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(57) Abstract

A method of treating a tumor which comprises transducing tumor cells with a nucleic acid sequence encoding a negative selective marker (such as a viral thymidine kinase gene), and contacting the cells with an interaction agent (such as acyclovir, ganciclovir, or a halogenated pyrimidine). The cells then are exposed to radiation. Such method provides for increased killing of tumor cells as compared with gene therapy or radiation treatments alone.

Treatment of Tumors by Transduction With Negative Selective Markers, Application of Interaction Agents and Subsequent Radiation Exposure

~~This application is a continuation-in-part of application Serial No. 08/368,602, filed January 4, 1995, the disclosure of which is incorporated herein by reference in its entirety.~~

This invention relates to the treatment of tumors. More particularly, this invention relates to the treatment of tumors such as, for example, malignant glioma of the brain, by transducing the tumor cells with an expression vehicle including a negative selective marker, contacting the transduced tumor cells with an interaction agent, and exposing the cells to radiation.

BACKGROUND OF THE INVENTION

Malignant glioma of the brain is one of the most lethal cancers in humans. The median survival time after diagnosis is 50 weeks for glioblastoma multiforme. The majority of patients die of local recurrence despite medical intervention including surgery, radiotherapy, and nitrosourea-based chemotherapy. Patient derived cultured malignant gliomas exhibit widely varying, although generally low, radiation sensitivity, and a high level of repair of radiation damage. (Taghian, et al., in Radiation Research: a Twentieth Century

Perspective, Chapman, et al., eds., Academic Press, New York, 1991, Vol. 1, pg. 175.).

Attempts have been made to improve the efficacy of radiation by chemical means. Most radiation sensitizers operate by their action on DNA either to modify the structure of DNA so as to make it more sensitive to the action of radiation, or to inhibit the repair of DNA lesions produced by irradiation. (Tubiana, et al., Introduction to Radiology, Taylor and Francis, London, 1991).

If the drug is to be an effective radiation sensitizer by the former mechanism hereinabove mentioned, it must be present prior to irradiation. For example, cells actively synthesizing DNA in the presence of halogenated pyrimidines, analogues of thymine such as 5-bromo (or chloro-, iodo, or fluoro-) deoxyuridine (BrdU, CldU, IdU, FdU), show an increased sensitivity to ionizing radiation. Radiosensitization is generally found to increase with increasing percentage replacement of thymine with the halogenated analogues.

Brown, et al., J.Nat. Cancer Inst., Vol. 47, pgs. 75-89 (1971) have demonstrated a differential radiosensitizing effect between a mouse sarcoma and skin. The results of clinical studies involving BrdU infusion and radiation treatment for brain tumors and tumors of the head and neck, however, have not been encouraging. Such results may be attributable in part to the dose limiting toxicity of proliferating normal tissues such as bone marrow and intestine. (Bagshaw, et al., Am. J. Roentgenol., Vol. 99, pgs. 889-894 (1967)).

If the drug is to be an effective radiation sensitizer by inhibiting the repair of DNA lesions, it must be present after irradiation. Several nucleoside analogues, such as arabino-furanosyl-cytosine (ara-C), and acyclovir (9-2-hydroxy-1-[hydroxymethyl] ethoxy methyl guanine, have been shown to increase the cytoxicity of radiation exposure,

(Iliakis, et al., Int. J. Radiation Oncology Biol. Phys., Vol. 16, pgs. 1235-1241 (1989); Iliakis, et al., Radiat. Res., Vol. 83, pgs. 537-552 (1982); Sougawa, et al., Int. J. Radiation Oncology Biol. Phys., Vol. 12, pgs. 1537-1540 (1986)).

The initial biochemical alteration of the anti-Herpes nucleoside analogue, which leads to its toxicity as an antiviral agent and as a sensitizer of radiation damage, is a specific phosphorylation by virus induced thymidine kinase. The phosphorylated compound may interact with DNA polymerase either as an inhibitor or a substrate. If acting as a substrate, it will be incorporated into DNA. Either event may lead to a fundamental disturbance of DNA integrity. (Clercq, Bioch. Pharmacol., Vol. 33, pgs. 2159-2169 (1984)).

At the drug concentrations that could be achieved in humans, however, anti-Herpes agents have been found not be effective radiation sensitizers in the many in vivo systems studied to date. A possible explanation involves the enzyme thymidine kinase. Mammalian thymidine kinase has a very narrow substrate range requirements; therefore, the nucleoside analogues are not phosphorylated in normal mammalian cells. In contrast, viral-derived thymidine kinase has a broad substrate range and thus is able to toxify the nucleoside analogue. Recent attention has been focused on using viral-derived thymidine kinase to toxify the anti-viral drug ganciclovir for the treatment of malignant brain tumors (Oldfield, et al., Human Gene Therapy, Vol. 4, pgs. 39-69 (1993)).

Malignant brain tumors may be susceptible to gene therapy involving retroviral vectors for reasons which include the following: (i) retroviral vectors integrate only in to the genome of dividing cells, and thus non-proliferating neuronal tissue will be spared; (ii) there are few limitations to the types of cells retroviruses can infect or to their host range; and (iii) the stereotactic

implantation technique can be carried out routinely in patients with brain tumors. This approach would offer a means to provide continuous production of the vector within the primary tumor site.

Retroviral vectors including the Herpes Simplex Virus thymidine kinase (TK) gene, offer a conditional killing mechanism for proliferating cells. The use of this gene, also sometimes referred to as a "suicide" gene, has been successful in developmental studies of transgenic mice (Borelli, et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 7572-7576 (1988)), as a selectable marker against non-homologous recombination events in cultured cells, for killing cells harboring wild-type Herpes viruses (Corey, et al., N. Engl. J. Med., Vol. 314, pgs. 749-756 (1986)), for killing tumor cells *in vitro* and *in vivo*, and more recently for treatment of malignant glioma of the brain in rats (Ezzedine, et al., New Biologist, Vol. 3, pgs. 608-614 (1991); Moolten, et al., J. Nat. Cancer Inst., Vol. 82, pgs. 297-300 (1990); Culver, et al., Science, Vol. 256, pgs. 1550-1552 (1993); Ram, et al., Cancer Res., Vol. 53, pgs. 83-88 (1993)). An advantage of employing the Herpes Simplex Virus TK gene to effect cell death, as compared with other gene products, is that the gene product, thymidine kinase, is not toxic by itself. The thymidine kinase enzyme is able to convert purine and pyrimidine derivatives (widely used anti-Herpes drugs such as acyclovir, ganciclovir, and bromovinyldeoxyuridine (BVdU)) into nucleoside intermediates, thereby disrupting cellular DNA replication, and eventually causing cell death.

The efficacy of the anti-Herpes agent ganciclovir in the treatment of sarcoma and lymphoma cells, transduced with a retroviral vector containing the Herpes Simplex Virus TK gene has been demonstrated, although many animals showed recurrence of tumors after treatment with ganciclovir was stopped. (Moolten, et al., 1990). Ezzedine, et al. (1991) showed selective killing of C6 glioma cells in culture and *in*

vivo by transducing the cells with a retrovirus including the Herpes Simplex Virus TK gene, and then contacting the transduced cells with ganciclovir. Recurrence of tumor growth after the ganciclovir treatment was stopped also was noted. The feasibility of *in vivo* gene transfer of the Herpes Simplex Virus TK gene through the use of direct injection of retroviral vector producer cells into 9L glioma cells of the rat brain also has been demonstrated. (Culver, *et al.*, *Science*, Vol. 256, pgs. 1550-1552(1992)). The combination of the Herpes Simplex Virus TK gene and ganciclovir selectively destroyed a rat glioma in the brain. Toxicity studies involving the direct injection of the vector producer cells IV or IP has demonstrated the safety of the approach because no evidence of illness with or without the drug therapy was found. (Ram, *et al.*, 1993). Based on such data, "suicide" gene therapy for the treatment of malignant glioma of the brain has been approved by the NIH Recombinant DNA Advisory Committee. A Phase I clinical trial has been completed. (Oldfield, *et al.*, 1993).

Detailed Description of the Invention

In accordance with an aspect of the present invention, there is provided a method of treating a tumor. The method comprises transducing tumor cells with a nucleic acid sequence encoding a negative selective marker. The transduced tumor cells then are contacted with a chemotherapeutic agent or interaction agent, which interacts with the negative selective marker in order to prevent, inhibit, or destroy the growth of the tumor cells. The transduced tumor cells also are exposed to radiation.

According to a first embodiment of the invention, there is provided a method of treating glioblastoma, comprising:

transducing tumor cells with a nucleic acid sequence encoding a viral thymidine kinase;

contacting said transduced tumor cells with an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and

exposing said transduced tumor cells to gamma-radiation.

According to a second embodiment of the invention, there is provided the use of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said viral vector to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

According to a third embodiment of the invention, there is provided the use of a producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said producer cell to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

According to a fourth embodiment of the invention, there is provided the use of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said interaction agent to a patient subsequent to the administration of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase and prior to, concurrent with or after the administration of gamma-radiation.

According to a fifth embodiment of the invention, there is provided a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase when used in the treatment of glioblastoma, wherein said treatment comprises administration of said viral vector to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

According to a sixth embodiment of the invention, there is provided a producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase, when used in the treatment of glioblastoma, wherein said treatment comprises administration of said producer cell to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

According to a seventh embodiment of the invention, there is provided an interaction agent when used in the treatment of glioblastoma, wherein said treatment comprises administration of said interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines to a patient subsequent to the administration of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase and prior to, concurrent with or after the administration of gamma-radiation.

The term "treating a tumor," as used herein, means inhibiting, preventing, or destroying the growth of the tumor cells.

The term "nucleic acid sequence" as used herein, means a DNA or RNA molecule, and more particularly a linear series of deoxyribonucleotides or ribonucleotides connected one to

the other by phosphodiester bonds between the 3′ and 5′ carbons of the adjacent pentoses. Depending upon the use herein, such term includes complete and partial gene sequences, and includes polynucleotides as well.

The nucleic acid sequence which encodes the negative selective marker is contained in an appropriate expression vehicle which transduces the tumor cells. Such expression vehicles include, but are not limited to, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial plasmids), and viral vectors.

In one embodiment, the expression vehicle is a viral vector. Viral vectors which may be employed include, but are not limited to, retroviral vectors, adenovirus vectors, adeno-associated virus vectors, and Herpes virus vectors. Preferably, the viral vector is a retroviral vector.

In a preferred embodiment, a packaging cell line is transduced with a viral vector containing the nucleic acid sequence encoding the negative selective marker to form a producer cell line including the viral vector. The producer cells then are administered to the tumor, whereby the producer cells generate viral particles capable of transducing the tumor cells.

In a preferred embodiment, the viral vector is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. Preferably, the retroviral vector is an infectious but non-replication competent retrovirus. However, replication competent retroviruses may also be used.

Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells. Retroviral vectors are generally constructed such that the

majority of sequences coding for the structural genes of the virus are deleted from the proviral DNA and replaced by the gene(s) of interest. Most often, the genes for the structural proteins (i.e., gag, and env), and for the viral enzymes (i.e., pol), are removed from the proviral DNA using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

The removal of the gag, pol and env genes results in a vector backbone, comprised of a 5' LTR, a packaging signal, one or more cloning sites, and a 3'LTR, into which the heerologous gene or genes of interest can be introduced. The preferred vector backbone is the G1 vector backbone, which is disclosed in McLachlin, et al., Virology, 195:1-5 (1993) and in PCT patent application WO 91/10728 for "Novel Retroviral Vectors," published on July 25, 1991.

The heterologous gene or genes are incorporated into the proviral backbone by standard techniques to form the retroviral vector. Techniques for the preparation of retroviral vectors are disclosed in PCT application WO 91/10728 as well as the following articles: Armentano, et al., J. Virol., 61:1647-1650 (1987), Bender, et al., J. Virol., 61:1639-1646 (1987), and Miller, et al., Biotechniques, 7:980-990 (1989). The most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal

promoter. Suitable promoters include the SV40 promoter, the human cytomegalovirus (CMV) promoter, the beta-actin promoter, the alpha fetoprotein promoter, and any promoter naturally associated with any heterologous gene of interest.

The retroviral vectors may be in the form of a plasmid, a segment of viral RNA, or a segment of viral DNA., For the instance invention, the preferred retroviral vector is G1TK1SvNa, which is disclosed in PCT patent application WO 95/06486 for "Treatment of Human Tumors by Genetic Transformation of Human Tumor Cells," published on March 9, 1995.

The retroviral vector is introduced into a packaging cell to form a producer cell. Packaging cells provide the gag, pol, and env genes in trans, which permits the packaging of the retroviral vector into a recombinant retrovirus that is infectious but replication defective. The vectors are transferred into the packaging cells by standard gene transfer techniques, which include transfection, transduction, calcium phosphate precipitation, electroporation, and liposome-mediated DNA transfer. Examples of packaging cells that may be used include, but are not limited to, the PE501, PA317, Psi-2, Psi-AM, PA12, T19-14X, VT-19-17-H2, Psi-CRE, Psi-CRIP, GP+E-86, GP+envAM12, and DAN cell lines. The preferred producer cell line for the instant invention is the producer cell line designated PA317/G1TK1SvNa7, which is disclosed in PCT application WO 95/06486.

