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(54) **METHODES ET COMPOSITIONS POUR LE TRAITEMENT DES CELLULES HUMAINES**

(54) **METHODS AND COMPOSITIONS FOR TREATING HUMAN CELLS**

(57) The present invention relates to methods and compositions for treating human cells. In particular, the invention relates to the use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition to render human cells sensitive to a nucleoside analog in vitro, ex vivo or in vivo. The invention can be used in experimental, biological, screening or therapeutical applications.

ABSTRACT

The present invention relates to methods and compositions for treating human cells. In particular, the invention relates to the use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition to render human cells sensitive to a nucleoside analog in vitro, ex vivo or in vivo.

The invention can be used in experimental, biological, screening or therapeutical applications.

METHODS AND COMPOSITIONS FOR TREATING HUMAN CELLS

The present invention relates to methods and composition for treating human cells in vitro, ex vivo or in vivo. More specifically, this invention provides improved methods and compositions for rendering human cells sensitive to nucleoside analogs. The invention relies in particular on the discovery of advantageous properties of certain polypeptides and nucleic acids as conditional suicide agents, more particularly of the thymidine kinase type. The invention can be used in various technical areas such as experimental biology, therapy, prophylaxy, screening methods, etc.

Mammalian cells that express a conditional suicide gene are selectively killed or destroyed by activation of a prodrug into a toxic metabolite. The Herpes Simplex virus type 1 thymidine kinase gene (HSV1-TK) is the most widely used suicide gene, both in experimental settings and clinical trials.[1] Expression of the HSV1-TK gene renders tumor cells sensitive to antiviral agents like acyclovir (ACV), ganciclovir (GCV) and bromovinyl-deoxyuridine (BVDU). These nucleoside analogs are efficiently converted to their monophosphate form by HSV1-TK, and are then converted to triphosphate compounds by host cellular kinases.[2-4] Incorporation of these metabolites into elongating DNA blocks elongation leading to cell death.[5-8] The HSV1-TK/GCV system has proved efficient for inducing the regression of transplanted tumors in various animal models,[9-11] as well as of carcinogen induced tumors.[12] Based on these favorable results, several clinical gene therapy trials are in progress aimed to assess safety and efficacy of this treatment for treating malignancies[13] or other proliferative diseases such as Graft versus Host diseases, and preliminary results have now been reported.[14-17]

Despite the effectiveness of the HSV1-TK/GCV system for killing proliferating cells (e.g., tumor cells), it remains important to try to improve it,

with the aim to maximize therapeutic efficacy and/or to facilitate treatment modalities. For example, GCV has the disadvantage of being administered by slow intra-venous perfusion, requiring patient hospitalization. It would thus be interesting to find nucleoside analogs with an oral administration having the same efficacy as the GCV prodrug. It would also represent a significant achievement to find ways to improve the efficacy of the killing effect observed with a TK. In this regard, it has been proposed in the art to use stronger promoter regions to increase the cellular levels of thymidine kinase. It has also been suggested to use a combination of several enzymes in order to increase the phosphorylation efficacy. It has furthermore been proposed to produce and screen for mutants or other derivatives of HSV-1 TK, with improved affinity or activity towards nucleoside analogs.

The present invention now provides alternative methods and compositions which address the above problems and, more particularly, which provide for an improved sensitivity of human cells to nucleoside analogs. More specifically, the Applicants have now unexpectedly shown that an increased sensitivity to nucleoside analogs can be obtained in human cells with a particular type of thymidine kinase, i.e., an Equine herpesvirus type-4 thymidine kinase polypeptide or nucleic acid. In particular, by using wild-type Equine herpesvirus type-4 thymidine kinase (wtEHV4-TK) or variants thereof, the Applicants have demonstrated that human cells become 3 to 12 fold more sensitive to nucleoside analogs than with HSV-1 TK. The results obtained show that EHV4-TK provides a very interesting alternative suicide gene to the HSV1-TK gene for many uses, including experimental, biological, screening or therapeutical applications.

One object of the present invention therefore resides in the use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition to render human cells sensitive to a nucleoside analog in vitro, ex vivo or in vivo.

Another object of the present invention lies in a method of rendering a human cell sensitive to a nucleoside analog comprising contacting said human cell in vitro, ex vivo or in vivo, with an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor.

Within the context of the present invention, the term Equine herpes virus type-4 thymidine kinase polypeptide ("EHV4-TK") designates either wild-type EHV4-TK or any biologically active fragment or variant thereof. The wild type EHV4-TK has been isolated, characterized and its amino acid sequence has been published in the art.[18] This polypeptide can be obtained by any conventional method known in the art such as recombinant production, artificial synthesis or purification from EHV4-infected cells. A biologically active fragment or variant of wild-type EHV4-TK means inter alia a polypeptide having the ability to phosphorylate a nucleoside analog as defined below such as, for instance, GCV, ACV or BVDU.

