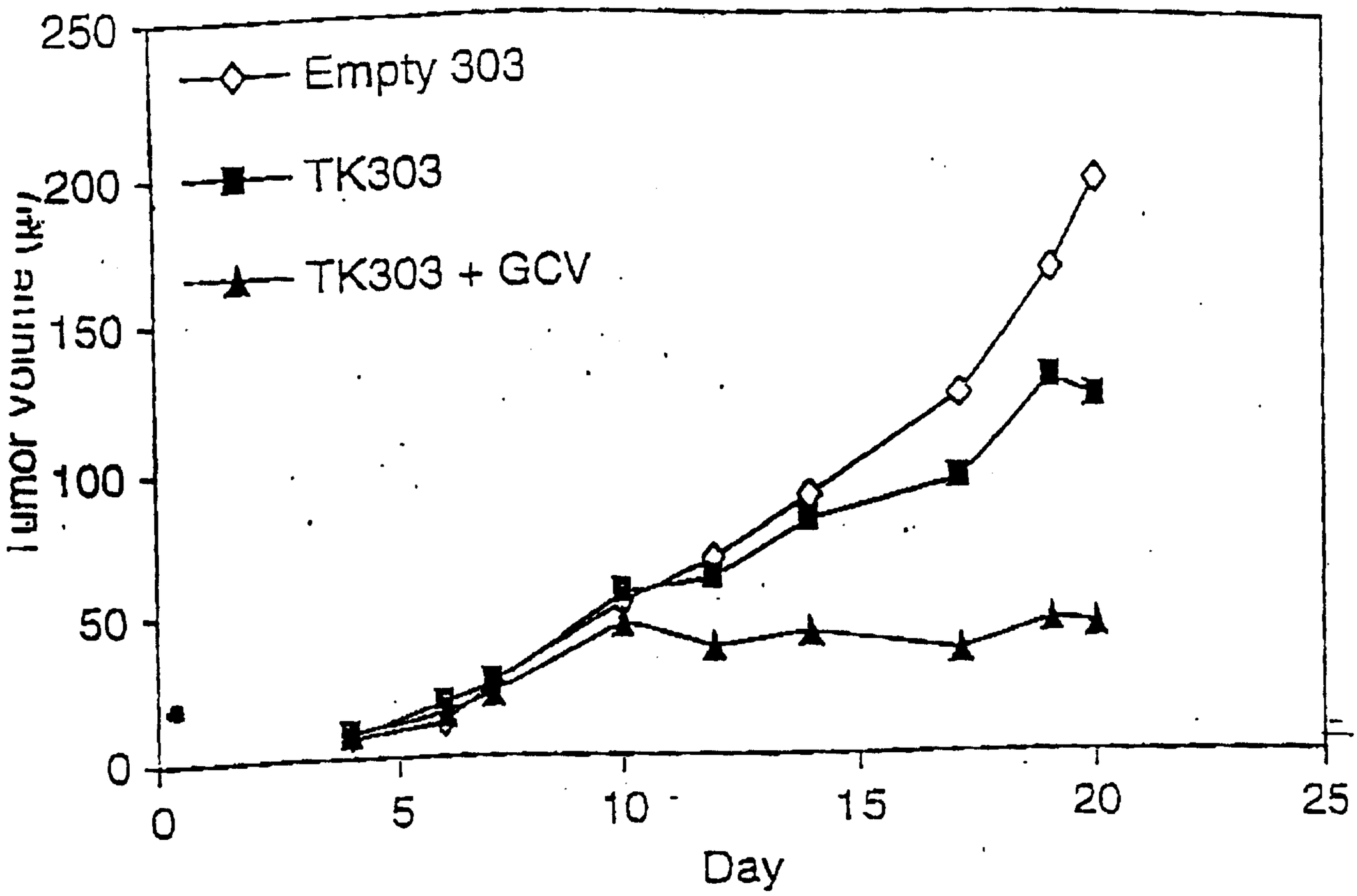
Assessment of Tumor Growth
Empty 303
TK303
TK303 + GCV
Tumor volume (µl)
250
200
150
100
50
0
0
5
10
15
20
25
Day