The producer cells then are administered directly to or adjacent to the tumor in an amount effective to inhibit, prevent, or destroy the growth of the tumor. In general, the producer cells are administered in an amount of at least 10^3 cells per cc of tumor. In general, the amount of cells administered does not exceed 10^6 cells per cc of tumor; however, greater amounts may be used. The exact amount of producer cells to be administered is dependent upon various

factors, including but not limited to, the type of the tumor and the size of the tumor.

In one embodiment, the producer cells may be administered directly to or adjacent to the tumor by injection. For example, the cells may be administered by use of a CT or MRI guided stereotaxic system which permits representation of a tumor mass on a two dimensional implantation grid, such as one containing 89 holes. The system can provide the exact coordinates, positions and injection tracts to optimize the distribution of producer cells into a tumor mass.

The producer cells are administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier such as, for example, a saline solution or a buffer solution or other isomolar aqueous solution.

Upon administration of the producer cells to the tumor, the producer cells generate viral particles. The viral particles then transduce the surrounding tumor cells. Because tumor cells, and in particular cancerous tumor cells, in general are actively replicating cells, the retroviral particle would be integrated into and expressed preferentially or exclusively in the tumor cells as opposed to normal cells.

Tumors which may be treated in accordance with the present invention include malignant and non-malignant tumors.

Malignant (including primary and metastatic) tumors which may be treated include, but are not limited to, those occurring in the adrenal glands; bladder; bone; breast; cervix; endocrine glands (including thyroid glands, the pituitary gland, and the pancreas); colon; rectum; heart; hematopoietic tissue; kidney; liver; lung; muscle; nervous system; brain; eye; oral cavity; pharynx; larynx; ovaries; penis; prostate; skin (including melanoma); testicles; thymus; and uterus.

Upon transduction of the tumor cells with the negative selective marker, an interaction agent is administered to the human host. The interaction agent interacts with the negative selective marker in order to prevent, inhibit, or destroy the growth of the tumor cells.

Negative selective markers which may be employed include, but are not limited to, thymidine kinase, such as Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase; and cytosine deaminase.

In one embodiment, the negative selective marker is a viral thymidine kinase selected from the group consisting of Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase. When such viral thymidine kinases are employed, the interaction or chemotherapeutic agent preferably is a nucleoside analogue, for example, one selected from the group consisting of ganciclovir, acyclovir, 1,2-deoxy-2-fluoro-B-D-arabinofuranosil-5-iodouracil (FIAU), and halogenated pyrimidines such as 5-bromo, 5-chloro, 5-iodo, or 5 fluoro-deoxyuridine (BrdU, CldU, IdU, or FdU), or bromovinyldeoxyurdine (BVdU).

Such interaction agents are utilized efficiently by the viral thymidine kinases as substrates, and such interaction agents thus are incorporated lethally into the DNA of the tumor cells expressing the viral thymidine kinases, thereby resulting in the death of the tumor cells.

In another embodiment, the negative selective marker is cytosine deaminase. When cytosine deaminase is the negative selective marker, a preferred interaction agent is 5-fluorocytosine. Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is highly cytotoxic. Thus, the tumor cells which express the cytosine deaminase gene convert the 5-fluorocytosine to 5-fluorouracil and are killed.

The interaction agent is administered in an amount effective to inhibit, prevent, or destroy the growth of the transduced tumor cells. For example, the interaction agent may be administered in an amount from about 1 μM to about 200 μM, depending on overall toxicity to a patient. The interaction agent preferably is administered systemically, such as, for example, by intravenous administration, by parenteral administration, by intraperitoneal administration, or by intramuscular administration. The interaction agent may be administered before irradiating the tumor cells, after irradiating the tumor cells, or before and after irradiating the tumor cells.

The transduced tumor cells also are subjected to radiation. The transduced tumor cells may be irradiated before or after contacting the transduced tumor cells with the interaction agent.

Radiation to which the transduced tumor cells may be subjected includes any type of photon beam radiation, such as X-rays and gamma-radiation.

In one embodiment, the radiation is gamma-radiation. The gamma-radiation may be administered in an amount of from about 100 cGy (or 1 Gy) to about 2,500 cGy (or 25 Gy), preferably from about 200 cGy (or 2Gy) to about 1,500 cGy (or 15Gy).

Applicants have found that, when tumor cells are transduced with a negative selective marker and then contacted with an interaction agent, the interaction agent enhances the radiation cytotoxicity of tumor cells transduced with the negative selective marker, and that the addition of radiation improves the effectiveness of Herpes Simplex Virus TK gene therapy for the treatment of tumors.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention now will be described with respect to the drawings, wherein:

Figure 1 is a schematic of the construction of plasmid pG1;

Figure 2 is a multiple cloning site found in plasmid pG1;

Figure 3 is a map of plasmid pG1;

Figure 4 is a map of plasmid pBG;

Figure 5 is a schematic of the construction of plasmid pG1TkSvNa;

Figure 6 is a map of plasmid pG1TkSvNa;

Figures 7A through 7F are graphs which show the survival of 9L glioma cells transduced with the Herpes Simplex Virus thymidine kinase (TK) gene upon exposure to BVdU, acyclovir, or ganciclovir, as compared with 9L glioma cells which were not transduced with the Herpes Simplex Virus TK gene;

Figure 8 shows a Southern blot analysis of 9L glioma cells transduced with the Herpes Simplex TK gene, and the radiation sensitivity of such cells;

Figures 9A and 9B are graphs which show the survival of 9L glioma cells transduced with the Herpes Simplex TK gene which were exposed to BVdU, followed by gamma-radiation;

Figures 10A through 10E are graphs showing the survival of 9L glioma cells transduced with the Herpes Simplex Virus TK gene, and exposed to aciclovir before and/or after exposure to gamma-radiation;

Figures 11A and 11B are graphs showing the survival of U-251 cells which were not transduced with the Herpes Simplex Virus TK gene, and which were transduced with the Herpes Simplex Virus TK gene, respectively, upon exposure of such cells to BVdU;

Figures 12A and 12B are graphs showing the survival of U-251 cells which were exposed to BVdU 24 hours before or after irradiation at a dose of 8 Gy;

Figures 13A and 13B are graphs showing the survival of U-251 cells which were exposed to 40 μg/ml of BVdU 24 hours before or after irradiation doses up to 12 Gy; and

Figure 14 is a graph of the median survival time of rats, with the number of rats in each group shown in parentheses, which were implanted with 9L glioma cells transduced with the Herpes Simplex TK gene, and were treated with radiation alone, or with radiation and BvdU.

EXAMPLES

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

Generation of Producer Cell Lines

PA317/G1TkSvNa.53

A. Construction of pG1TkSvNa

The following describes the construction of pG1TkSvNa, a map of which is shown in Figure 6. This vector contains the Thymidine Kinase (hTK) gene from Herpes Simplex Virus I regulated by the retroviral promoter and the bacterial gene, neomycin phosphotransferase (Neo^R) driven by an SV40 promoter. The hTK gene confers sensitivity to the DNA analogs acyclovir and ganciclovir, while the Neo^R gene product confer resistance to the neomycin analogue, G418.

To make pGITkSvNa, a three step cloning strategy was used. First, the Herpes Simplex thymidine kinase gene (Tk) was cloned into the G1 plasmid backbone to produce pG1Tk. Second, the Neo^R gene (Na) was cloned into the plasmid pSvBg to make pSvNa. Finally, SvNa was excised from pSvNa and ligated into pG1Tk to produce pG1TkSvNa.

Plasmid pG1TkSvNa was derived from plasmid PG1 (Figure 3). Plasmid pG1 was constructed from pLNSX (Palmer, et al., Blood, Vol. 73, pgs. 438-445. The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6kb EcoRI fragment, containing the 5' Moloney Murine Sarcoma Virus (MoMuSV) LTR, and the 3.0kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance

gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 (Figure 3) by the insertion of the 1.6kB EcoRI fragment containing the 5′ LTR into the unique EcoRI site of pGO. Thus, pG1 (Figure 3) consists of a retroviral vector backbone composed of a 5′ portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender, et al. 1987), a 54 base pair multiple cloning site (MCS) containing, from 5′ to 3′ the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindIII, ApaI, and ClaI and a 3′ portion of MoMuLV from base pairs 7764 to 7813 (numbered as described (Van Beveren, et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985) (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo^r gene, the ß-galactosidase gene, the $hygromycin^r$ gene, and the SV40 promoter.

To construct pBg (Figure 4) the 3.0 kb BamHI/EcoRI lacZ fragment that encodes β-galactosidase was isolated from pMC1871 (Pharmacia). This fragment lacks the extreme 5′ and 3′ ends of the β-galactosidase open reading frame. Linkers that would restore the complete lacZ open reading frame and add restriction sites to each end of the lacZ gene were synthesized and ligated to the BamHI/EcoRI lacZ fragment. The structure of the 5′ linker was as follows: 5′ - 1/2 NdeI - SphI - NotI - SnaBI - SalI - SacII -AccI - NruI - Bg lII - III 27 bp ribosomal binding signal -Kozak consensus sequence/NcoI - first 21 bp of the lacZ open reading frame - 1/2 BamHI - 3′. The structure of the 3′ linker was as follows: 5′ - 1/2 mutated EcoRI - last 55 bp of the lacZ open reading frame - XhoI - HindIII - SmaI - 1/2 EcoRI - 3′. The restriction sites in the linkers were chosen because they are not present in the neomycin resistance gene, the β-

galactosidase gene, the hygromycin resistance gene, or the SV40 promoter. The 27 bp ribosomal binding signal was included in the 5' linker because it is believed to enhance mRNA stability (Hagenbuchle, et al., Cell, Vol. 13:551-563, 1978 and Lawrence and Jackson, J. Mol. Biol., Vol. 162:317-334, 1982). The Kozak consensus sequence (5'-GCCGCCACCATGG-3') has been shown to signal initiation of mRNA translation (Kozak, Nucl.Acids Res., Vol. 12:857-872, 1984). The Kozak consensus sequence includes the NcoI site that marks the ATG translation initiation codon.

pBR322 (Bolivar et al., Gene, Vol. 2:95, 1977) was digested with NdeI and EcoRI and the 2.1 kb fragment that contains the ampicillin resistance gene and the bacterial origin of replication was isolated. The ligated 5' linker - lacZ - 3' linker DNA described above was ligated to the pBR322 NdeI/EcoRI vector to generate pBg. pBg has utility as a shuttle plasmid because the lacZ gene can be excised and another gene inserted into any of the restriction sites that are present at the 5' and 3' ends of the lacZ gene. Because these restriction sites are reiterated in the pG1 plasmid, the lacZ gene or genes that replace it in the shuttle plasmid construct can easily be moved into pG1.

A 1.74 kB Bg1II/PvuII fragment containing the Herpes Simplex Virus Type I thymidise kinase gene (GenBank accession no. V00467, incorporated herein by reference) was excised from the pX1 plasmid (Huberman, et al., Exptl. Cell Res. Vol. 153, pgs 347-362 (1984) incorporated herein by reference), blunted with the large (Klenow) fragment of DNA polymerase I, and inserted into the unique SnaBI site in the pG1 multiple cloning site, to form plasmid pG1TK. (Figure 5).

A 339 bp PvuII/HindIII SV40 early promoter fragment obtained from the plasmid pSV2Neo (Southern et al, Journal of Molecular and Applied Genetics 1:327-341(1982)) was then inserted into pBg in the unique NruI site to generate the plasmid pSvBg (Figure 5). The pSvBg plasmid was digested

with BglII/XhoI to remove the lacZ gene, and the ends were made blunt using the Klenow fragment. An 852 bp EcoRI/AsuII fragment containing the coding sequence of the neomycin resistance gene was removed from pN2 (Armentano, et al., J. Virol., Vol. 61, pgs. 1647-1650 (1987)), blunted with Klenow fragment and ligated into the 2.5 kb blunted BglII/XhoI fragment generated hereinabove, resulting in pSvNa. The SV40 promoter/neomycin resistance gene cassette was then removed from pSvNa as a 1191bp SalI/HindIII fragment. The pG1Tk plasmid was then digested with SalI/HindIII and ligated with the SV40/neo^r fragment to generate pG1TkSvNa. (Figure 6).

B. Generation of Producer Cell Line

A producer cell line was made from vector plasmid and packaging cells. The PA317/G1TkSvNa producer cell was made by the same techniques used to make previous clinically relevant retroviral vector producer cell lines. The vector plasmid pG1TkSvNa DNA was transfected into a ecotropic packaging cell line, PE501. Supernatant from the PE501 transfected cells was then used to transinfect the amphotropic packaging cell line (PA317). Clones of transinfected producer cells were then grown in G418 containing medium to select clones that contain the Neo^R gene. The clones were then titered for retroviral vector production. Several clones were then selected for further testing and finally a clone was selected for clinical use.