Fragments of EHV4-TK can be prepared by any conventional method such as chemical or enzymatic cleavage followed by separation of the fragments, recombinant production from a truncated nucleic acid, artificial polypeptide synthesis, etc. The fragment can be an internal fragment or a C-ter or N-ter fragment of wild-type EHV4-TK, or a combination thereof. Preferably, a fragment according to the present invention retains at least 40%, more preferably at least 50% of the entire amino acid sequence of wild-type EHV4-TK. In a preferred embodiment, a fragment comprises at least 75% of the entire amino acid sequence of wild-type EHV4-TK, more preferably at least 85% thereof. Variants of wild-type EHV4-TK comprise any biologically active polypeptide derived from wild-type EHV4-TK by any mutation(s), substitution(s), addition(s) and/or other modification(s) of the primary or secondary structure. Variants include any natural variants resulting from mutations or polymorphism in EHV4, as well as, for instance, hybrid polypeptides comprising EHV4-TK fused to a heterologous amino acid sequence. The term heterologous means any amino acid sequence

comprising one or several amino acid residues, having an origin different from EHV4. Such a heterologous amino acid sequence can be any artificially created sequence providing for instance for an increased stability, or a secretion of the polypeptide, or a marking of the polypeptide. In this regard, the present invention also discloses fusion polypeptides comprising an EHV4-TK moiety fused to a marker protein such as the Enhanced Green Fluorescent Protein (E-GFP), Luciferase, β-Gal, Thy-1 protein, chloramphenicol-acetyl-transferase (CAT), and cell proteins conferring resistance to antibiotic(s). The fusion is generally prepared by genetically coupling a nucleic acid region coding for the EHV4-TK polypeptide and a nucleic acid region coding for the heterologous amino acid sequence, e.g., the marker moiety. The nucleic acids are fused in frame, so that the two regions are expressed simultaneously and yield a chimeric polypeptide.

Mutation or substitution variants may be prepared by known techniques such as specifics mutagenesis, amplification with specific primers and the like. Deletions may also be introduced by conventional techniques using for instance restriction enzymes. Preferably, the variants retain at least 80% of the primary structure of wtEHV4-TK. More preferred variants contain at most 5 modified amino acid residues compared to the wtEHV4-TK.

Other variants may comprise unnatural glycosylation or other post-translational modifications.

As indicated, the variants of EHV4-TK may comprise only one or several modifications as described above (deletion(s), mutation(s), addition(s), etc.).

The biological activity of the above fragments or variants of wild-type EHV4-TK can be assessed by conventional techniques. In particular, the ability of said polypeptides to phosphorylate nucleoside analogs can be measured by contacting said polypeptide with a nucleoside analog such as

Acyclovir or Gancyclovir and detecting the presence of phosphated Acyclovir or Gancyclovir. Preferably, the test is conducted by (i) introducing, in a cell, a nucleic acid construct encoding the polypeptide under conditions allowing expression of said polypeptide in said cell, (ii) contacting said cell with a nucleoside analog such as Acyclovir or Gancyclovir, and (iii) assessing the toxicity of the nucleoside analog on said cell. If appropriate, the level of toxicity observed can be compared to a standard toxicity level observed in a similar experiment with a nucleic acid encoding wild-type EHV4-TK. As an example, said test can be performed with any dividing cell such as fibroblasts, for instance. Preferred biologically active fragments or variants retain 75% at least of the toxicity of wild-type EHV4-TK in a test as disclosed above.

As indicated above, the present invention can be performed by using an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor. The use of a nucleic acid represents a preferred embodiment. Nucleic acids of this invention can be DNA or RNA, preferably cDNA or an artificial DNA comprising synthetic regions. The nucleic acid encoding wild-type EHV4-TK has been cloned and sequenced.[18] A nucleic acid encoding a EHV4-TK polypeptide of this invention can be obtained by conventional techniques such as library screening, artificial synthesis, cloning from genomic DNA, etc. A particular nucleic acid encoding a EHV4-TK polypeptide of this invention is any nucleic acid which hybridizes with the nucleic acid encoding wild-type EHV4-TK and codes for a biologically active EHV4-TK polypeptide as defined above. Hybridization is preferably performed under stringent conditions, which are known to the skilled artisan (Sambrook et al.), such as for instance 40°C, 50% formamide, 5 x SSC; 1 x Denhardt, with washings at between 55 and 65°C. Another particular nucleic acid of this invention is a nucleic acid encoding wild-type EHV4-TK modified so as to improve the expression conditions. In particular, a preferred nucleic acid of this invention

is modified to inactivate any cryptic promoter contained therein. Indeed, it has been observed that thymidine kinase genes contain cryptic promoters which interfere with their expression in mammalian cells. In particular, the presence of cryptic promoters often leads to the expression of truncated versions of thymidine kinase, with lower or no biological activity, and potential side effects (in this respect, see references 19-20). A preferred nucleic acid of this invention is a nucleic acid encoding a EHV4-TK polypeptide, which nucleic acid is devoid of a cryptic promoter. Such a nucleic acid can be prepared by (i) identifying the cryptic promoter in the nucleic acid sequence and (ii) modifying the nucleic acid sequence, for instance by translationally neutral modifications and/or by deletion(s). The resulting nucleic acid can be tested for improved expression in mammalian cells, in particular in human cells, as disclosed in the examples. A particular nucleic acid of this invention comprises a deletion in the 5' region of a EHV4-TK gene, said deletion providing for an increased expression in mammalian cells.

Preferably, the nucleic acid encoding a EHV4-TK polypeptide of this invention is included in a nucleic acid construct comprising for instance a promoter region, located 5' of the coding sequence, and a termination signal located 3' thereof. Such a nucleic acid construct can be part of a vector, such as a plasmid vector, a viral vector (i.e., a recombinant virus), an artificial chromosome, an episome, etc.

In a preferred embodiment, the invention uses a vector comprising a nucleic acid encoding a EHV4-TK polypeptide as defined above. Even more preferably, such a vector is a plasmid or a viral vector, such as a retroviral vector, an adenoviral vector, an AAV, an herpesvirus vector or a vaccinia virus vector. In this regard, one particular embodiment of the invention uses a culture supernatant comprising a recombinant virus which contains a nucleic acid encoding a EHV4-TK polypeptide. Another particular way of providing a nucleic acid of this invention comprises the use of cells which

produce a recombinant virus containing a nucleic acid encoding a EHV4-TK polypeptide. In this regard, any composition comprising virus packaging cells can be used, in particular retrovirus or adenovirus packaging cells. The use of a viral supernatant or a virus packaging cell represents a preferred way of carrying out the present invention.