FIGURE 8A

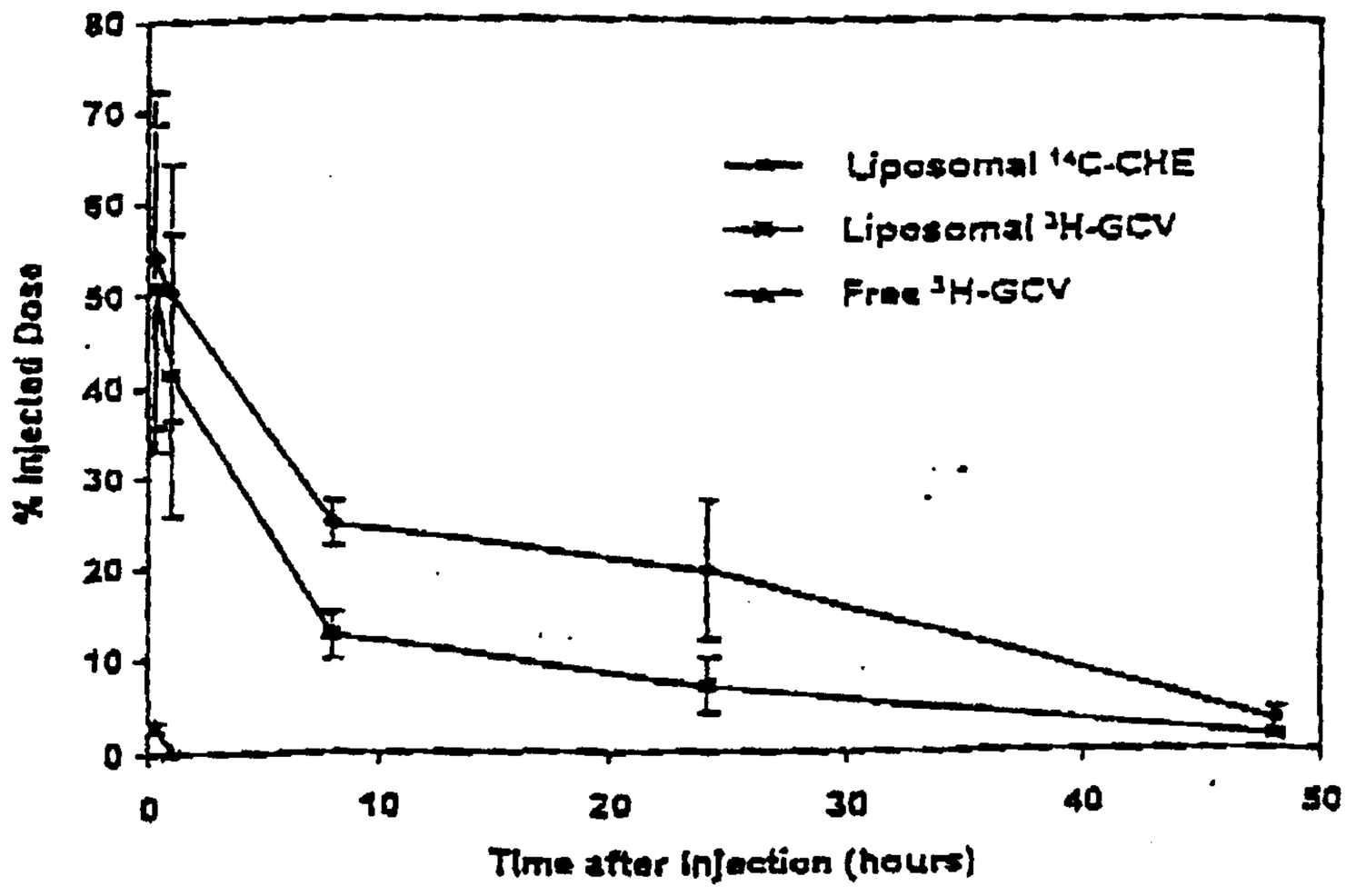
Liposomal ^{14}C-CHE
Liposomal ^{3}H-GCV
Free ^{3}H-GCV
% Injected Dose
Time after injection (hours)
0
10
20
30
40
50
60
70
80

FIGURE 9

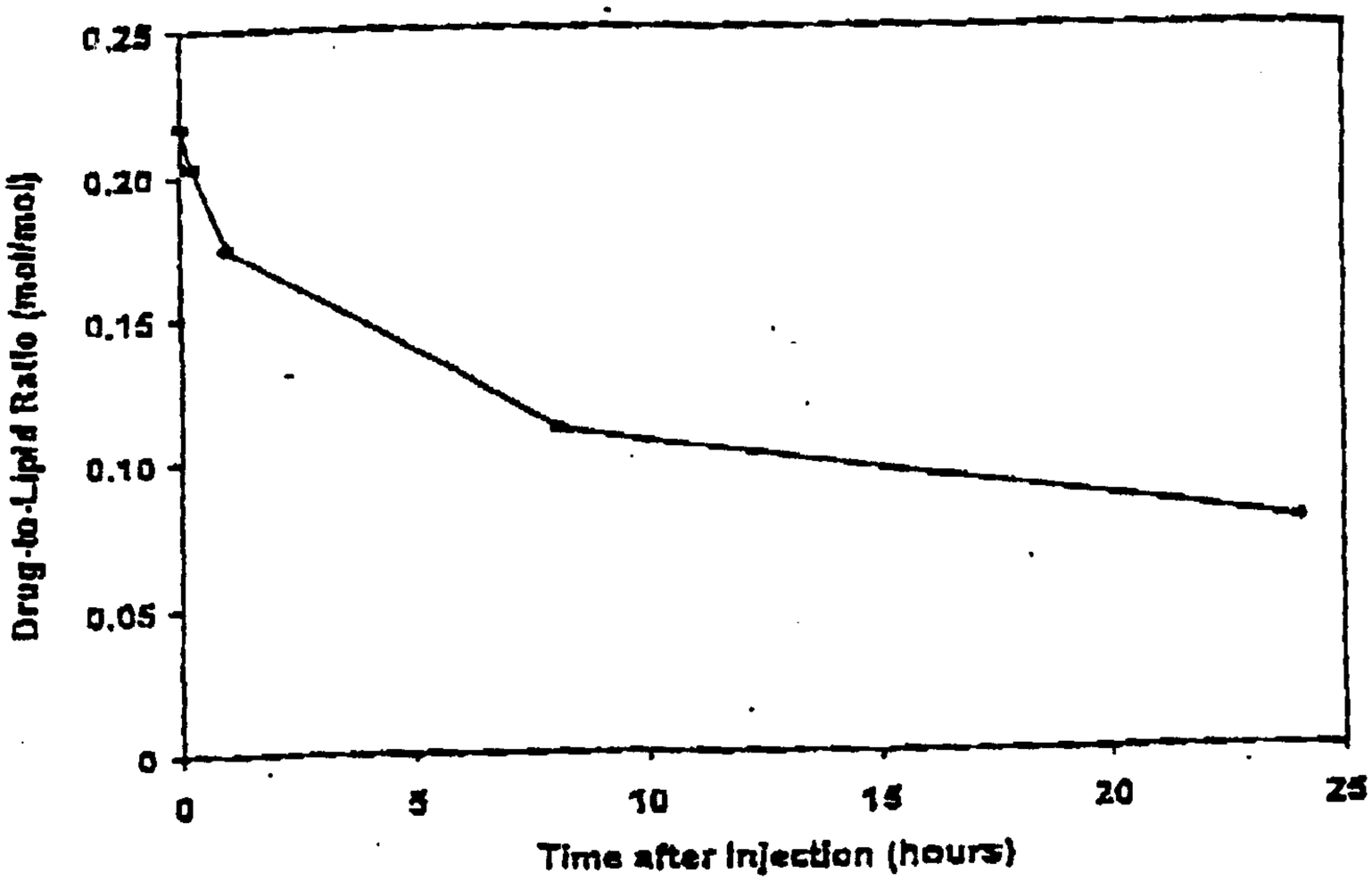
0.25
0.20
0.15
0.10
0.05
0
Drug-to-Lipid Ratio (mol/mol)
0
5
10
15
20
25
Time after injection (hours)