5 x 10^5 PE501 cells (Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989), incorporated herein by reference) were plated in 100 mm dishes with 10 ml high glucose Dulbecco's Modified Essential Medium (DMEM) growth medium supplemented with 10% fetal bovine serum (HGD10) per dish. The cells were incubated at 37°C, in 5% CO_2/air overnight.

The plasmid pG1TKSvNa then was transfected into PE501 cells by $CaPO_4$ precipitation using 50 μg of DNA by the following procedure.

50 μg of DNA, 50 μl 10 x $CaCl_2$, and 450μl of sterile H_2O was mixed in a 15 ml polypropylene tube to yield a 0.25M Ca Cl_2 solution containing 50 μg DNA, 0.5 ml 2x BBS (containing 50 mM N-N-bis- (2-hydroxyethyl)- 2-aminoethane-sulfonic acid, 280 mM Na Cl, 1.5 mM Na_2 HPO_4, and 50 mM Hepes, pH6.95). The DNA solution then was left at room temperature for about 20 minutes to 1 hour. The dishes then were incubated at 35°C in a 3% CO_2 atmosphere overnight.

A culture dish(es) with optimum precipitate following the overnight incubation then was (were) selected. The dish(es) then was (were) washed again with PBS to remove the salt and the salt solution. 10 ml of HGD10 medium then was added to the dish(es), and the dish(es) incubated at 37°C in a 5% CO_2 atmosphere for about 48 hrs.

After 48 hours, supernatant was collected from the transfected cells. The dish(es) then was (were) rinsed with 5 ml PBS. The PBS then was removed, and cells were removed with trypsin-EDTA. Serial dilutions of the cells were then inoculated into six 100 mm dishes in medium containing HGD10 and 0.8 mg/ml G418.

The six plates of cells were examined daily. The medium was changed as needed to remove dead cells. Live cells or colonies were allowed to grow to a size such that the colonies are large enough to clone (i.e., the colonies are visible to the naked eye). PE501 ecotropic containing supernatants from such colonies of PE501 cells were collected in volumes of from about 5 to 10 ml, placed in cryotubes, and frozen in liquid nitrogen at -70°C.

PA317 cells (Miller et al. Mol. Cell. Biol. 6:2895-2902 (1986)) then were plated at a density of 5 x 10^4 cells per 100 mm plate on Dulbecco's Modified Essential Medium (DMEM) including 4.5 g/l glucose, glutamine supplement, and 10% fetal bovine serum (FBS).

The PE501 supernatant then was thawed, and 8 μg/ml of polybrene was added to the supernatant. The medium was

aspirated from the plates of PA317 cells, and 7 to 8 ml of viral supernatant was added and incubated overnight.

The PE501 supernatant then was removed and the cells refed approximately 18-20 hours with fresh 10% FBS. One day later, the medium was changed to 10% FBS and G418 (800 μg/ml). The plate then was monitored, and the medium was changed to fresh 10% FBS and G418 to eliminate dying or dead cells as necessary. The plate was monitored for at least 10 to 14 days for the appearance of G418 resistant colonies.

Cloning rings were placed around all selected colonies. The cells were tyrpsinized and incubated into wells in a six well dish in 5 ml of HGD10 plus 1x hypoxanthine aminopterin thymidine (HAT).

If the clones grew to confluency, they were trypsidized and incubated in a 100 ml dish. As a clone in the 100 ml dish approached confluency, its amphotropic vector-containing supernatant was removed and centrifuged at 1,200 to 1,500 rpm for 5 minutes to pellet out cells.

Supernatants were aliquoted into six cryovials (1 ml/vial) and stored in liquid nitrogen. 5 ml of PBS were added to the dish, and the cells were rinsed, and refed with HGD-10 and frozen in 1 ml aliquots with 10% DMSO in liquid nitrogen. The different clones were monitored to determine the one with the highest titer of retroviral vector.

The clone with the highest titer, designated as producer cell line PA317/G1TkSvNa.53, was used to produce a master cell bank.

Example 2

9L rat glioma tumor cells were divided into two groups. Each group was divided into six subgroups. Subgroup 1 in each group included about 500 cells; subgroup 2 in each group included about 1,000 cells; subgroup 3 in each group included about 2,000 cells; and each of subgroups 4, 5, and 6 in each group included about 5,000 cells. One group of cells served as a control, and the other group was transduced with from

about 0.5 x 10^6 to about 1 x 10^6 retroviral vector particles generated from the PA317/G1TkSvNa.53 cell line. Cells in each group then were exposed to BVdU, acyclovir, or ganciclovir. The cells in subgroup 1 of each group were exposed to BVdU, acyclovir, or ganciclovir in an amount of 0.5 μg/ml. The cells in subgroup 2 of each group were exposed to BVdU, aciclovir, or ganciclovir in an amount of 1.0 μg/ml. The cells in subgroup 3 of each group were exposed to BVdU, acyclovir, or ganciclovir in an amount of 2.0 μg/ml. The cells in subgroup 4 of each group were exposed to BVdU, acyclovir, or ganciclovir in an amount of 5.0 μg/ml. The cells in subgroup 5 of each group were exposed to BVdU, acyclovir, or ganciclovir in an amount of 10.0 μg/ml. The cells of subgroup 6 of each group were exposed to BVdU, acyclovir, or ganciclovir in an amount of 20.0 μg/ml. The cells were cultured for an additional 50 hours, and the percent survival of the cells was measured over this time period. The results are shown in Figures 7A through 7F.

As shown in Figures 7A, 7C, and 7E, the 9L cells which were not transduced with the retroviral vector particles were not sensitive to BVdU, acyclovir, or ganciclovir. In contrast, as shown in Figures 7B, 7D, and 7F, the antiviral agents were toxic to the cells transduced with the retroviral vector particles. The survival of the cells decreased. Cell toxicity, in general, increased with increased drug concentration, particularly with acyclovir. (Figure 7F)

Example 3

Prior to the combined experiments wherein 9L-TK cells are exposed to radiation and an anti-Herpes drug as described hereinbelow, a control experiment was carried out to determine whether the introduction of the Herpes Simplex Virus TK gene would alter the intrinsic radiosensitivity of 9L cells. In this experiment, genomic DNA (10μg) was digested with 5 units/μg of a restriction endonuclease at

37°C overnight. The DNA was subjected to electrophoresis in 1% agarose and transferred to Gene Screen overnight (Southern, J. Mol. Biol., Vol. 98, pgs. 503-517 (1975)). Prior to the transfer, the gel was irradiated with ultraviolet light for 5 minutes to facilitate the transfer of large DNA fragments. Prehybridization was carried out in 6X standard saline citrate (SSC), 5X Denhardt's, and 0.25 mg/ml of denatured salmon sperm DNA at 68°C for 12 hours. Hybridization was carried out in a solution of the same formulation, but, in addition, it contained 10% (wt./vol.) dextran sulfate and $2x10^5$ cpm/lane of ^{32}P-labeled TK gene. Hybridization was allowed to proceed at 68°C for 36 hours. The filter was washed in 2XSSC at room temperature for 15 minutes, then twice in 1XSSC, 1% sodium dodecyl sulfate at 68°C for 1 hour. The filter was blot dried and radioactive bands were visualized by autoradiography using an intensifying screen. (Cronex® Lightning Intensifying Screen, DuPont, Wilmington, Delaware.)

The left panel of Figure 8 shows the Southern blot analysis of 9L cells transduced with the TK gene. The blot illustrates that the TK gene is incorporated stably into the transduced cells, and that they contain, on average, approximately three copies of provirus per cell. The right panel of Figure 8 demonstrates that intrinsic radiation sensitivity (that is, in the absence of antiviral agent) was not altered when cells were transduced with the TK gene.

Example 4

9L glioma cells which were transduced with the retroviral vectors generated from the PA317/G1TkSvNa.53 cell line were exposed to BVdU in amounts up to 20μg/ml. 24 hours after exposure to BVdU, one group of cells was exposed to gamma-radiation in an amount of 8Gy, another group of cells was exposed to gamma-radiation in an amount of 12Gy, and another group of cells was not irradiated. (Control) The results are shown in Figure 9A.

As shown in Figure 9A, enhancement of the effects of radiation increased, with increasing radiation doses when exposure to BVdU preceded irradiation.

In another experiment, one group of cells transduced with retroviral vector particles generated from the PA317/G1TkSvNa.53 cell line was exposed to 20μg/ml BVdU, and another group of such cells was not exposed to BVdU. After 24 hours, each group of cells was exposed to gamma-radiation in amounts up to 12Gy. The results are shown in Figure 8B. As shown in Figure 9B, cell survival was lower in those cells which were exposed to BVdU, as compared with the control cells. From the data plotted in Figure 9B, the sensitizer enhancement ratio, or SER, was calculated for BVdU using the least square curve fitting routine developed by Albright, Radiat. Res., Vol. 112, pgs. 331-340 (1982). The SER is the ratio of the radiation dose necessary to achieve a given level of cell kill in the absence of drug relative to the radiation dose necessary to produce the same level of cell kill in the presence of the drug. The SER for 20 μg/ml of BVdU exposure before gamma radiation was calculated to be 1.4.

Example 5

9L glioma cells transduced with retroviral vector particles generated from the PA317/G1TkSvNa.53 producer cell line were exposed to acyclovir in concentrations up to 20μg/ml for 24 hours prior to or following gamma-radiation in amounts of 8Gy or 12Gy. Figures 10A and 10B show the results for the cells exposed to acyclovir prior to irradiation, and Figures 10C and 10D show the results for the cells exposed to acyclovir following irradiation. Figure 10E shows the results for cells exposed to 10μg/ml acyclovir prior to and following irradiation. Control groups of cells were exposed to acyclovir, but were not irradiated, or were irradiated but not exposed to acyclovir.

As shown in Figures 10B and 10D, additional cytotoxicity due to radiation sensitization was found either when cells were treated with acyclovir prior to irradiation or when acyclovir exposure followed irradiation. Exposure to acyclovir either before irradiation (Figures 10A and 10B) or following irradiation (Figures 10C and 10D) resulted in significant additional cytotoxicity.

The SER was calculated for 20 μg/ml acyclovir exposure before or after gamma-radiation. For acyclovir exposure before gamma-radiation, the SER was 1.27. For acyclovir exposure after gamma-radiation the SER was 1.36.

The radiation enhancement from the two 24-hour acyclovir exposures, the first before irradiation, the second after irradiation, resulted in an SER of 1.6 ± 0.1. The drug-alone cytotoxicity for the two 24-hour 10μg/ml acyclovir exposures was approximately the same as the cytotoxicity of a single 24-hour 20μg/ml exposure (30% and 35% survival relative to no treatment controls, respectively).

Example 6

Materials and Methods

The colony-forming ability of human glioma cells growing in cultured media was used to assess cytotoxicity of antiviral drug and radiation treatment to cells containing a viral thymidine kinase gene. Experiments were performed using the human glioma U-251 cell line (Bigner, et al., J. Neuropath. Exp. Neurol., Vol. 40, pgs. 201-229 (1981)) which included the Herpes Simplex Virus thymidine kinase (TK) gene. A control U-251 cell line, which did not include the Herpes Simplex Virus TK gene, also was employed. These cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum.

The vectors which were employed were generated from the PA317/G1TKSVNa.53 producer cell line hereinabove described. This cell line generates a supernatant with a titer of 5.0 x

10^5 colony forming units/ml. The producer cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

U-251 cells then were exposed to supernatant from the PA317/G1TKSVNa.53 producer cell line, which was added with Polybrene to a concentration of 8 μg/ml for about 4 hours. In order to achieve an enriched population of transduced cells, the tumor cells were selected in G418 (800 μg/ml) for 7 days. Surviving colonies were cloned for subsequent radiation and cytotoxic studies of antiviral agents.

For the following experiments, the antiviral drug BVdU was purchased from Sigma Chemical Company, St. Louis, MO. Cells were irradiated using a 5,000Ci ^{137}Cs source (Model Mark I; J. L. Shepherd, Inc., San Fernando, CA) at a dose rate of 1.61 Gy/min. Cell survival data were analyzed using a non-linear least-square algorithm developed by Albright, Radiat. Res., Vol. 112, pgs. 331-340 (1993).

Figures 11A and 11B depict the effects on cellular survival of exposure to BVdU as a function of exposure time in the absence of radiation. Non-infected U-251 cells were minimally sensitive to BVdU up to 100 μg/ml. (Figure 11A.) In contrast, U-251 cells transduced with the retroviral vectors generated from the PA317/G1TKSvNa.53 producer cell line were more sensitive to BVdU, even at lower concentrations such as 10μg/ml when exposure to BVdU was prolonged for up to 72 hours. The cytotoxicity of the U-251 cells transduced with the TK gene which were exposed to BVdU increased with increasing exposure time and generally increased with increasing drug concentration. (Figure 11B.)