As indicated above, the invention resides in methods and compositions for rendering human cells sensitive to a nucleoside analog. The term nucleoside analog means any compound which, when phosphorylated, can be incorporated into an elongating DNA chain to block DNA replication and/or transcription. Preferably, a nucleoside analog of this invention is any compound that can be converted into a monophosphated nucleoside analog by a thymidine kinase, in particular by HSV-1 TK. Examples of such nucleoside analogs include for instance gancyclovir (GCV), acyclovir (ACV), bromovinyl-deoxyuridine (BVDU). These nucleoside analogs are efficiently converted to their monophosphated form by HSV-1 TK, and are then converted to triphosphorylated compounds by host cellular kinases. Incorporation of these compounds into elongating DNA blocks elongation leading to an apoptotic cell death (Balzarini, 1989 ; Furman, 1980 ; St Clair, 1987). Other examples of nucleoside analogs are all compounds derived from pyrimidine or purine nucleosides that can be phosphorylated by EHV4-TK (known antiviral drugs or synthetic products).

The invention thus provides alternative compositions and methods of rendering cells sensitive to nucleoside analogs. Sensitivity means that, in the presence of said nucleoside analogs, the treated cells will be destroyed or killed, generally by apoptosis. The invention therefore also provides an improved method of destruction of targeted cells. One advantage of the present invention resides in the unexpectedly high sensitivity of the treatment that can be obtained. Indeed, the inventors have now shown that when polypeptides or nucleic acid constructs of the instant invention are contacted with human cells, a very efficient destruction of said cells can be

obtained in the presence of a nucleoside analog. In particular, the results presented in the examples show that human cells expressing a EHV4-TK polypeptide are from 3 to 12 fold more sensitive to nucleoside analogs such as GCV than human cells expressing HSV1-TK. In other words, the invention allows to significantly reduce the doses of nucleoside analogs, e.g., GCV, which are used to destroy the target cells.

In this regard, another object of the present invention resides in the use of an EHV4-TK polypeptide or a nucleic acid coding therefor, for the manufacture of a composition for the destruction (e.g., killing) of human cells (in vitro, ex vivo or in vivo). The invention also lies in a method of killing human cells comprising contacting said human cells in vitro, in vivo or ex vivo with a EHV4-TK polypeptide or a nucleic acid coding therefor, and further contacting said cells or their progeny with a nucleoside analog.

The present invention can be used in vitro, in vivo or ex vivo. It can be used to kill determined cells, either for selection purposes, depletion purposes, experimental uses or therapeutic applications.

The present invention can be performed with various types of human cells, including naturally, pathologically, or induced diving cells. Naturally dividing cells are cells which divide regularly during their life cycle. Induced dividing cells are cells which would not divide naturally but can be stimulated to divide. A preferred therapeutic use of the instant invention lies in the treatment of proliferating human cells, more preferably abnormally proliferating human cells. A particular illustration is the treatment (i.e., the destruction and/or killing) of human tumor cells. In this regard, various solid tumor cells can be mentioned, such as, for instance, head and neck cancer, lung cancer (in particular non small cell lung cancer), glioblastoma, melanoma, breast cancer, liver cancer, ovarian cancer, prostate cancer, brain tumors and the like. Another example of human cells which can be treated according to this invention is represented by specific clones of T lymphocytes which proliferate abnormally, for instance clones of T

lymphocytes involved in autoimmune diseases or in graft versus host disease.

Accordingly, in a particular embodiment, the invention resides in the use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition for the killing of human tumor cells in vitro, ex vivo or in vivo. The present invention also lies in a method of killing a human tumor cell comprising contacting said human tumor cell in vitro, ex vivo or in vivo, with an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor, and further contacting said cells or their progeny with a nucleoside analog.

In another particular embodiment, the invention resides in the use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition for the killing of human T lymphocytes in vitro, ex vivo or in vivo. The present invention also lies in a method of killing a human T lymphocyte comprising contacting said human T lymphocyte in vitro, ex vivo or in vivo, with an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor, and further contacting said cells, or their progeny, with a nucleoside analog.

As explained above, the present invention can be performed in vitro, in vivo or ex vivo. For in vitro or ex vivo uses, the invention can be carried out according to the following procedure.

A population of cells to be treated (i.e., rendered sensitive to a nucleoside analog) is provided. The cells can be maintained in culture in any appropriate device (e.g., a plate, dish, flasc, bottle, etc.), preferably under sterile conditions, in any conventional culture medium (RPMI, DMEM, etc.). The cells are then contacted with the EHV4-TK polypeptide or nucleic acid as defined above. In this regard, the cells can be contacted by direct

incubation of the cells with the EHV4-TK polypeptide or nucleic acid. This incubation allows the EHV4-TK polypeptide or nucleic acid to be taken up by the cells, or at least a portion of the cells. This incubation can be performed in the presence of facilitating agents or conditions, which increase the uptake of the EHV4-TK polypeptide or nucleic acid by the cells. Such agents are for instance any cationic lipid, polymer, liposome, calcium phosphate precipitation, etc. The incubation can also comprise physical treatments such as electrical fields, gene gun, and the like. In a particular embodiment of this invention, a EHV4-TK nucleic acid is used, contained in a vector. In a more preferred embodiment, the nucleic acid is contained in a viral vector (or a recombinant virus produced therefrom). The use of a recombinant virus is particularly advantageous because of the natural infectious power of these particles. Preferred recombinant viruses include recombinant retroviruses, adenoviruses, AAV or herpesviruses, for instance. Methods of preparation of these vectors are known to the skilled artisan.