FIGURE 10

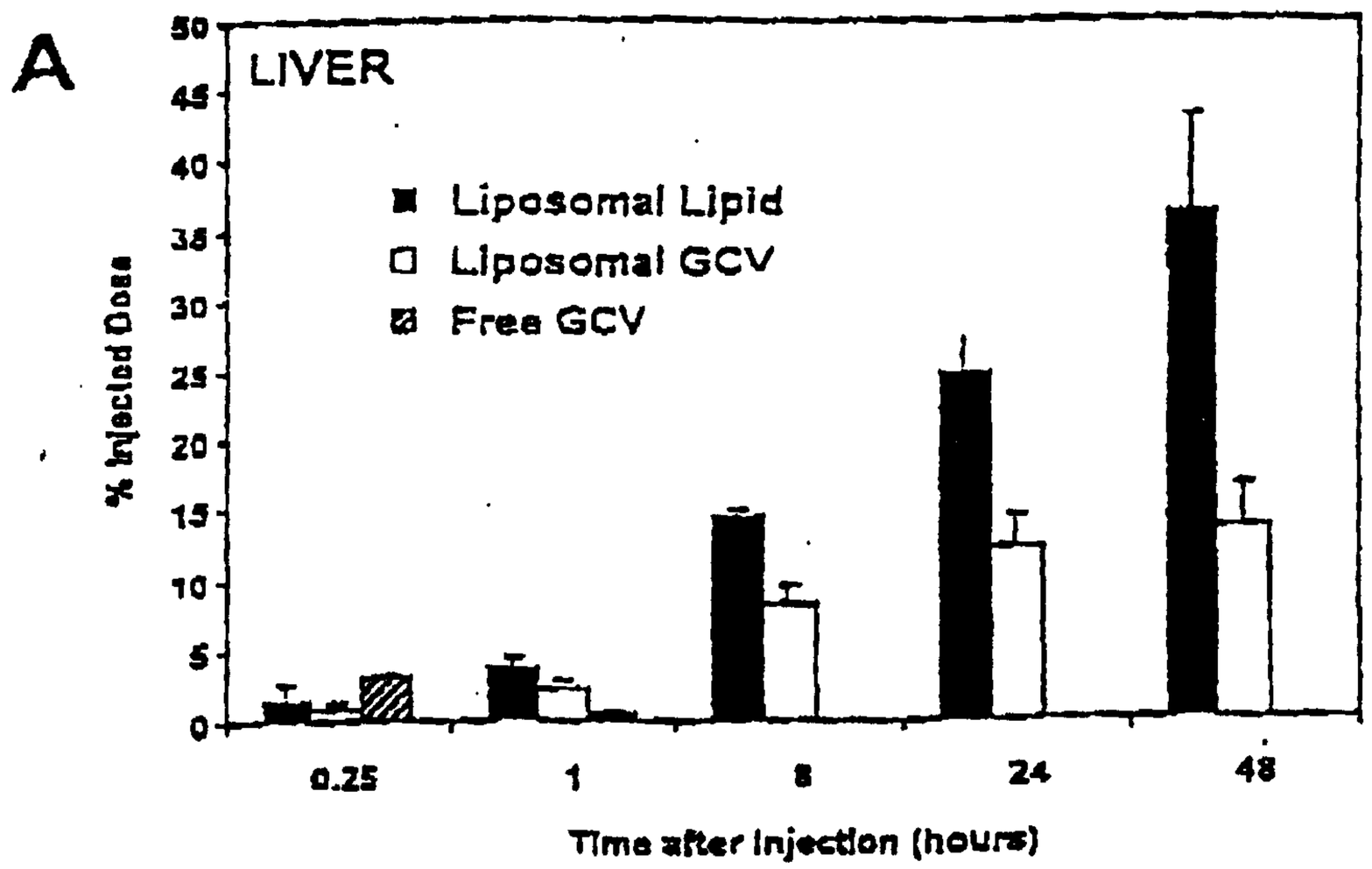
A
LIVER
% Injected Dose
50
45
40
35
30
25
20
15
10
5
0
Liposomal Lipid
Liposomal GCV
Free GCV
0.25
1
8
24
48
Time after injection (hours)