On the basis of the above cytotoxicity data, the effect of BVdU on the radiation response of U-251 cells transduced with the TK gene was evaluated. Cells were exposed to BVdU for 24 hours prior to (Figure 12A) or following (Figure 12B) a single dose of 8 Gy radiation. BVdU exposure for

twenty-four hours prior to or subsequent to radiation did not affect cell survival of control U-251 cells regardless of drug concentration (up to 200 μg/ml).

U-251 cells which were transduced with the TK gene displayed a marked enhancement in radiation sensitivity when exposed to BVdU for 24 hours prior to or subsequent to irradiation. The enhancement was dependent on the concentration of drug. A concentration of at least 40 μg/ml achieved the maximum effect. The radiation enhancement at 8 Gy contributed an additional log of cell kill in U-251 cells transduced with the TK gene relative to non-transduced U-251 cells.

Figure 13 depicts the effect of BVdU on the radiation response observed in non-transduced U-251 cells and in U-251 cells transduced with the TK gene. Cells were exposed to 40 μg/ml BVdU prior to irradiation in an amount up to 12 Gy. The sensitizer enhancement ratio was 1.9 in the transduced cell line (Figure 13B), as assessed by the ratio of the slope of the "straight line" portion of the survival curves using a least-square curve fitting method. (Albright, 1987). Figure 13 also indicates that incorporation of the TK gene by itself, in the absence of BVdU, resulted in no radiation enhancement. Also, no enhancement of radiation cytotoxicity was observed in the non-transduced U-251 cells.

Example 7

In vivo treatment of brain tumors in rats

The tumor implantation techniques used in this example have been described previously in Kim, et al., Endocuric/Hyperth. Oncol., Vol. 3, pgs. 47-53 (1987) and in Kim, et al., J. Neurooncol., Vol. 9, pgs. 9-15 (1990). Briefly, the 9L-tk cells were maintained as exponential cultures in Dulbecco's minimum essential medium with 10% fetal calf serum. Immediately prior to each implantation, the cells were trypsinized and resuspended for cell counting.

A final dilution of 2 x 10^6 cells per ml was made in the presence of Dulbecco's medium without serum.

Adult male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) weighing between 220g and 240g were used in all experiments. The rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and positioned in a stereotactic head frame. Aseptic surgical techniques were used to open the scalp in the midline and expose the frontal and temporalis bones. A 1.0mm burr hole for implanting tumor cells was made through the skull. Care was taken not to damage the dura. The position of the injection site was in the right cerebral hemisphere of the rat brain, 2.5mm anterior to the bregma and 2.0mm to the right of midline. Twenty-thousand 9L-tk tumor cells were implanted stereotactically at a 3mm depth in the cerebral parenchyma with a 10μl syringe (Hamilton Company Inc., Reno, Nevada) with a 26S needle. The volume injected was 5μl. The burr hole was sealed with sterile bone wax.

12 days after implantation of the tumor cells, a group of rats received irradiation. In this example, the rats were anesthetized as hereinabove described, and placed in a position identical to that used at the time of tumor implantation. The tumors were treated with a range of radiation doses delivered by means of a single dorso-ventral collimated beam, centered stereotactically over the burr hole. A Therac-20 accelerator (Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada) was used to deliver 18MV x-rays using a 7mm secondary collimator. The dose rate was 2.54 Gy/min. using a 75cm source-to-surface distance. Doses were prescribed to the 90% isodose line, which encompassed a cylinder 5mm in diameter. A tissue equivalent bolus was used to produce a more homogenous dose distribution throughout the treatment volume. Single doses ranged from 15 to 25 Gy.

One-half of the rats that received irradiation also received BVdU (15 mg/kg) three times a day for 3 days

intraperitoneally prior to the irradiation. All rats with the transduced tumor cells that did not receive radiotherapy died within 35 days after implantation of the tumor cells, with or without BVdU administration (median survival time of 21 days.) Figure 14 shows the median survival time (days) as a function of single-dose radiotherapy with and without BVdU. There was a clear therapeutic advantage with the drug-treated group following a single dose of 25 Gy. Preliminary histopathology of the irradiated brains showed no enhanced radiation effects on the normal brain parenchyma following the combined treatment.

The above results show that radiation-resistant brain tumors may benefit from the three-step therapy of infection with a retrovirus including a gene encoding a negative selective marker, administration of an interaction agent, and radiation treatment.

The disclosure of all patents, publications (including published patent applications), and database entries referenced in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

The claims defining the invention are as follows:

1. A method of treating glioblastoma, comprising:

transducing tumor cells with a nucleic acid sequence encoding a viral thymidine kinase;

contacting said transduced tumor cells with an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and

exposing said transduced tumor cells to gamma-radiation.

2. The method of claim 1 wherein said viral thymidine kinase gene is the Herpes Simplex Virus thymidine kinase gene.

3. The method of claim 1 or 2 wherein said interaction agent is ganciclovir.

4. The method of claim 1 or 2 wherein said interaction agent is acyclovir.

5. The method of claim 1 or 2 wherein said interaction agent is a halogenated pyrimidine.

6. The method of claim 5 wherein said halogenated pyrimidine is BVdU.

7. The method of any one of claims 1 to 6 wherein said viral thymidine kinase is contained within a viral vector.

8. The method of claim 7 wherein said viral vector is a retroviral vector.

9. The method of any one of claims 1-8 wherein said transduced tumor cells are exposed to said gamma-radiation in an amount of from about 100cGy to about 2,500 cGy.

10. The method of claim 9 wherein said transduced tumor cells are exposed to said gamma-radiation in an amount of from about 200 cGy to about 1,500 cGy.

11. The use of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said viral vector to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

12. The use of a producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said producer cell to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

13. The use of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines in the manufacture of a medicament for the treatment of glioblastoma, wherein said treatment comprises administration of said interaction agent to a patient subsequent to the administration of a viral vector containing a

nucleic acid sequence encoding a viral thymidine kinase and prior to, concurrent with or after the administration of gamma-radiation.

14. The use according to claim 13 wherein said viral thymidine kinase gene is the Herpes Simplex virus thymidine kinase gene.

15. The use according to claim 13 or 14 wherein said interaction agent is ganciclovir.

16. The use according to claim 13 or 14 wherein said interaction agent is acyclovir.

17. The use according to claim 13 or 14 wherein said interaction agent is a halogenated pyrimidine.

18. The use according to claim 17 wherein said halogenated pyrimidine is BVdU.

19. The use according to any one of claims 12 to 18 wherein said viral vector is a retroviral vector.

20. The use according to any one of claims 12 to 19 wherein said gamma-radiation is administered in an amount of from about 100 cGy to about 2,500 cGy.

21. The use according to claim 20 wherein said gamma-radiation is administered in an amount of from about 200 cGy to about 1,500 cGy.

22. A viral vector containing a nucleic acid sequence encoding a viral thymidine kinase when used in the treatment of glioblastoma, wherein said treatment comprises administration of said viral vector to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

23. A producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase, when used in the treatment of glioblastoma, wherein said treatment comprises administration of said producer cell to a patient prior to the administration of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines; and gamma-radiation.

24. An interaction agent when used in the treatment of glioblastoma, wherein said treatment comprises administration of said interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines to a patient subsequent to the administration of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase and prior to, concurrent with or after the administration of gamma-radiation.

25. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said viral thymidine kinase gene is the Herpes Simplex virus thymidine kinase gene.

26. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said interaction agent is ganciclovir.

27. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said interaction agent is acyclovir.

28. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said interaction agent is a halogenated pyrimidine.

29. The viral vector, producer cell or interaction agent of claim 28, wherein said halogenated pyrimidine is BVdU.

30. The viral vector of claim 22, producer cell of claim 23, or interaction agent of claim 24, wherein said viral vector is a retroviral vector.

31. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said gamma-radiation is administered in an amount of from about 100 cGy to about 2,500 cGy.

32. The viral vector of claim 22, producer cell of claim 23 or the interaction agent of claim 24, wherein said gamma-radiation is administered in an amount of from about 200 cGy to about 1,500 cGy.

33. A method of treating glioblastoma, substantially as hereinbefore described with reference to Example 7.

34. The use of a viral vector containing a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

35. The use of a producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase in the manufacture of a medicament for the treatment of glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

36. The use of an interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines in the manufacture of a medicament for the treatment of glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

37. A viral vector containing a nucleic acid sequence encoding a viral thymidine kinase when used in the treatment of glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

38. A producer cell capable of producing a viral vector which contains a nucleic acid sequence encoding a viral thymidine kinase, when used in the treatment of glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

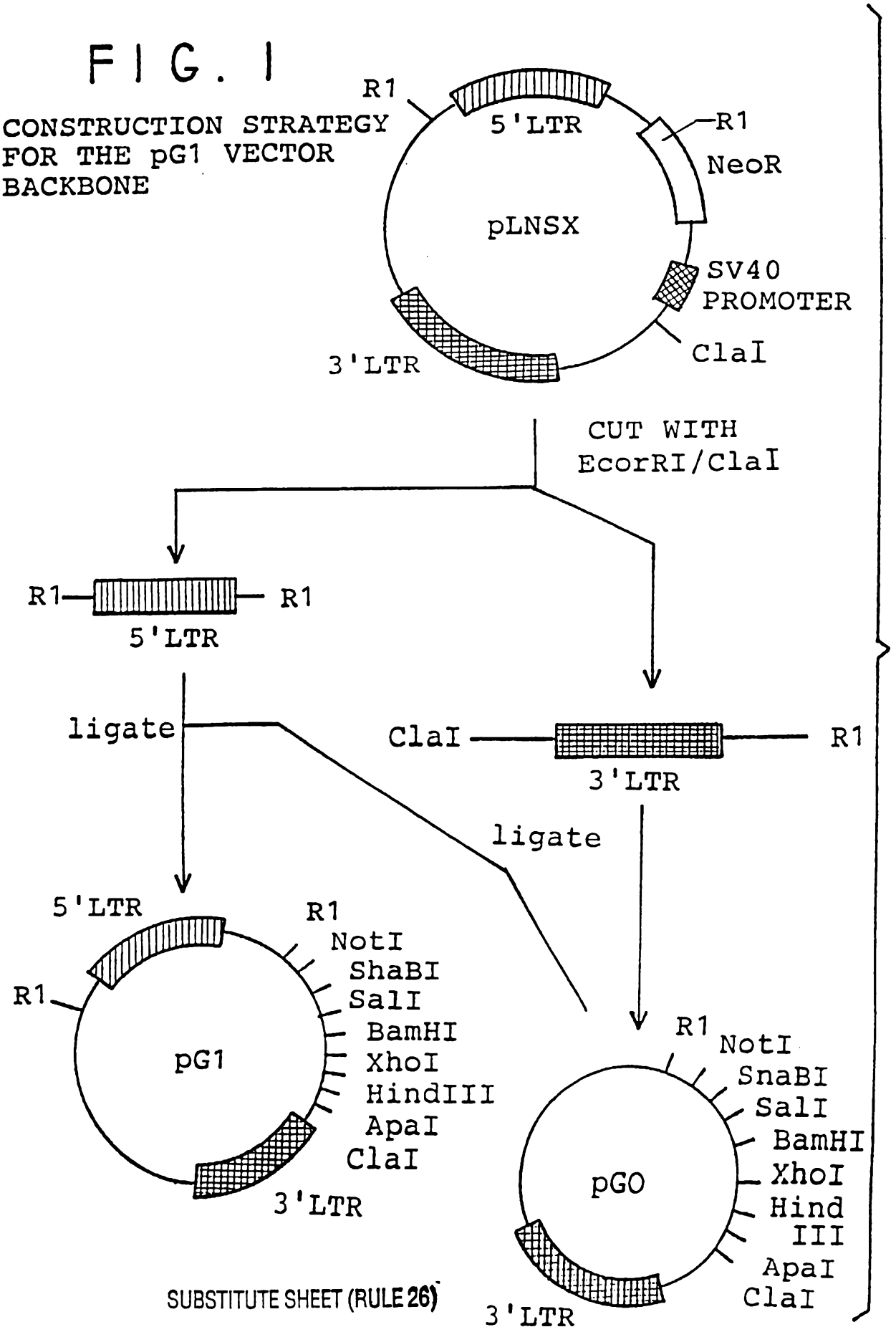
39. An interaction agent selected from the group consisting of ganciclovir, acyclovir, and halogenated pyrimidines when used in the treatment of glioblastoma, substantially as hereinbefore described with reference to any one of the examples.