In a particular embodiment, the cells are thus rendered sensitive to a nucleoside analog by contacting said cells with a vector comprising an EHV4-TK nucleic acid, more preferably a recombinant virus comprising an EHV4-TK nucleic acid.

In this respect, the invention relates more particularly to a method of rendering a human cell sensitive to a nucleoside analog, in vitro, ex vivo or in vivo, said method comprising contacting the human cell with a vector comprising an EHV4-TK nucleic acid, more preferably a recombinant virus comprising an EHV4-TK nucleic acid. The cells which, upon the incubation, have incorporated the EHV4-TK nucleic acid are sensitive to a nucleoside analog, as well as other surrounding cells, due to the by-stander effect of TK.

Contacting the cells with a recombinant virus as disclosed above can be accomplished by incubation of the cells with a culture supernatant, or an

enriched solution or suspension, such as a purified solution, comprising the recombinant virus, or by coculture of the cells with producer cells, which produce the recombinant virus.

The contacting step can last for periods of time of up to 72 hours or more, depending on the incubation conditions. The cells can then be recovered according to any known methods. If desired, they can be subjected to a selection step in order to obtain a population of cells comprising essentially cells which actually contain the EHV4-TK polypeptide or nucleic acid. In this regard, depending on the incubation conditions and the cell population, the percentage of transfected cells can vary from 5 to almost 99%, before any selection step. The recovered cells can be washed and placed in fresh medium, or stored in any appropriate conditions (freezing, lyophilisation, etc.).

For killing the treated cells, the cells are then further contacted with a nucleoside analog, i.e., a compound that is converted by the treated cells into a toxic molecule (by incorporation into elongating DNA). For in vitro use, the cells can be contacted at any appropriate dosage of the nucleoside analog, in any suitable device. The treatment can be performed to screen for proliferating cells, eliminate specific cell populations (i.e., depletion), assess the activity of a promoter region, etc. For ex vivo indications, the nucleoside analog treatment can also be performed once the treated cells have been injected in a subject. In this embodiment, the dosages are essentially as discussed below for in vivo uses.

Indeed, for performing the invention in vivo, cells are contacted with the EHV4-TK polypeptide or nucleic acid by direct administration to a subject in need thereof. Administration can be performed by any appropriate route (intra arterial, intravenous, intra muscular, intra tumoral, cutaneous, etc.). For treatment of proliferating cells, in particular tumor cells, the EHV4-TK polypeptide or preferably, nucleic acid, is preferably administered by intravenous, intra arterial, or intra tumoral injections, more

preferably intra tumoral injection, according to known techniques. The dosage of EHV4-TK nucleic acid can be adjusted by the skilled artisan, based on the teaching of the prior art clinical trials with HSV1-TK gene.

In this respect, nucleic acids can be injected from 0,1 to 1000 µg/dose, for instance. With regard to recombinant virus, 10^3 - 10^9 pfu can be injected at dose. When packaging cells are being used, 10^4 - 10^9 cells are preferably injected.

Once the target cells have become sensitive to a nucleoside analog, they can be killed by further contact with a nucleoside analog.

In this regard, the nucleoside analog can be administered to the subject according to various protocols and different timeframes. Generally, the nucleoside analog is administered by perfusion, intra arterial, intravenous, or cutaneous routes. Conventional dosage for nucleoside analogs range from 1 to 50 mg/kg per day, in particular about 10 mg/kg, twice a day. The nucleoside analog can be GCV, ACV, BVDU, or any other compound as described above. Oral administration is also envisioned. For treatment of tumor cells for instance, the nucleoside analog is usually administered shortly after the EHV4-TK polypeptide or nucleic acid, and for a period of time comprised between 1 to 15 days. For other cells, such as specific T cell clones, the nucleoside analog is administered only when these particular targeted T cell clones proliferate, for instance (i.e., GVHD, auto immune diseases, etc.)

The invention also relates to a pharmaceutical composition for the treatment of human cell proliferative disorders comprising an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor and a pharmaceutically acceptable excipient. In a preferred embodiment, the pharmaceutical composition comprises a vector comprising the above nucleic acid, such as a viral vector as defined above. In another embodiment, the pharmaceutical composition comprises a

recombinant virus producing cell, such as a retrovirus producing cell, for instance.

Preferably, the pharmaceutical composition of the instant invention further comprises a nucleoside analog, to be administered simultaneously or separately.

The invention also relates to a composition comprising human cells containing an EHV4-TK nucleic acid as defined above.

Other aspects and advantages of the present invention will be presented in the following experimental section, which should be regarded as illustrative and not limiting the scope of the invention.

LEGEND TO THE FIGURES

Figure 1: Map of the expression vectors.

(A) Plasmids as described in Cazaux et al. harbor the cDNA encoding an herpesvirus TK (HSV1, EHV4, EBV or VZV) fused in frame with the *She ble* gene conferring resistance to zeocin. (B) Map of the vector coding for the fusion protein EHV4-TK/E-GFP. Underlined sequences correspond to the 5' to 3' arm between the thymidine kinase and the Zeocin resistance protein or the E-GFP.

Figure 2: Analysis of fusion TK/ZEO protein expression in transfected cells.

(A) Western blot analysis showing HSV1- and EHV4-TK protein levels in tk(-) 3T3 stable clones. (B) Dot blot analysis of TK expression in transfected and non-transfected tk(-) 143B cells. Fusion proteins were revealed with purified rabbit anti-Sh polyclonal antibody.