FIGURE 11A

B

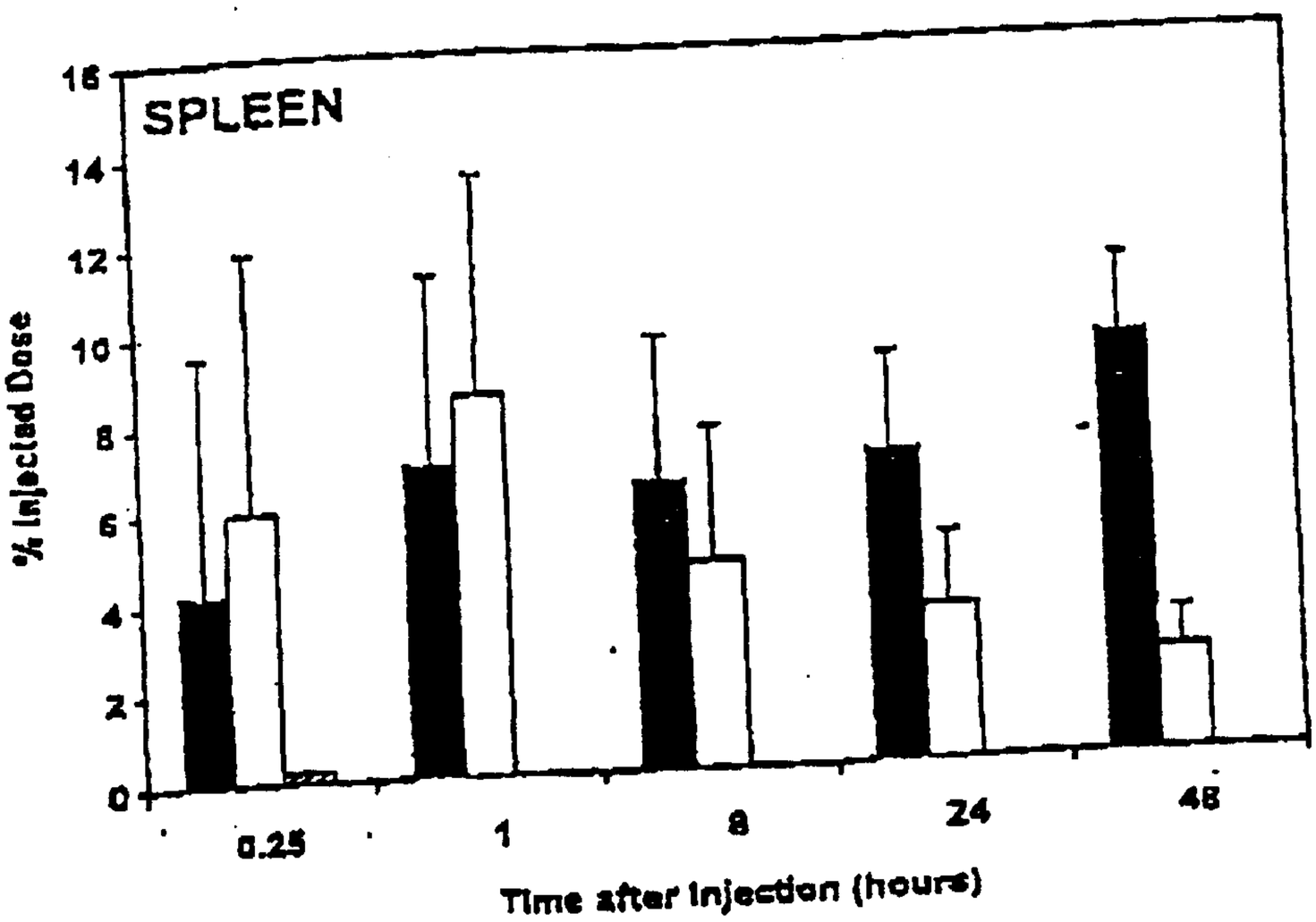
SPLEEN
% Injected Dose
0
2
4
6
8
10
12
14
16
0.25
1
8
24
48
Time after injection (hours)

FIGURE 11B

C

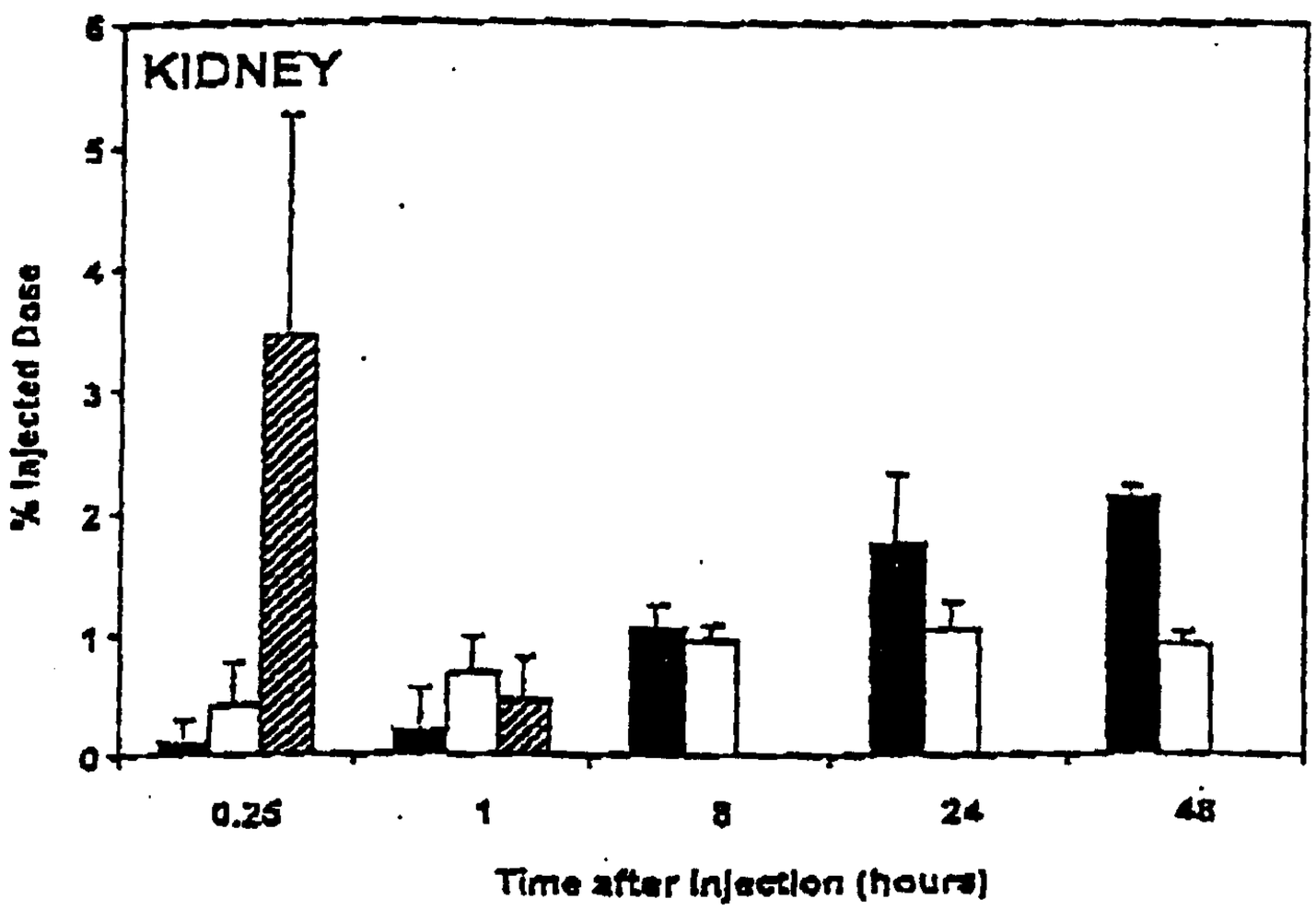
KIDNEY
% Injected Dose
0
1
2
3
4
5
6
0.25
1
8
24
48
Time after injection (hours)