Dated 9 November, 1999
Henry Ford Hospital

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

FIG. 1

CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE



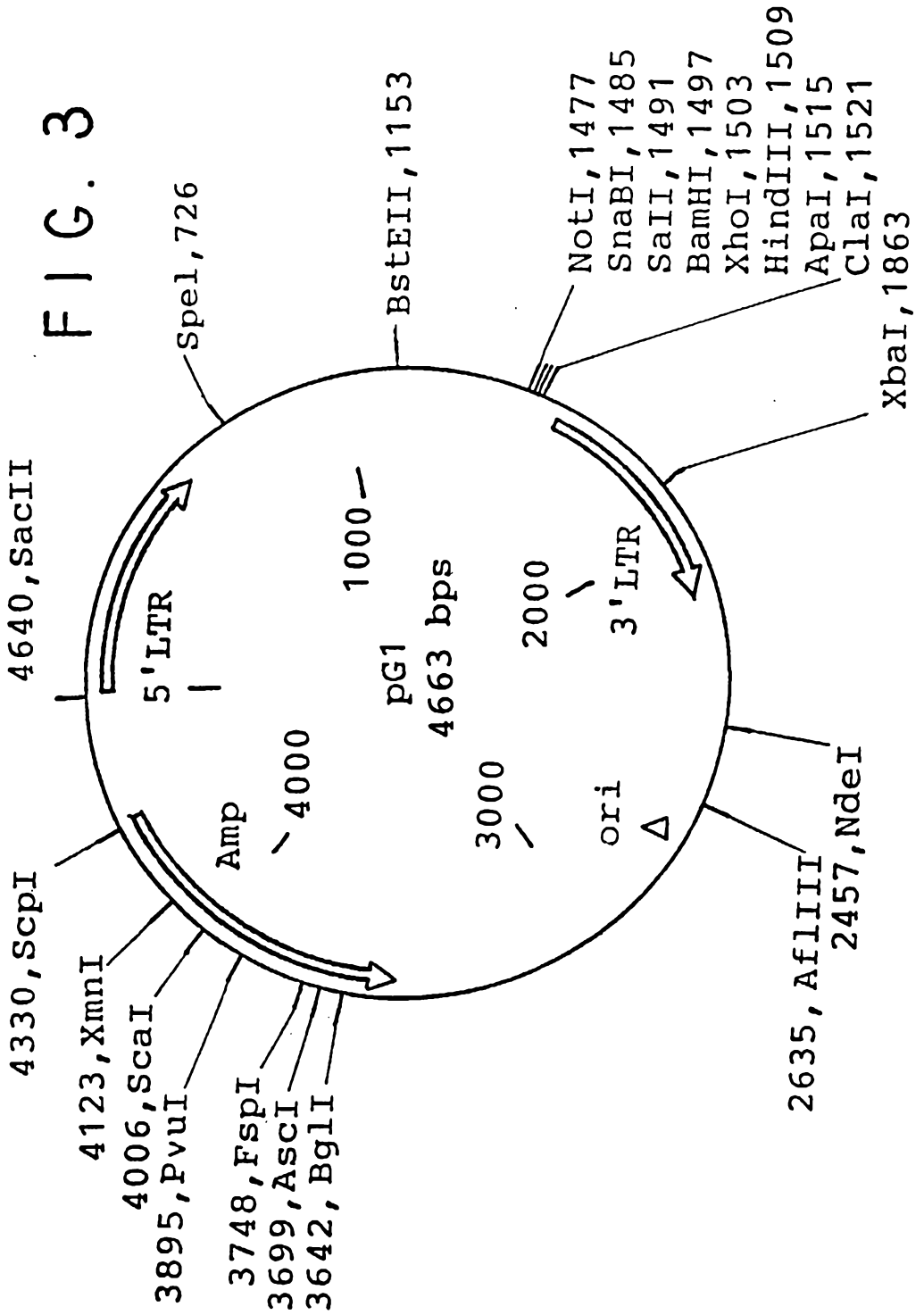
SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

1/2 EcoRI	NotI	SnaBI	SalI	BamHI	XhoI	HindIII	ApaI
AATTC	GCGGCCGC	TACGTA	GTCGAC	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	CAGCTG	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2ClaI
AT
TAGC

FIG. 2

FIG. 3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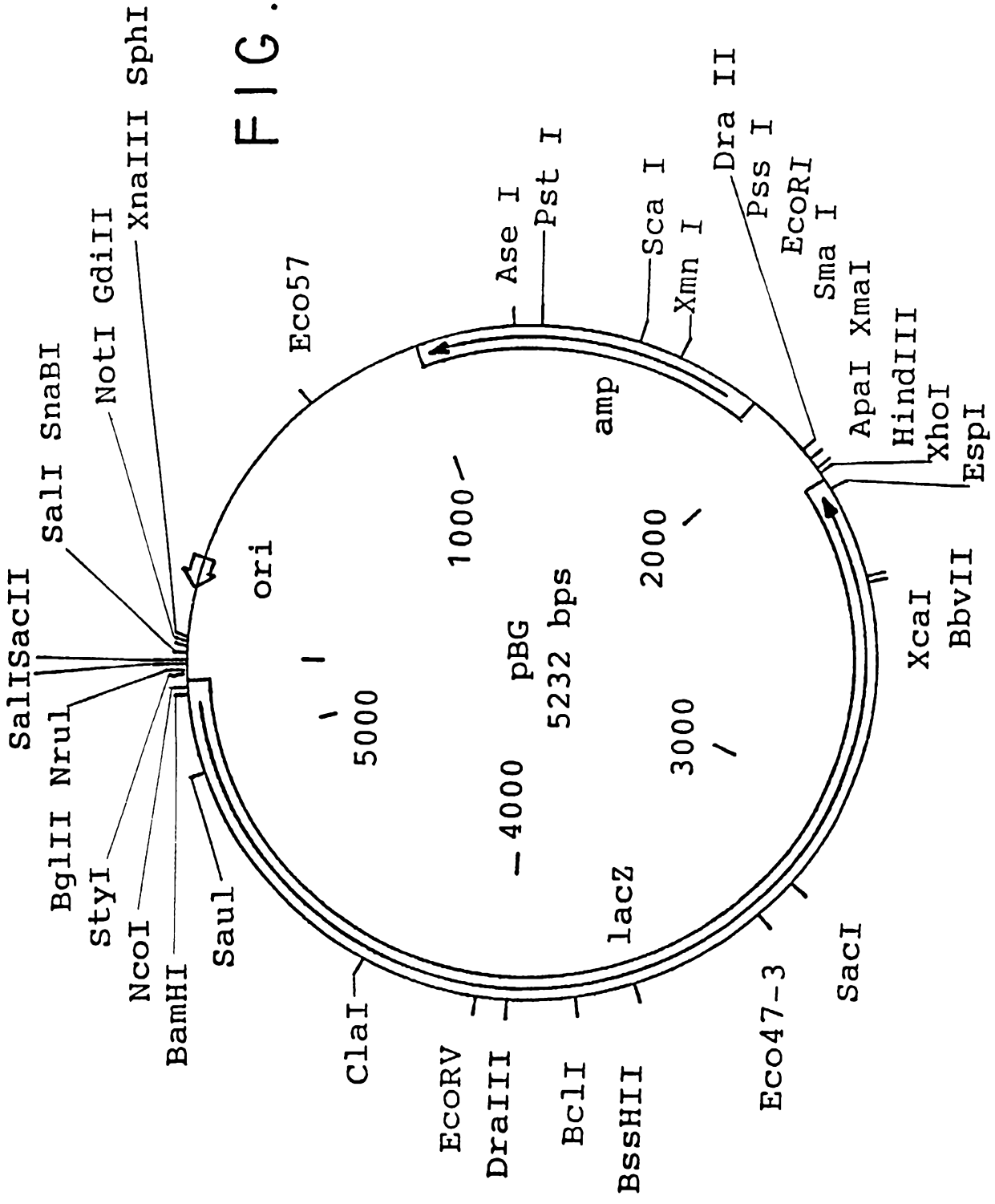
SpeI,726
BstEII,1153
NotI,1477
SnaBI,1485
SalI,1491
BamHI,1497
XhoI,1503
HindIII,1509
ApaI,1515
ClaI,1521
XbaI,1863
5'LTR
1000
pG1
4663 bps
2000
3'LTR
4000
Amp
3000
ori
4330,ScpI
4123,XmnI
4006,ScaI
3895,PvuI
3748,FspI
3699,AscI
3642,BglI
2635,AflIII
2457,NdeI

FIG. 4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SalI SnaBI
NotI GdiII
XnaIII SphI
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Pst I
Sca I
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Dra II
Pss I
EcoRI
Sma I
ApaI XmaI
HindIII
XhoI
EspI
XcaI
BbvII
SacI
Eco47-3
BssHII
BclI
DraIII
EcoRV
ClaI
SauI
BamHI
NcoI
StyI
BglII NruI
ori
amp
lacZ
pBG
5232 bps
1000
2000
3000
4000
5000