Figure 3: Cytotoxic activity of ACV, GCV and BVDU against human tk(-) 143B cells transfected with HSV1- or EHV4-TK. Toxicity is presented as mean of IC50 values ± SD. The means, determined on 3 independent experiments, are indicated on the top of each histogram.

Figure 4: Thymidine and GCV phosphorylation in transfected and non-transfected tk(-) 143B cells.

Cells were incubated with radiolabeled thymidine or GCV, and the different phosphorylated forms were separated by FPLC analysis. The percentage of thy-MP, thy-DP and thy-TP were respectively 29.3, 27.5 and 39.2% in HSV1-TK expressing cells and 36.8, 27.5 and 28.8% in EHV4-TK expressing cells. The percentage of GCV-MP, GCV-DP and GCV-TP were respectively 15.5, 36.5 and 45% in HSV1-TK expressing cells and 23.9, 29.3 and 45% in EHV4-TK expressing cells.

Figure 5: Analysis of the dual functionality of the EHV4-TK/E-GFP fusion protein.

(A) E-GFP expression in tk(-) 3T3 transfected and non-transfected cells. EHV4-TK gene fused with E-GFP gene (Figure 1-B) was transfected in tk(-) 3T3 cells, and E-GFP positive cells were detected by FACS analysis. Expression of E-GFP was determined a week after transfection (1) and after 2 months of culture in HAT medium (2). (B) GCV toxicity in transfected and non-transfected cells. Results were expressed as % of viable cells compared to cells grown without GCV.

MATERIALS AND METHODS

Chemicals

Acyclovir (ACV, [9-(2-hydroxyethoxymethyl)guanine] Zovirax®), Ganciclovir (GCV, [9-(1,3-dihydroxy-2-propoxymethyl)guanine] Cymevan®), Bromovinyldeoxyuridine, (BVDU (E)-5-(2-bromovinyl)-2'-deoxyuridine), were

purchase from Wellcome (Issy les-Moulineaux, France), Syntex (Palo Alto, CA, USA) and Sigma Chemical Co. (St Louis, MO, USA), respectively.

Cell lines and Plasmids

Murine NIH 3T3 cells lacking an endogenous TK (tk(-) 3T3) activity were obtained from the American Type Culture Collection (Rockville, MD, USA) as well as human tk(-) 143B (ATCC CRL 8303) and HCT116 (ATCC CCL 247) cells; murine pancreatic tumor cell line, PANC-O2, was a gift from Rhone-Poulenc (France). All cells were grown in Dubelcco's modified Eagle's medium (DMEM) supplemented with 1% L-glutamine (GibcoBRL), 1% penicillin, streptomycin and neomycin mixture (GibcoBRL) and 10% new born calf serum (GibcoBRL) for NIH 3T3 cells or 10% fetal calf serum (GibcoBRL) for other cell lines.

Expression vectors harboring the cDNA encoding herpesvirus TK (HSV1, EHV4, EBV or VZV) fused in frame with the *Sh Ble* gene conferring resistance to zeocin, have been previously described by Cazaux et al.[21] The fused genes are under the transcriptional control of the HSV1-TK viral promoter and the viral polyoma pYF441 enhancer (Figure 1-A).

Plasmid containing HSV1-TK gene fused in frame with the E-GFP gene (Figure 1-B) was constructed from plasmid described by PCR replacement of the *Sh Ble* gene with the E-GFP sequence (Clontech, Palo Alto, California, USA).

DNA Transfection:

Cells were transfected by the various plasmids using a calcium phosphate precipitation kit (GibcoBRL). After 48 h of phenotypic expression, cells were grown in selective medium with zeocin (Cayla, Toulouse, France): 25 µg/ml (3T3 cells), 50 µg/ml (143 B cells) or 100 µg/ml (PANC-O2 and HCT 116 cells).

Transfection was performed in 2 petri-dishes (Ø 100mm). Independent clones were picked-up and individually expanded, or were left in culture to obtain a cell bulk.

Cytotoxicity assay:

ACV, GCV and BVDU toxicity were quantitatively assessed by measuring inhibition of cellular proliferation. Cells were seeded at $2.5x10^3$ per wells in 24-wells plates. 24 h later they were grown in drug-containing medium for 7 days. Drug concentrations ranging from 0.001 nM to 100 µM were tested in triplicate. The medium was changed with fresh DMEM every 2 days. On day 6, 1.5 µCi of [methyl-^{3}H]thymidine (Amersham, UK) was added per well. After washes with phosphate-buffered saline (PBS pH 7.3), incorporated radioactivity was determined with a Micro-Beta Plus counter (EG&G Instruments and Wallac, Turku, Finland). Cytotoxic doses were expressed as IC50 values (the drug concentration required to reduce the [methyl-^{3}H]thymidine incorporation by 50%), and selectivity indexes were calculated as the IC50 ratios of parental to transfected cells.

Viability assay:

Parental and viral-TK expressing cells were plated at $5x10^3$ per wells in 6-wells dishes. After 24 h, drugs was added to the medium at various concentrations ranging from 0.01 to 100 µM. Medium containing drugs was changed every 2 days. After 7 days of culture, cell viability was assayed by counting viable cells by trypan blue exclusion after 7 days of culture with GCV.