FIGURE 11C

D

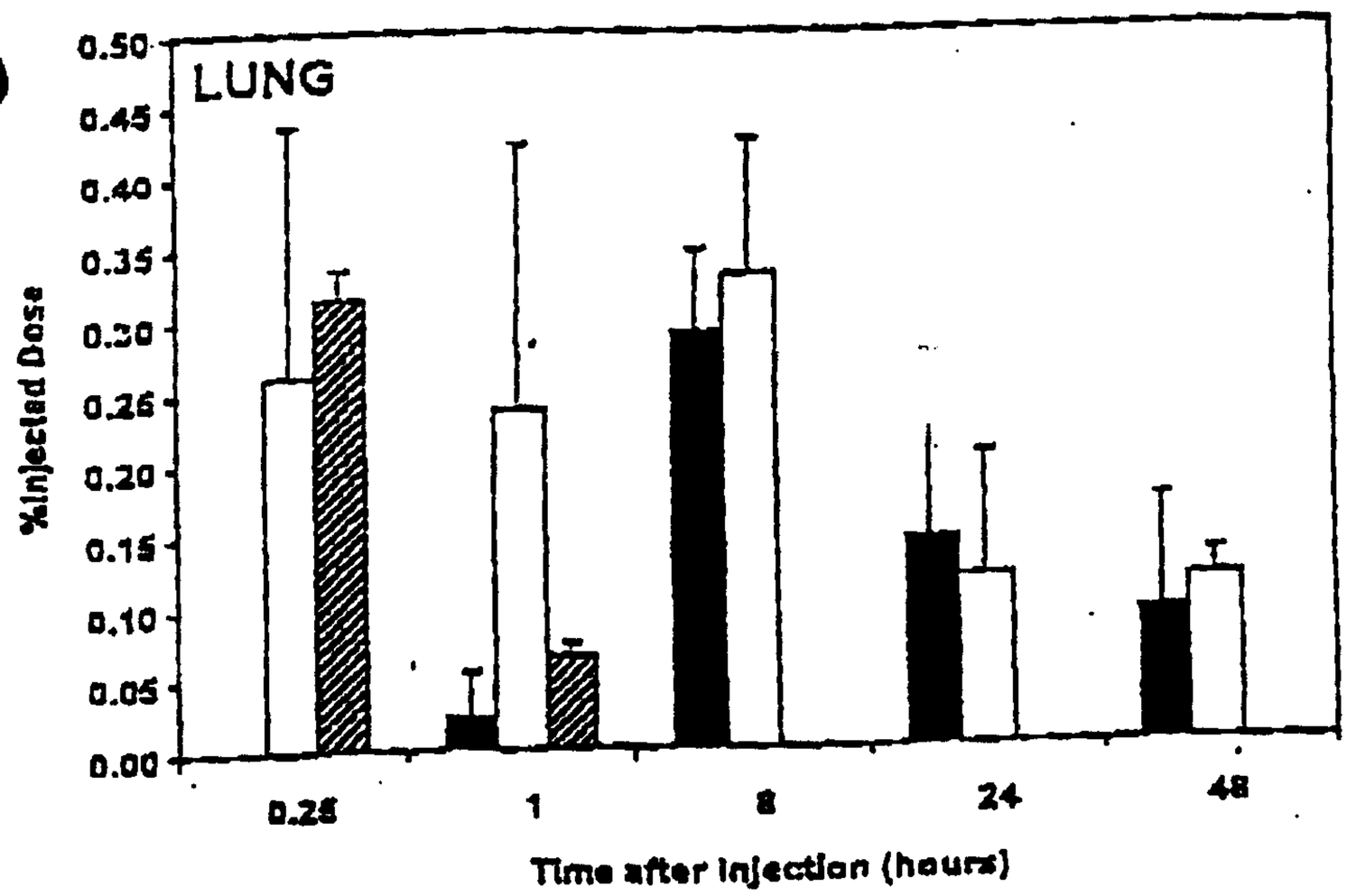
LUNG
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
%Injected Dose
0.25
1
8
24
48
Time after injection (hours)