FIG. 5

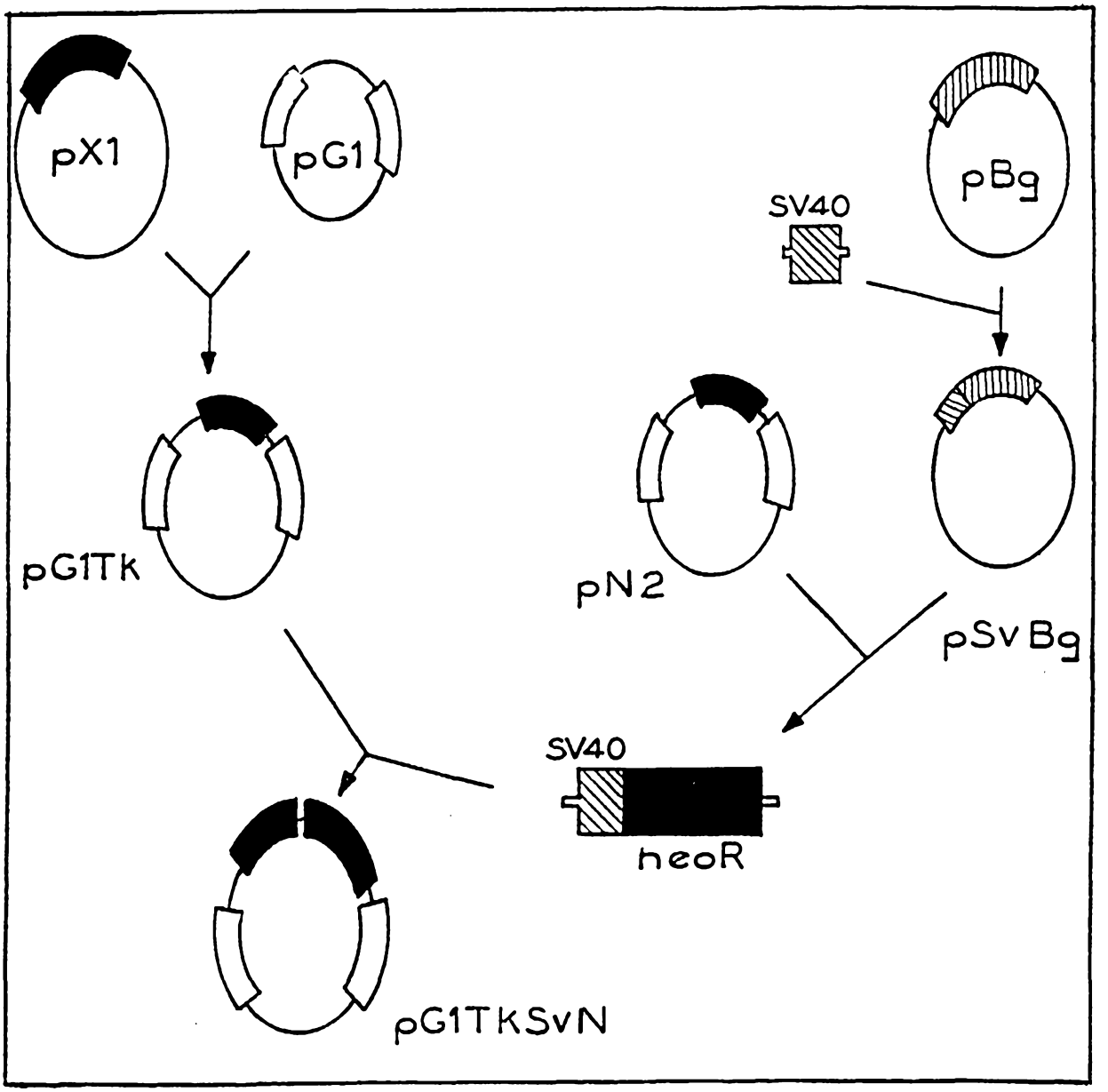


FIG. 6

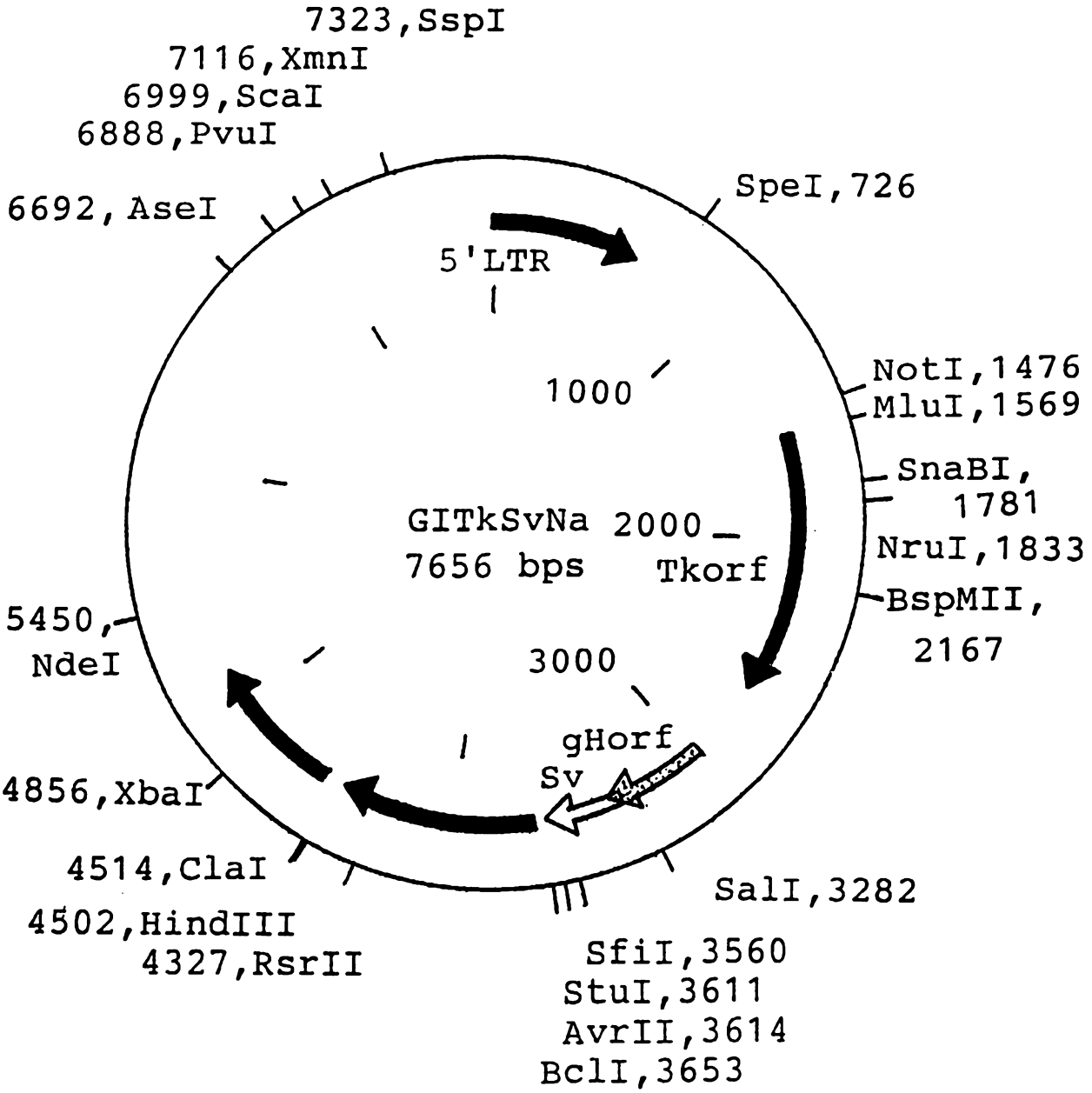

7323,SspI
7116,XmnI
6999,ScaI
6888,PvuI
6692,AseI
SpeI,726
5'LTR
1000
NotI,1476
MluI,1569
SnaBI,
1781
NruI,1833
BspMII,
2167
GITkSvNa
7656 bps
2000
Tkorf
5450,
NdeI
3000
gHorf
Sv
4856,XbaI
4514,ClaI
4502,HindIII
4327,RsrII
SalI,3282
SfiI,3560
StuI,3611
AvrII,3614
BclI,3653

FIG. 7

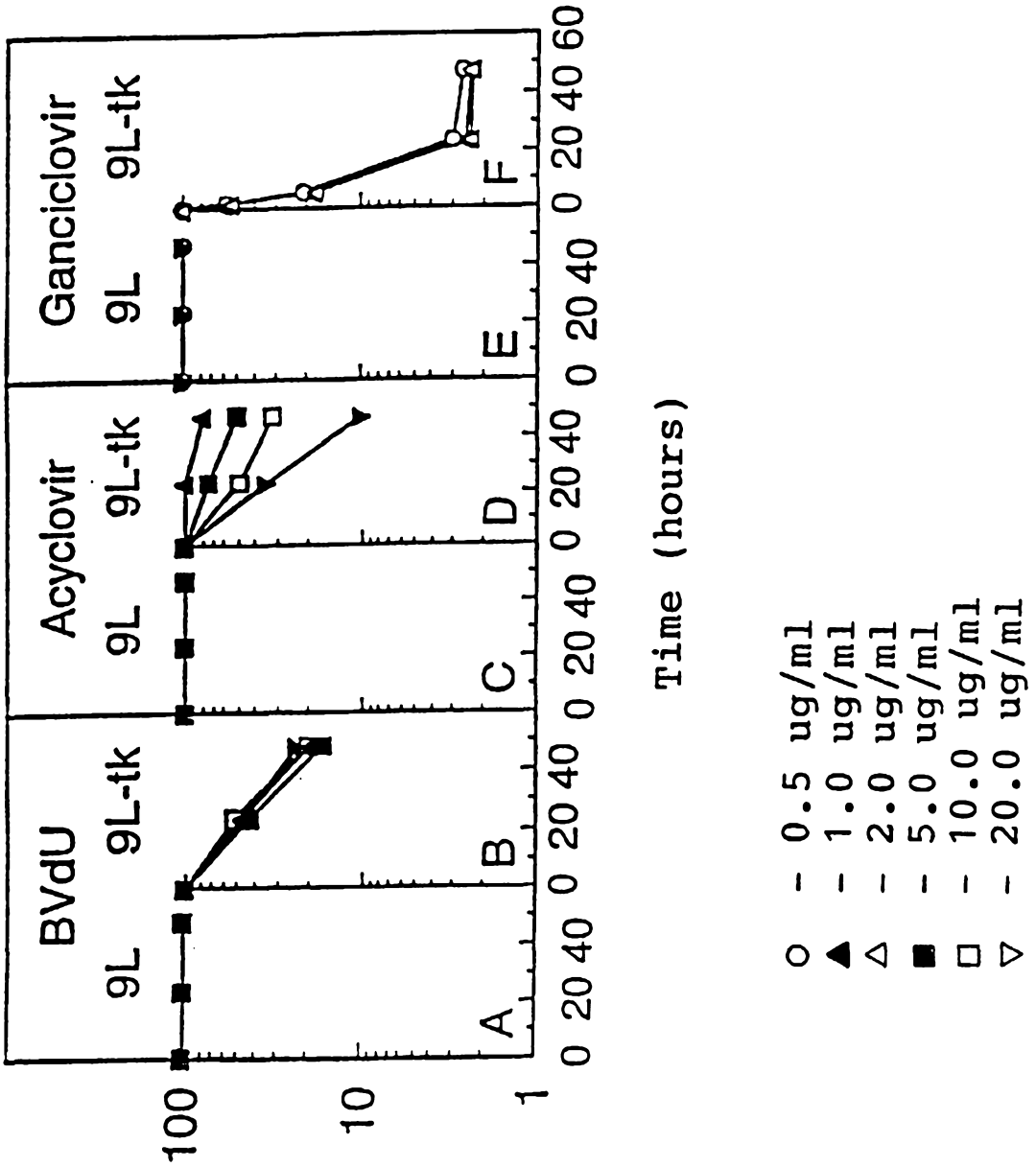

BVdU
Acyclovir
Ganciclovir
9L
9L-tk
A
B
C
D
E
F
100
10
1
0 20 40 0 20 40 0 20 40 0 20 40 0 20 40 0 20 40
Percent Survival
Time (hours)
○ - 0.5 ug/ml
◀ - 1.0 ug/ml
△ - 2.0 ug/ml
■ - 5.0 ug/ml
□ - 10.0 ug/ml
▽ - 20.0 ug/ml