Western Blot analysis:

Cells were washed three times in ice-cold PBS, and 10^6 transfected cells were incubated in lysis buffer [37] Lysates from $1x10^5$ cells were loaded on each line on a 10-12% trycine-SDS polyacrylamide gel electrophoresis (Novex, San Diego, CA, USA), and separated proteins were transferred

onto nitro-cellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting. To ensure equivalent protein loading in each line and correct transfer, bands were made visible by staining with 0.3% Ponceau Rouge staining. Zeo-fusion proteins were revealed with purified rabbit anti-Zeo polyclonal antibodies (Cayla, Toulouse, France) followed by incubation with antibody to rabbit IgG, conjugated to horseradish peroxydase. The reaction was visualized using an BM chemiluminescence detection method (Boehringer Mannheim, France SA).

Dot Blot analysis:

Successive dilutions of cellular extracts were dropped on nitro-cellulose membrane. Zeo-fusion proteins were revealed with rabbit anti-Zeo polyclonal antibodies (Cayla, Toulouse, France) followed by incubation with antibody to rabbit IgG conjugated to horseradish peroxydase (Boehringer Mannheim, France SA). The reaction was visualized as described in Western Blot analysis.

Nucleotide analysis by fast protein liquid chromatography(FPLC):

$3x10^6$ cells were incubated for 7 hours with 1 µM of [8-^{3}H] GCV (3 Ci/mmol, Isotopchim, France) or [methyl-^{3}H] Thymidine (5 Ci/mmol, Amersham, UK) in a final volume of 5 ml of medium. Cells were detached by trypsin treatment, and washed twice with PBS. The nucleotides were extracted with 1 ml of ice cold methanol 60% over the night at -20°C. The samples were analyzed by fast liquid chromatography (FPLC, Pharmacia-LKB Instruments, Sweden) on a anion-exchange column.[38,39] Radioactivity was counted at the column exit with a radiamatic Flo-one Beta A-500 apparatus (Packard, Meriden, CT), and peaks were analyzed with Flo-one/Data software.

Flow cytometric analysis:

tk(-) 3T3 cells were transfected with plasmid encoding the EHV4-TK/E-GFP fusion protein, and selected in HAT medium. After 1 week of culture, fluorescence of transfected cells were analyzed. The fluorescence analysis was repeated 2 months after selection. Cells were washed twice with PBS, and intensity of fluorescence was directly determined on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) with a 525 nm filter and analyzed with CellQuest software.

RESULTS

Nucleoside analogs toxicity on cell bulks expressing different herpesviruses thymidine kinases

We used expression vectors with herpesvirus TK genes fused in frame with the *Sh Ble* gene conferring resistance to zeocin (pUT-TKx) (Figure 1A).[21] The use of such vectors facilitates the selection of transduced cells and the detection of the chimeric protein by immunodetection with anti-*Sh* polyclonal antibodies. The functionality of the viral TK expressed by these vectors has been previously demonstrated in bacteria and yeast lacking endogenous TK or thymidylate kinase activities.[21] We first expressed these genes in murine fibroblasts deficient in cellular TK (tk(-) 3T3 cells) and selected the stable transfected cells based on their resistance to zeocin. The functionality of the viral TK in cells was then tested by analyzing cell growth in selective HAT medium and by [methyl-^{3}H]thymidine (^{3}HT) incorporation assays. As expected, while all untransfected cells died in HAT, all transfectants survived and incorporated ^{3}HT (data not shown).

Selected bulk populations were then analyzed for their *in vitro* sensitivity to nucleoside analogs by measuring inhibition of cell proliferation

in comparison with NIH-3T3 parental cells (Table 1). ACV IC50 values were approximately fifteen fold lower in HSV1-, EHV4- and VZV-TK expressing cells compared to the parental NIH-3T3 cells, and only four fold lower for EBV-TK expressing cells. HSV1- and EHV4-TK expressing cells were 350 and 700 fold more sensitive to GCV than NIH-3T3 cells, compared to only 7 fold for VZV- or EBV-TK expressing cells. Unlike ACV and GCV, IC50 values for BVDU were in the same range for all herpesvirus-TK expressing cell bulks, about 1300 to 3600 fold lower than for NIH-3T3 cells.

These observations were extended using an additional murine tumor cell line, the pancreatic tumor PANC-O2 cells. Again, it was found that IC50 values were 3 fold lower for EHV4-TK than for HSV1-TK expressing cell bulk, 0.55 ± 0.07 and 1.7 ± 0.5 µM respectively (means of two independent experiments, data not shown).

Table 1: Cytotoxicity of ACV, GCV and BVDU on bulk transfected and non transfected murine tk(-) 3T3 cells. Drug toxicity was assessed by measuring inhibition of cell proliferation. Cells were grown in drug-containing medium for 7 days. [methyl-^{3}H]-thymidine was added at day 6 and incorporated radioactivity was measured. Cytotoxic doses were expressed as mean IC50 values (50% inhibitory concentration required to reduce the [methyl-^{3}H]-thymidine incorporation by 50%) of 2 or 3 experiments and selectivity indexes were calculated as the IC50 ratios of parental to transfected cells.

Table 1 Cytotoxic activity (IC50) of ACV, GCV and BVDU against murine tk(-) 3T3 transfected and non-transfected cells

	ACV					GCV					BVDU				
	IC50 (μM	s.d	n	electivity Index		IC50 (μM	s.d	n	electivity Index		IC50 (μM	s.d	n	electivity Index	
NIH-3T3	165	49.5	2			70	14.1	2			55	7.1	2		
HSV1-TK	8.7	5.1	3	19		0.2	0.11	3			0.015	0.007	2	5500	
EHV4-TK	11.1	16.4	3	15		0.1	0.02	3			0.02	0.03	2	2750	
VZV-TK	11	5.7	3	15		8	1.4	2			0.016	0.02	2	2750	
EBV-TK	43.3	5.8	3	4		6	3.6	3			0.04	0.05	3	1375	

s.d: standard deviation to the mean

n: number of experiments

TABLE 1

Nucleoside analog toxicity on HSV1-TK or EHV4-TK expressing cell clones.