FIGURE 11D

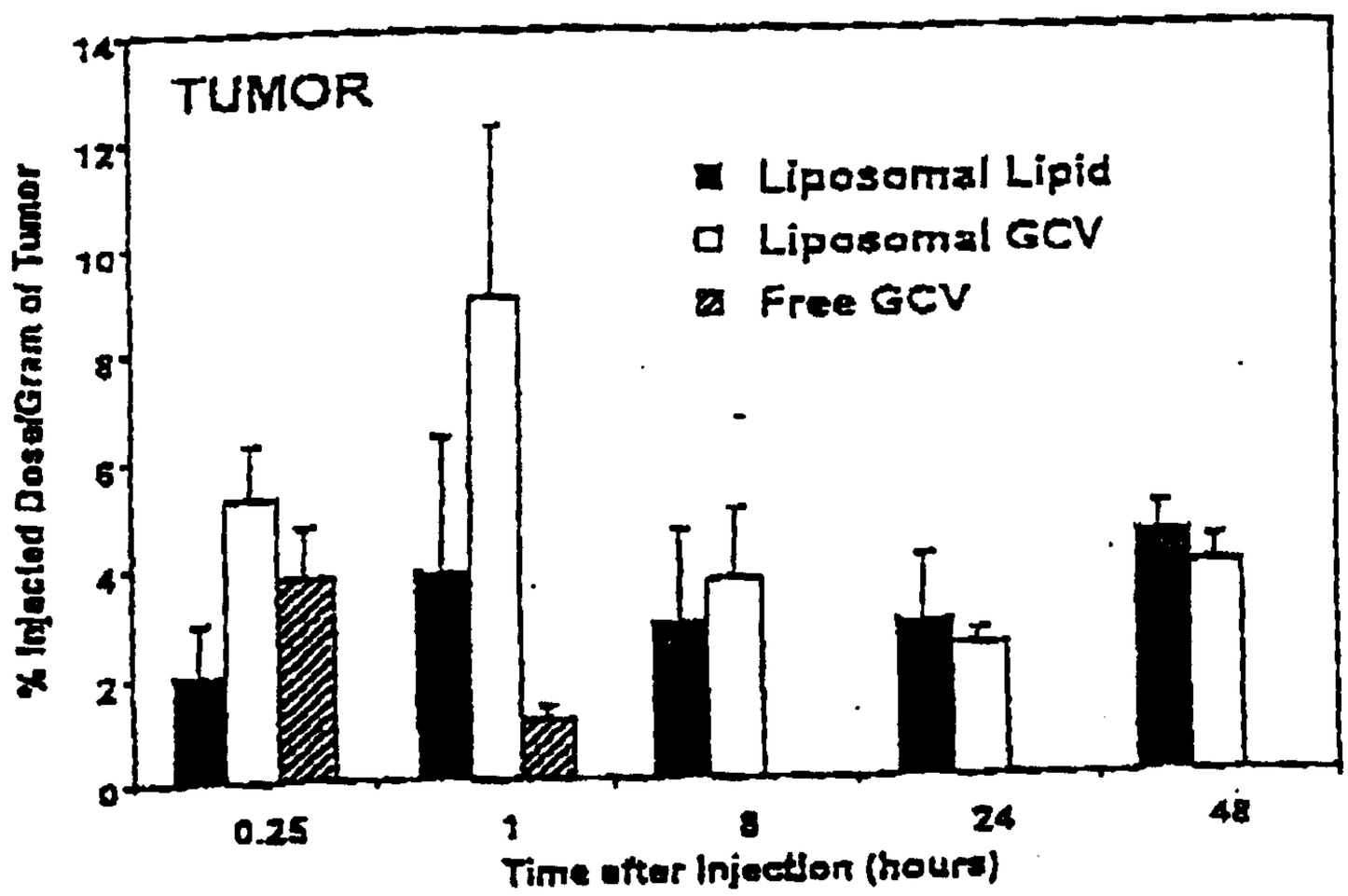
TUMOR
Liposomal Lipid
Liposomal GCV
Free GCV
% Injected Dose/Gram of Tumor
0
2
4
6
8
10
12
14
0.25
1
8
24
48
Time after Injection (hours)