FIG. 8B

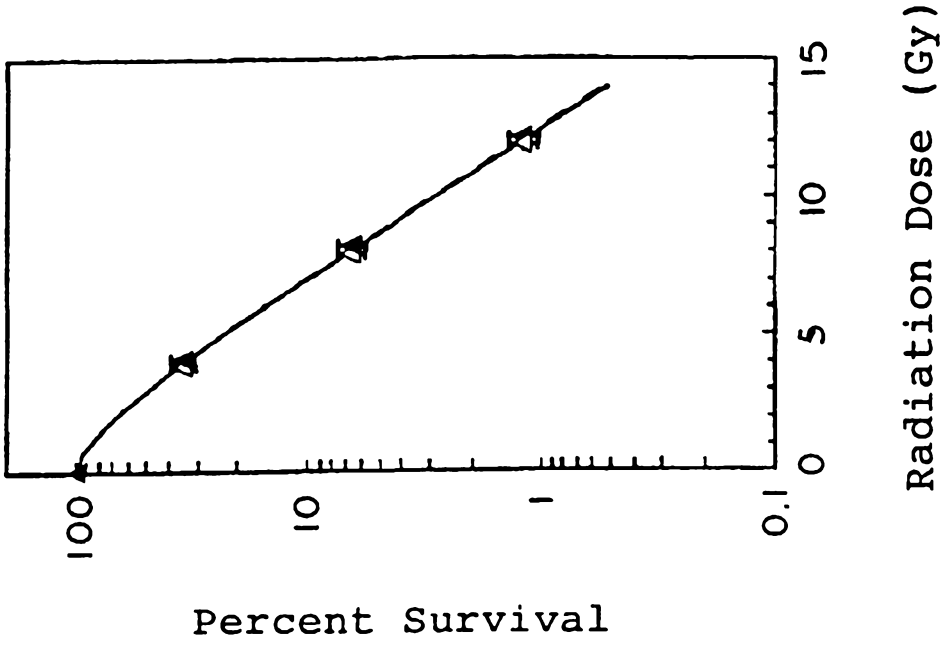

100
10
1
0.1
0
5
10
15
Radiation Dose (Gy)
Percent Survival

FIG. 8A

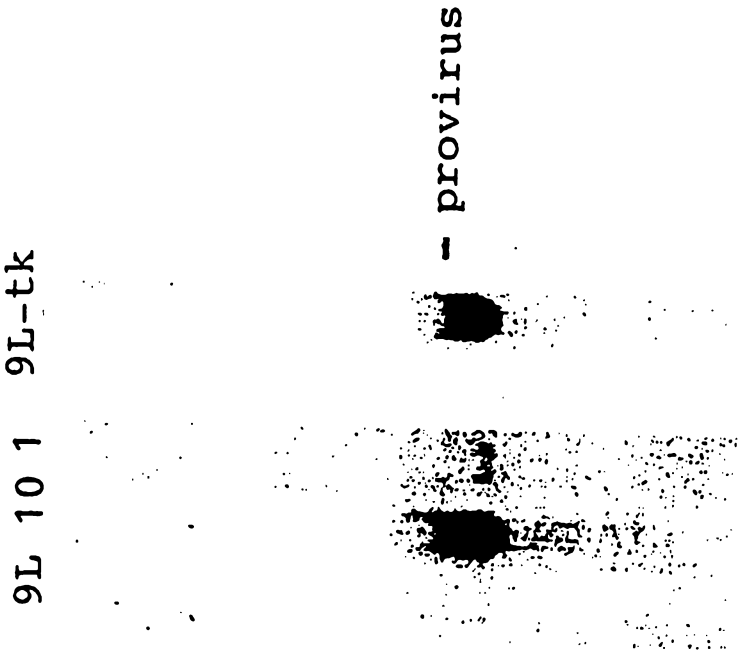

GE
9L 10 1 9L-tk
- provirus

FIG. 9A

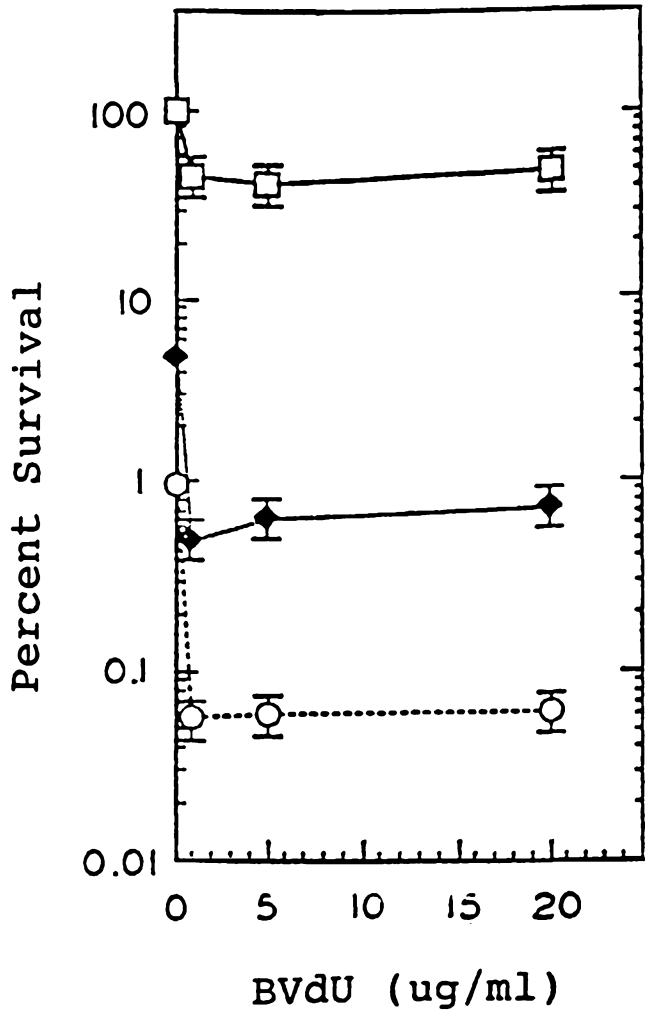
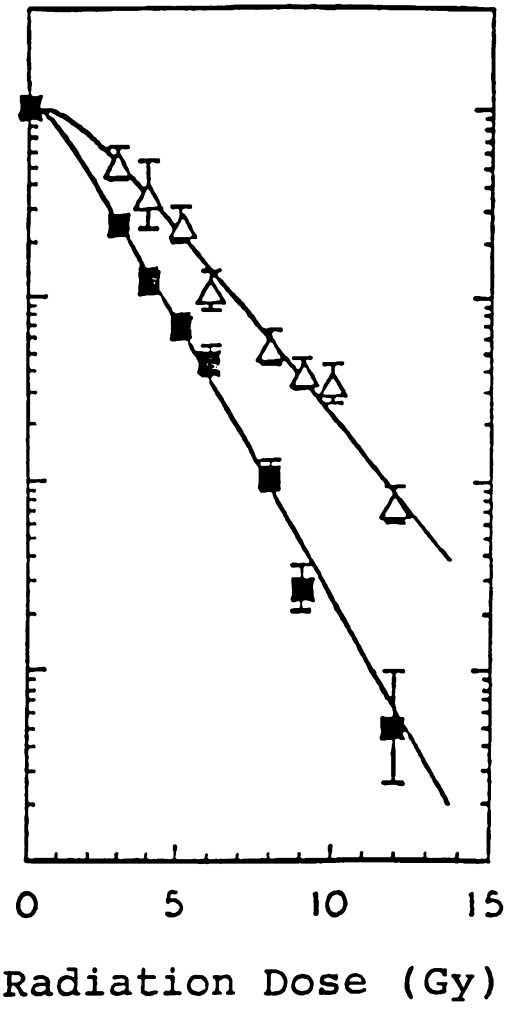


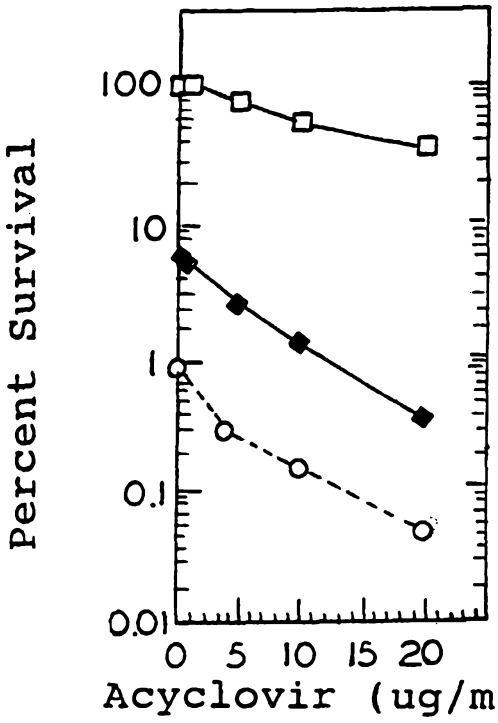
FIG. 9B



◆ - 8 Gy
○ -12 Gy
□ -control

■- 20 ug/ml BVdU exposure
△ - control

FIG. 10A

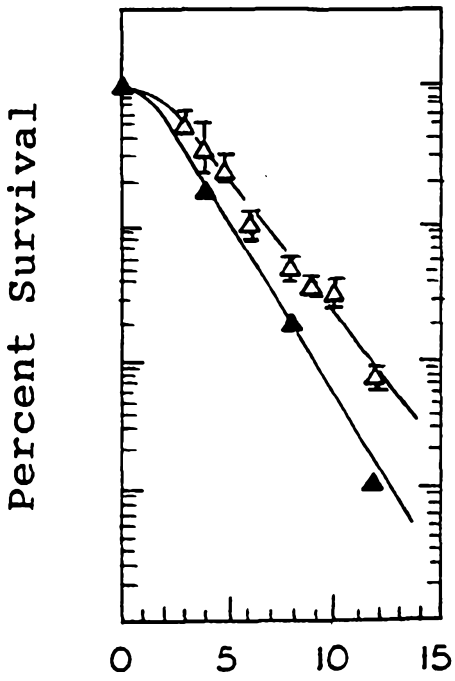


□ - control

◆ - 8 Gy

○ - 12 Gy

FIG. 10B



▲ -20 ug/ml acyclovir exposure

△ - control

FIG. 10C

□ - control

◆ - 8 Gy

○ - 12 Gy

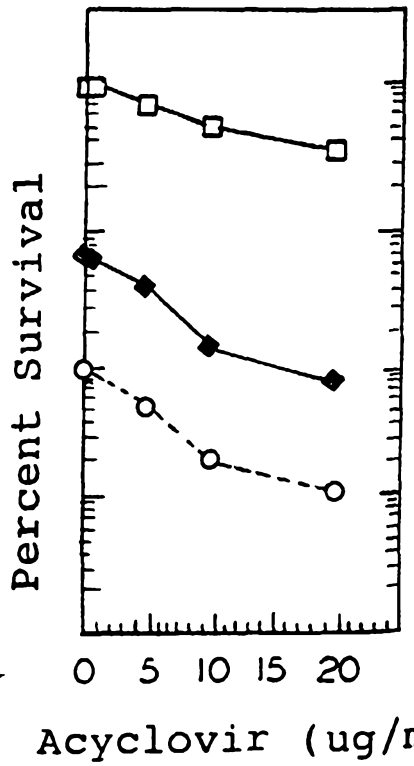
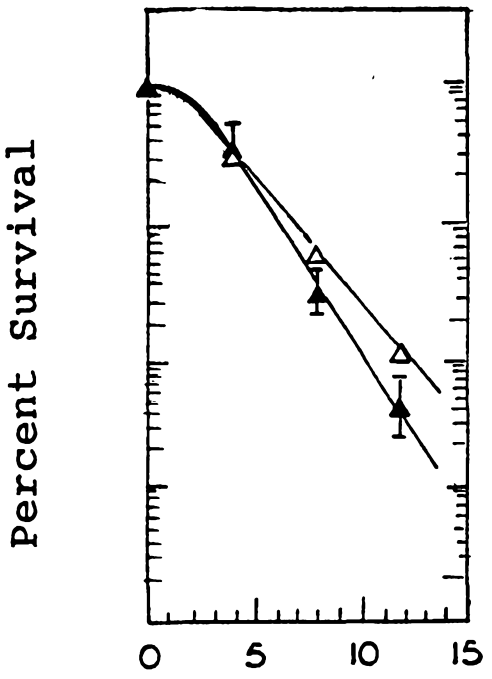


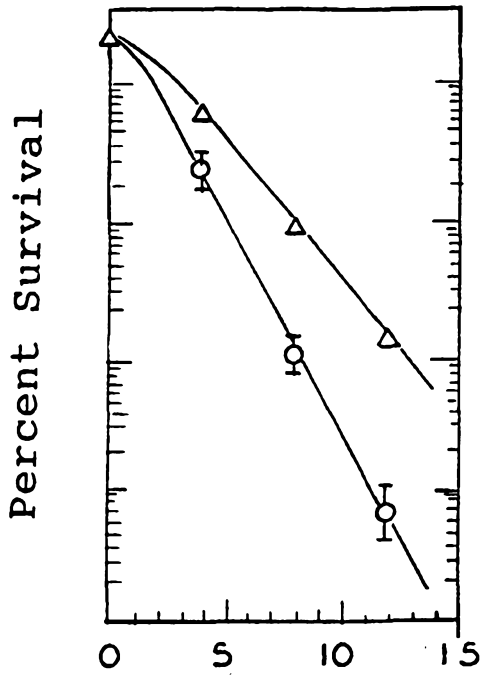
FIG. 10D



▲ -20 ug/ml acyclovir exposure

△ - control

FIG. 10E



○ -Two 10 ug/ml acyclovir exposures

△ -- control

FIG. IIB

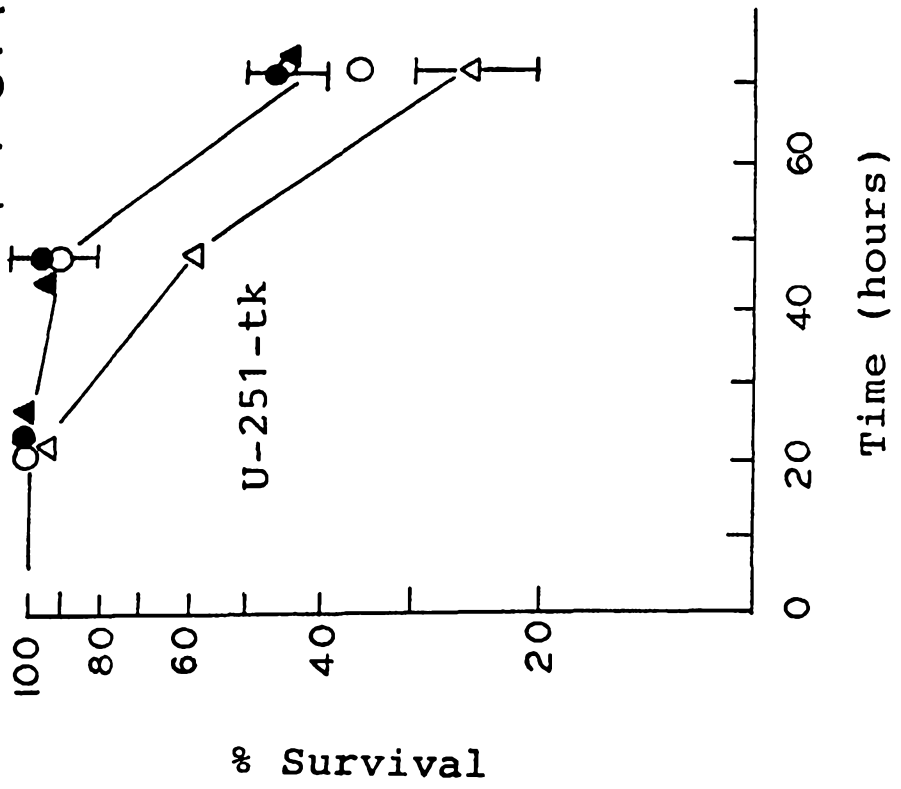
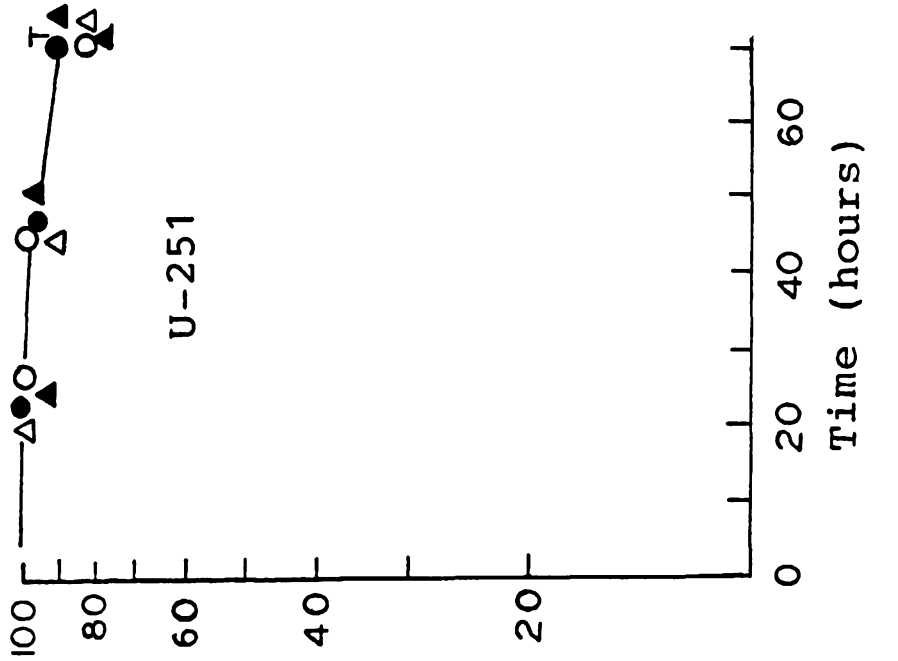