Among the four herpesviruses TK tested for their capacity to sensitize cells to nucleoside analogs toxicity, HSV1- and EHV4-TK demonstrated the more interesting selectivity indexes. Because cell bulks represent a mixture of clones with different sensibilities, we next analyzed the drug cytotoxicity on individual clones expressing either of these two TK.

We first analyzed the herpesvirus-TK protein expression level for several clones by western-blot using polyclonal antibodies against *Sh* protein. Cellular extract of 10^5 cells were loaded to electophoresis. Bands corresponding to EHV4-TK/ZEO (53 KDa), and to HSV1-TK/ZEO (55 KDa) were observed with similar intensity in the four EHV4-TK+ and four HSV1-TK+ clones tested (Figure 2-A). There was no detectable band corresponding to Sh protein alone (20Kda) in these cell extracts (data not shown). A weaker band of lower PM could also be observed with all extracts of HSV1-TK(+) cells. This band corresponds to a transcription initiation at a known cryptic internal promoter resulting in the translation of a truncated but functional protein.[19-20,22]

We then analyzed the toxicity of ACV, GCV and BVDU, on these eight clones (Table 2). For ACV and GCV, the sensitivity of EHV4-TK expressing cells was statistically more important than the one of HSV1-TK expressing cells ($p < 0.05$ and 0.01 respectively). HSV1- or EHV4-TK expressing clones displayed the same BVDU sensitivity.

Table 2: Cytotoxicity of ACV, GCV and BVDU on HSV1-TK and EHV4-TK expressing murine tk(-) 3T3 clones. Drug sensitivity of TK expressing clones were tested as described in Table 1. Cytotoxic doses were expressed as mean IC50 values of 3 experiments.

Table 2 Selectivity indexes of the cytotoxic activity (IC50) of ACV, GCV and BVDU against murine tk(-) 3T3 HSV1- and EHV4-TK expressing clones

	ACV			GCV			BVDU		
	IC50 (μM)	SD	electivity index	IC50 (μM	SD	electivity index	IC50 (μM	SD	electivity index
NIH-3T3	53.3	4.4		36.7	15.5		43.3	11,0	
HSV1-TK	15 (1)	7,0	4	0.16 (1)	0.06	230	0.013 (1	0.01	3331
EHV4-TK	7.3 (1)	2.9	7	0.06 (1)	0.03	612	.035 (1)	0.03	1237

SD: standard deviation to the mean

(1) means values were different from parental values with the Mann-Whitney U test

* means were different from HSV1-TK cells values with the Mann-Whitney U test

** means values were not different from HSV1-TK cells values with the mann-Whitney U test

TABLE 2

Nucleoside analog toxicity on human cell bulk expressing HSV1-TK or EHV4-TK.

Since nucleoside analog's metabolism might be different in cells of different species [23-25] we thus analyzed the efficiency of the system for killing human cells.

Human tk(-) osteosarcoma 143B cell bulks expressing HSV1- or EHV4-TK were thus generated. We first analyzed the herpesvirus-TK protein expression levels for each cell bulks by dot-blot using polyclonal antibodies against *Sh* protein (Figure 2-B). There was no detectable band corresponding to fused protein in tk(-) 143B cell extracts. Similar expression of the fused protein was observed in HSV1 or EHV4-TK expressing cells.

We then, studied cell sensitivity to ACV, GCV and BVDU (Figure 3). The toxicity of ACV was similar in HSV1- and EHV4-TK expressing cells. Noteworthy, EHV4-TK expressing cells were approximately twelve fold more sensitive to GCV than HSV1-TK expressing cells. On the contrary the toxicity of BVDU was greater in HSV1-TK than in EHV4-TK expressing cells (Figure 3).

These observations were extended to an additional tumor cell line, human colonic carcinoma HCT116 cells. IC50 values for GCV were 3 fold lower for EHV4-TK than for HSV1-TK expressing cells, 0.11 ± 0.02 and 0.32 ± 0.08 µM respectively (means ± SD for 3 independent experiments). EHV4- and HSV1-TK expressing cells were 485 and 170 fold more sensitive to GCV than HCT116 cells (mean of IC50 values for GCV was 53.4 ± 15 µM).

GCV phosphorylation in human cells expressing HSV1- or EHV4-TK.

In order to understand why GCV is more toxic in EHV4-TK compared with HSV1-TK expressing cells, we analyzed the GCV phosphorylation in tk(-) 143B and HSV1- or EHV4-TK expressing cells. After 7 hours of

incubation with radiolabeled GCV or thymidine, nucleotides were extracted and analyzed by FPLC.

While there was no detectable phosphorylation of thymidine in tk(-) 143B cells (data not shown), thymidine was similarly phosphorylated in both TK expressing cells (Figure 4). For GCV, the proportions of the different phosphorylated forms were the same in both TK cells, while the total amount of intracellular GCV was 5 fold higher for EHV4 than for HSV1-TK expressing cells.

Functionality of an EHV4-TK/E-GFP fusion protein.

To facilitate the transgene detection in transfected cells or tissues, the E-GFP gene was fused in frame at the 3' end of the EHV4-TK gene and cloned into an expression vector (Figure 1-B). This construction was then transfected into tk(-) 3T3 cells, followed by FACS analysis of E-GFP positive cells. A cell bulk containing 95% of E-GFP expressing cells was obtained. The stable expression and the dual functionality of the transgene could be demonstrated by analyzing fluorescence and survival in presence of HAT medium during 2 months of culture (Figure 5-A). Expression of the EHV4-TK/E-GFP fusion protein efficiently sensitized these cells to GCV for which the IC50 was 0.6 µM, compared to 30 µM for tk(-) 3T3 cells (Figure 5-B).