FIGURE 12

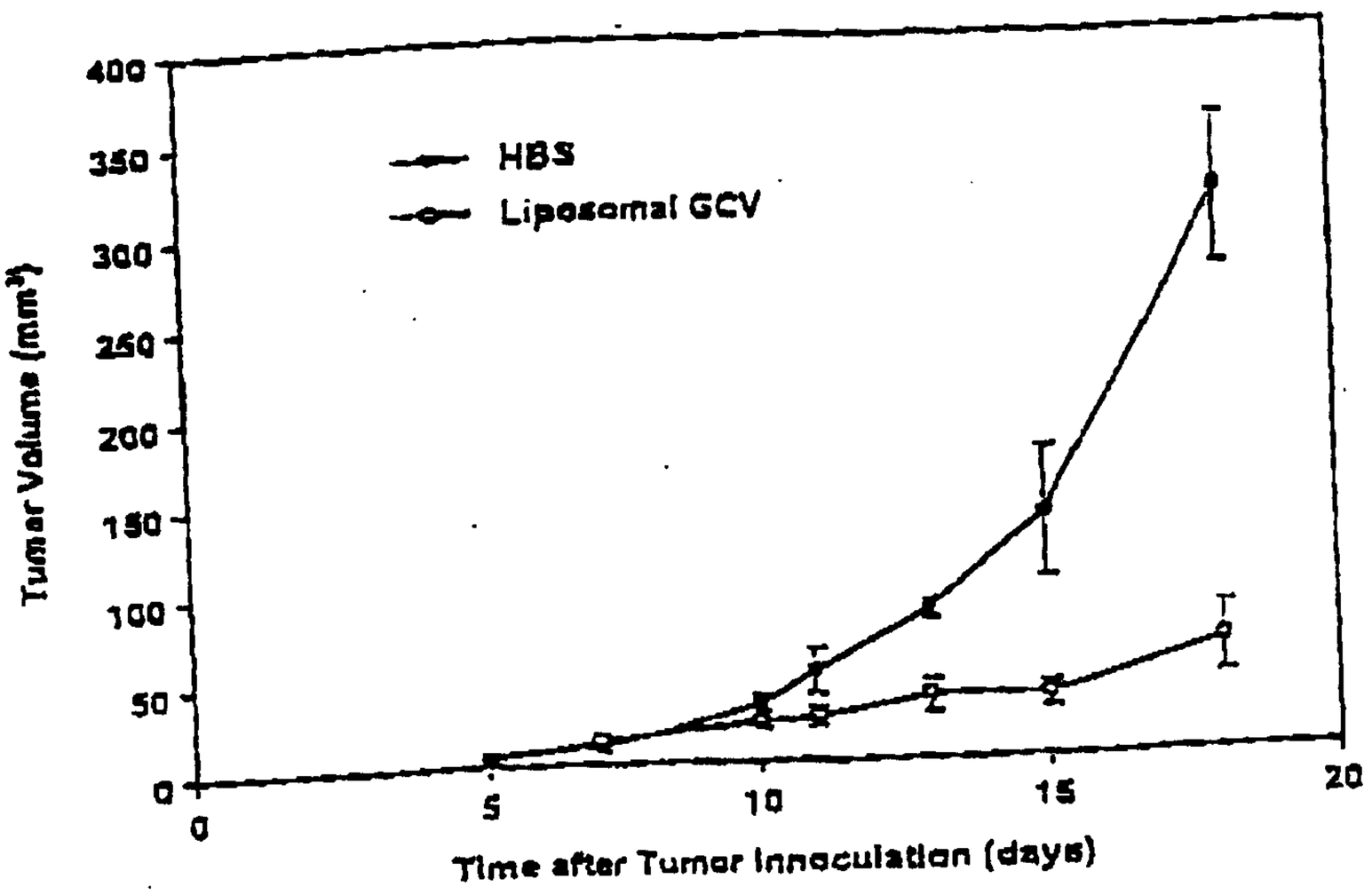
HBS
Liposomal GCV
Tumor Volume (mm³)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
Time after Tumor Innoculation (days)