FIG. IIA



BVdU concentrations

● - 10 ug/ml ○ - 40 ug/ml

▲ - 20 ug/ml △ -100 ug/ml

FIG. 12A

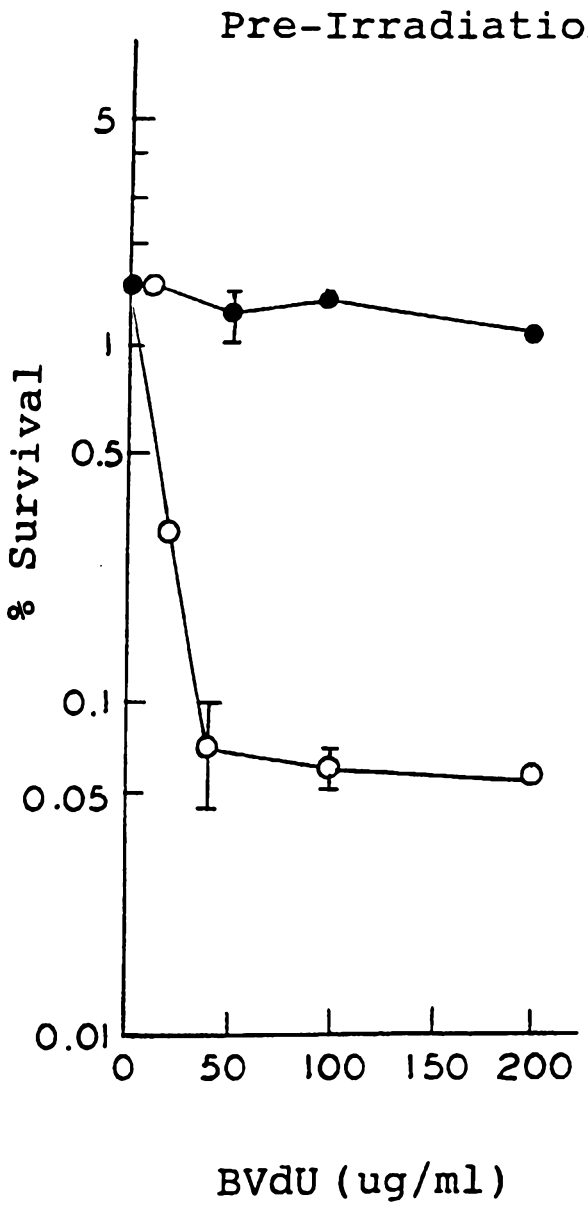
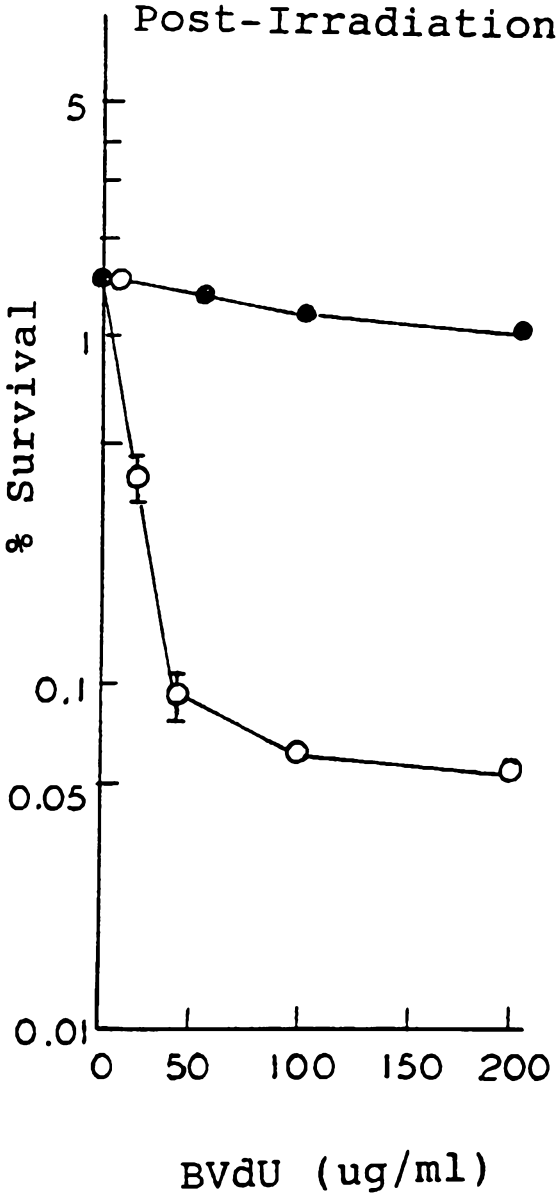


FIG. 12B



● - Control U-251 cells

○ - U-251 TK cells

FIG. 13A

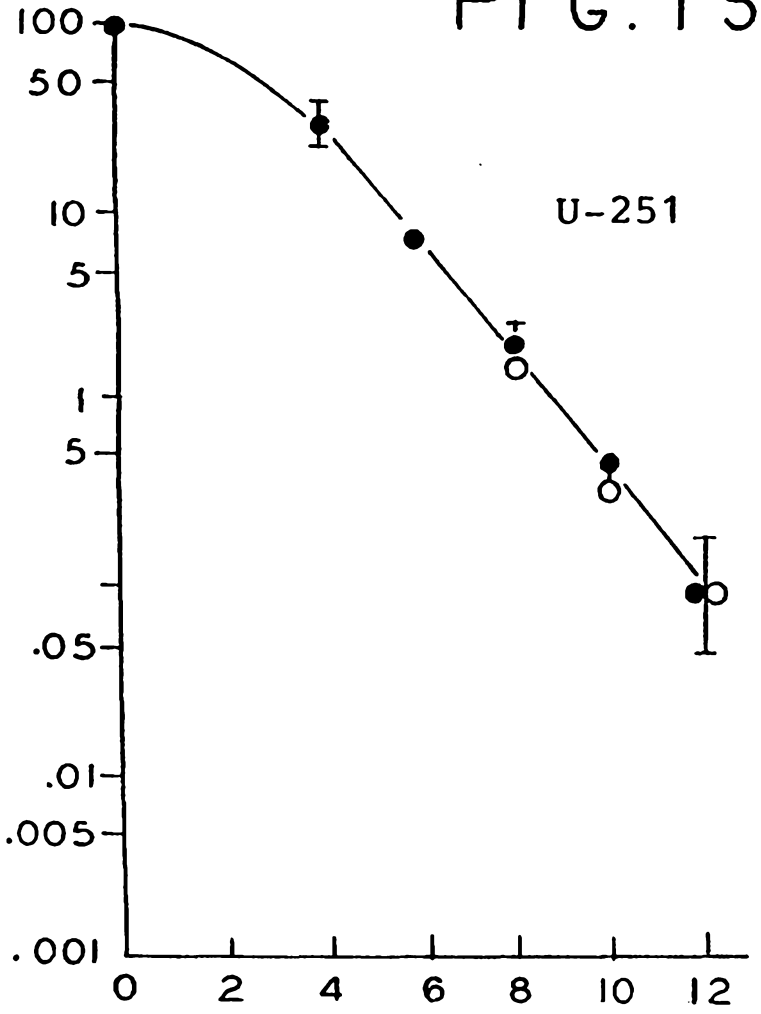
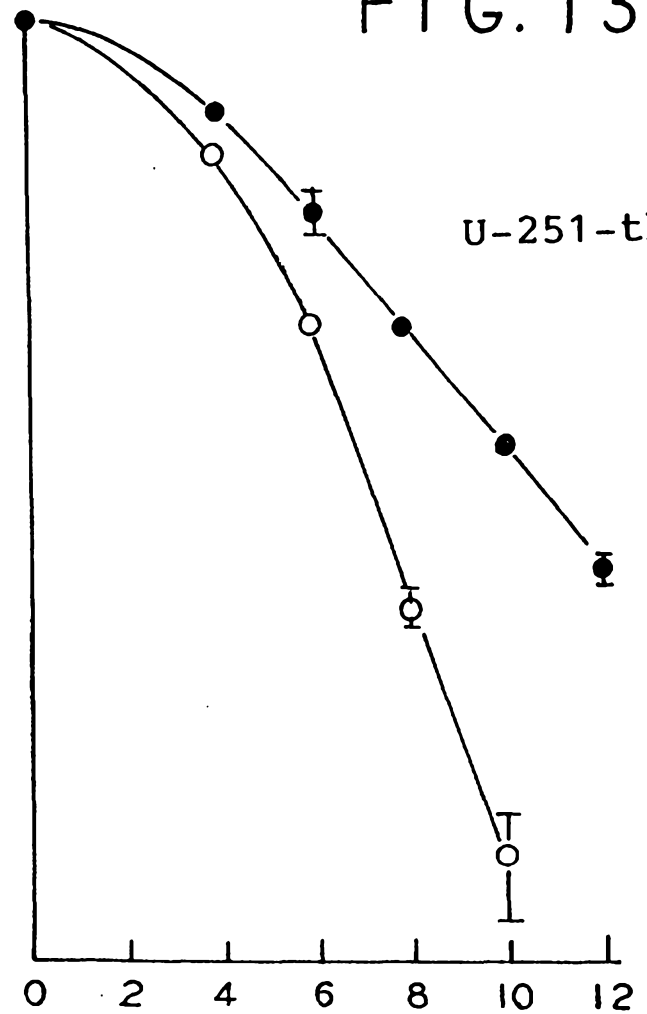


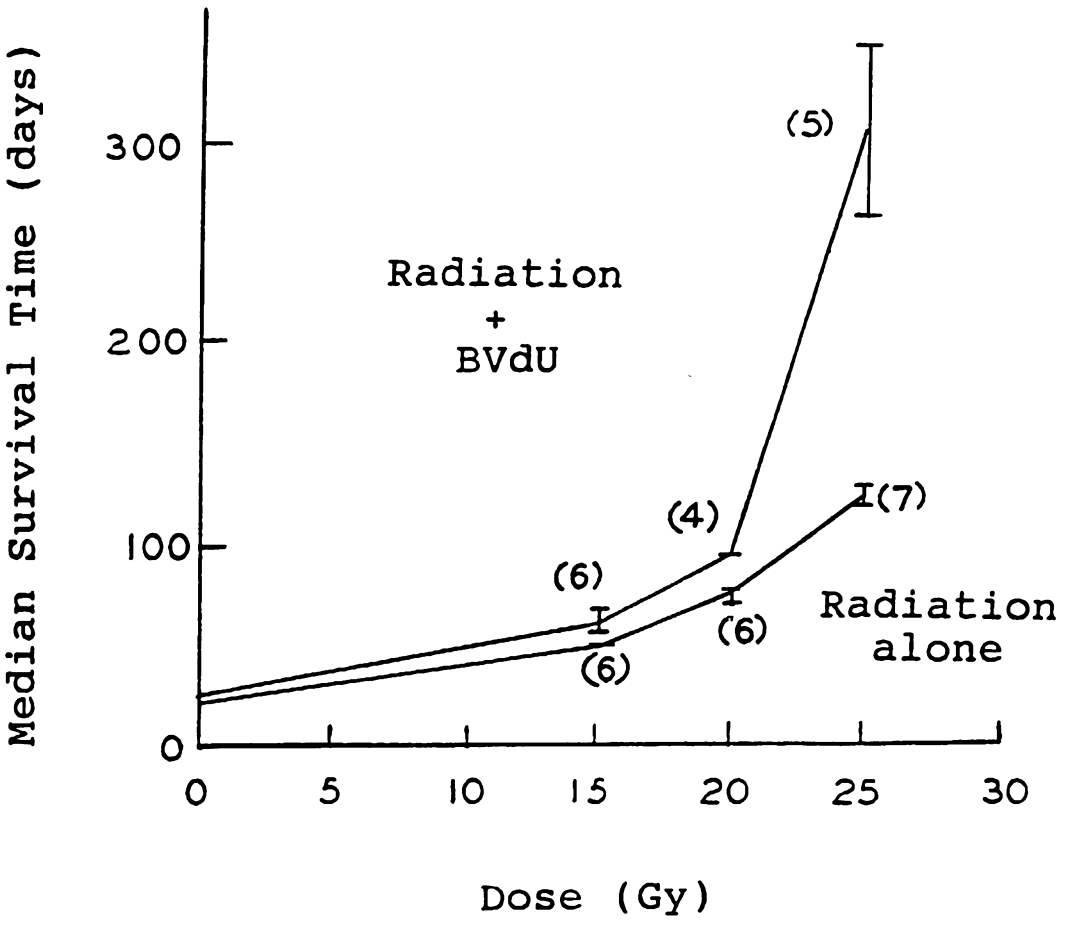
FIG. 13B



○ – EXPOSURE TO 40 ug/ml BVdU

● – NO BVdU EXPOSURE

FIG. 14


Median Survival Time (days)
300
200
100
0
0
5
10
15
20
25
30
Dose (Gy)
Radiation
+
BVdU
Radiation
alone
(5)
(4)
(6)
(6)
(6)
(7)