FIGURE 13

pINEX L018 Circular Map

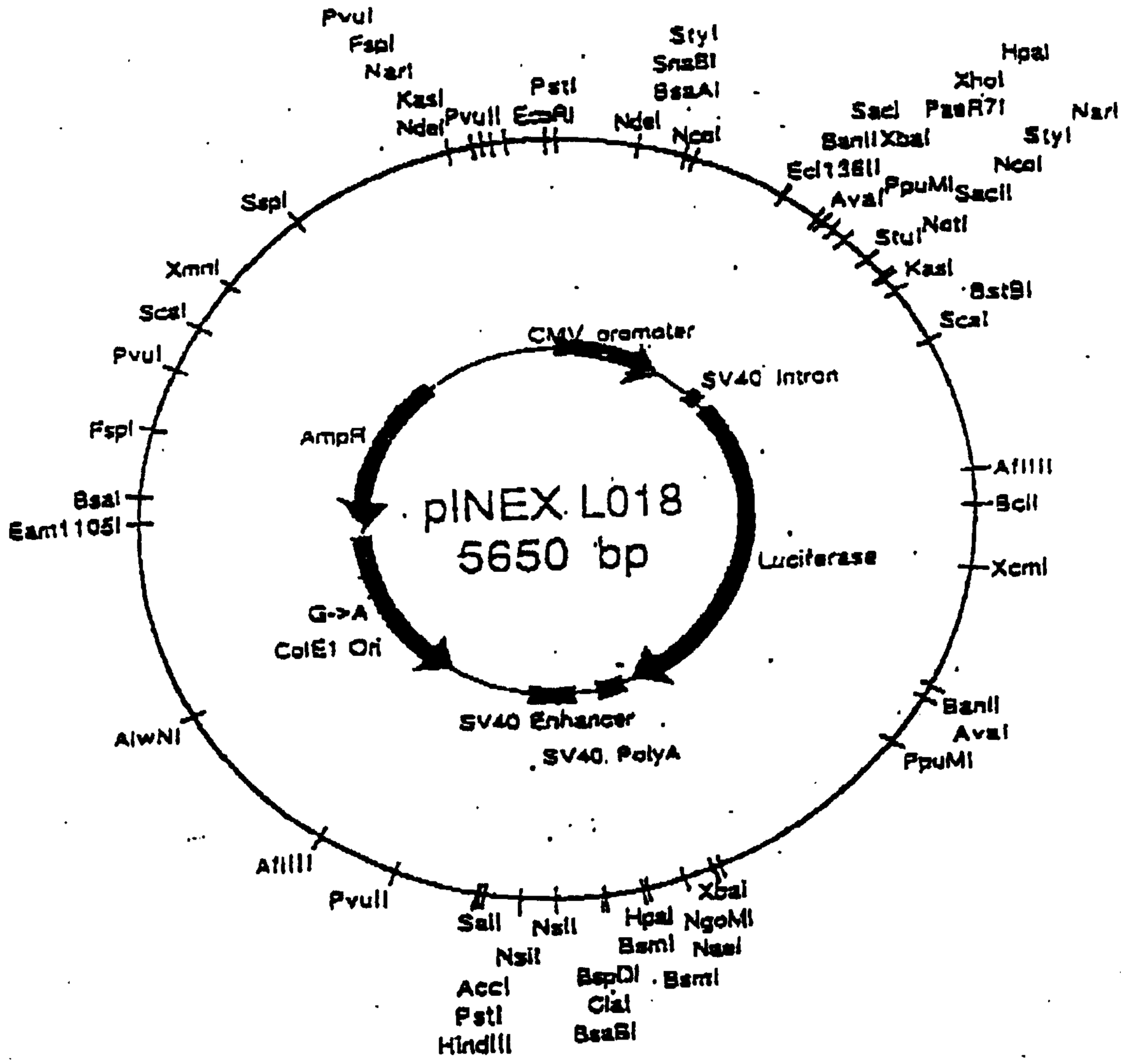

FIGURE 14A

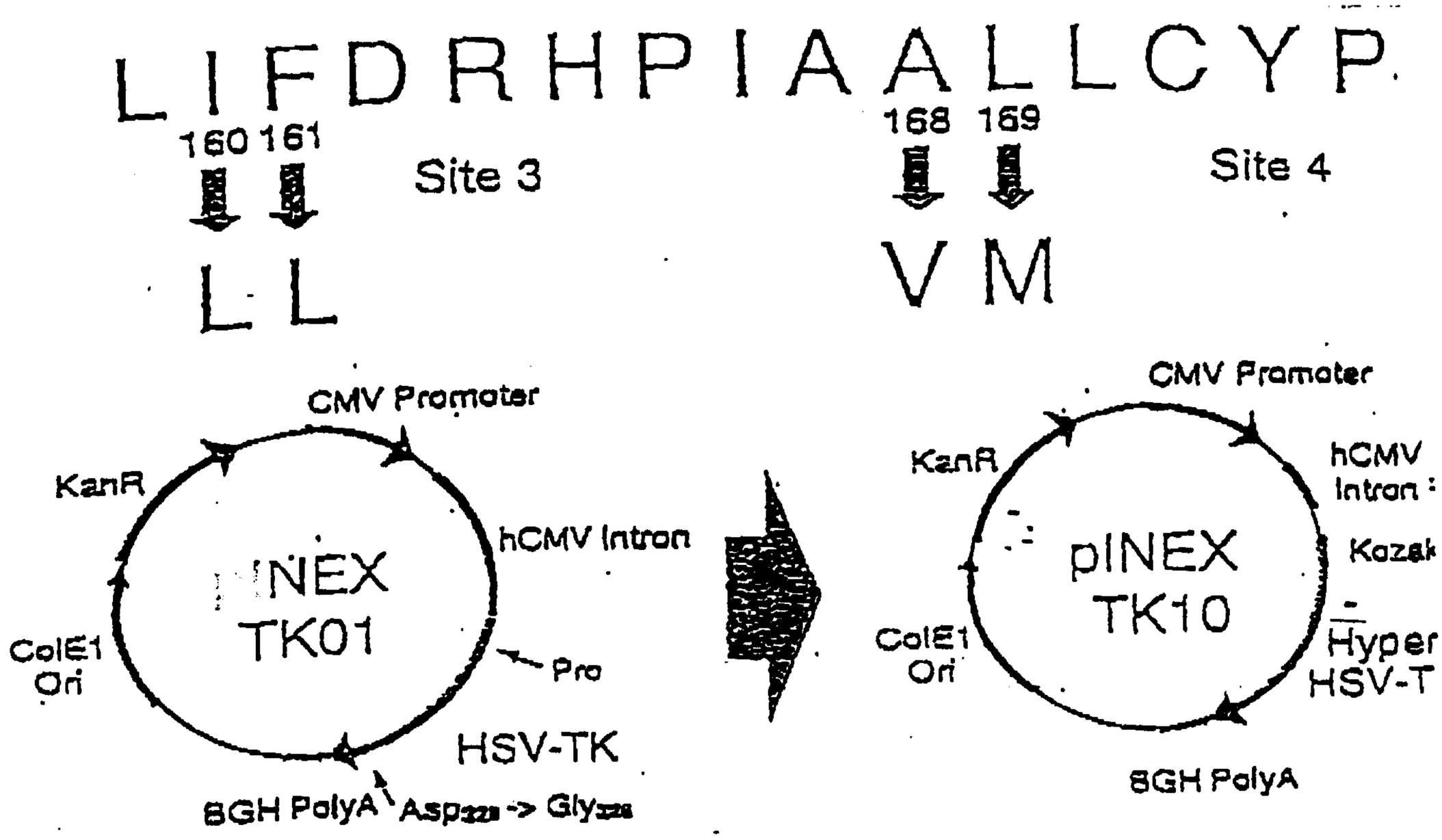
L I F D R H P I A A L L C Y P
160 161
Site 3
168 169
Site 4
L L
V M
CMV Promoter
KanR
hCMV Intron
pINEX
TK01
ColE1
Ori
Pro
HSV-TK
SGH PolyA
Asp222 -> Gly222
CMV Promoter
KanR
hCMV
Intron
pINEX
TK10
Kozak
Hyper
HSV-T
ColE1
Ori
SGH PolyA

FIGURE 14B

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

2319468

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈMŒ ÉTAGE AIRE 5 DANS LA SECTION DE LA PRÉPARATION DES DOSSIERS)