DISCUSSION

We aimed to improve the efficacy of current herpes virus derived TK systems for cell destruction. For that purpose, we chose to assess the efficacy of alternative TK genes in human cells. Since we needed to monitor the expression level of these genes, they were fused to *sh ble*, encoding a protein conferring resistance to zeocin, which can be detected by western blotting with available polyclonal antibodies.

We first verified the dual functionality of the different TK-ZEO fusions in eukaryotic cells by transfection of murine or human cells deficient for endogenous thymidine kinase activity. Selection with zeocin always generated resistant clones that all survived when later cultured in the presence of HAT media. Therefore we concluded that even if peptidic fusions were not cleaved into the cells, all the fusion proteins were bi-functional. This is in agreement with previous experiments showing that HSV1-TK retain its functionality after amino- or carboxy-terminal fusion to various proteins.[21, 24, 26-28]

Significant differences in the activity of the different herpes TK were observed. These differences were found both when analyzing cell bulks as well as independent clones. The bulk provides a better estimate of the overall enzyme activity in a polyclonal population, that mimics the *in situ* transfected population during pre-clinical and clinical setting, while the clones allow to analyze the activity in individual cells with respect to transgene expression levels. We studied the toxicity of ACV, GCV and BVDU.

VZV- and EBV-TK did not demonstrate any advantages over HSV1-TK, whatever the nucleoside analog used. Two recent studies showed the potential of VZV-TK as a suicide gene in human breast cancer cells[29] and in human osteosarcoma cells.[30] In these articles selectivity indexes and IC50 values for BVDU ranged from 0.06 to 0.6 µM, quite similar to our values, but no comparison between VZV and HSV1-TK was shown.

Noteworthy, EHV4-TK expressing cells were always more sensitive to GCV than HSV1-TK expressing ones, whether as bulks or clones. This enhanced toxicity was not due to a difference in TK expression levels as assessed by western blot. This is also further indicated by the observation of an opposite effect with BVDU that is more toxic for HSV1-TK expressing

cells, demonstrating a qualitative rather than a quantitative difference between the activity of these enzymes.

To investigate the possible mechanism explaining the improved sensitivity to GCV in EHV4-TK expressing cells compared to HSV1-TK expressing cells, we analyzed GCV phosphorylation by FPLC. The total amount of intracellular GCV was 5 fold higher in EHV4-TK than in HSV1-TK expressing cells, while proportions of the different phosphorylated forms were similar in both TK cells. Such a difference might be explained by the uptake of nucleoside and their analogs that has been reported to be correlated with TK expression. Indeed, the uptake of thymidine into tk(-) *Escherichia coli* is proportional to the amount of TK activity expressed from the heterologous HSV1-TK gene.[31] Furthermore, mammalian HSV1-TK expressing cells showed higher GCV uptake and phosphorylation than control cells.[32] As we detected similar TK expression in both TK(+) cells (Figure 2-B), the greater amount of intracellular GCV in EHV4-TK expressing cells could be correlated to a greater EHV4-TK activity towards GCV compared to HSV1-TK activity.

Gene transfer detection could be greatly facilitated by markers that allow rapid and efficient detection of successfully transduced cells. This specially applies to cytoplasmic proteins. However, no suitable anti-EHV4-TK monoclonal or polyclonal antibodies are available. We thus used the Enhanced-Green-Fluorescent Protein (E-GFP) gene to generate a EHV4-TK/E-GFP fusion protein. Expression of E-GFP remains stable for months in transfected cells and can be easily monitored by flow cytometry. Transfection of the fusion plasmid to mammalian cells revealed that the construct is functional, cells being both fluorescent (95%) and sensitive to GCV. This result is in line with the recent study of Liomas *et al* who reported the dual functionality of a HSV1-TK-green fluorescent protein fusion gene.[28] This chimaeric suicide/marker gene should dramatically facilitate

the detection of transduced cells. This would be particularly important in experimental models. In this regard, suicide genes are frequently used in transgenic mice to generate models of conditional ablation of specific cell populations.

Depending on the cell lines, EHV4-TK was three to twelve fold more potent than HSV1-TK. Such a difference appears significant when contemplating the clinical use of suicide genes since the treatment of experimental tumors in mice or rats demonstrate that the GCV dosage is often critical for obtaining efficacy. When administered at 10 mg/kg twice a day, the usual dosage in humans, the GCV plasmatic concentration range between 0.44 to 2.2 µg/ml,[33] and the cerebral spinal fluid (CSF) concentrations are approximately 3 fold lower.[34, 35] It is therefore possible that CSF GCV concentrations are sub-optimal for the treatment of brain tumors transduced with HSV1-TK. In any case, a better efficiency of the enzyme should lead to a better treatment efficiency and/or a decrease of the GCV dose used. This later point is also significant since GCV toxicity in human appears cumulative depending on the overall amounts administered (Cytovene® product monograph).[36]

Altogether, these experiments underline the potential advantages of the EHV4-TK as a suicide gene, for both experimental, and therapeutical approaches, in vitro, ex vivo or in vivo.

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CLAIMS

1. The use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition to render human cells sensitive to a nucleoside analog in vitro, ex vivo or in vivo.

2. The use of an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor for the manufacture of a composition for the killing of human proliferative cells, in particular human tumor cells in vitro, ex vivo or in vivo.

3. The use of an EHV4-TK polypeptide or a nucleic acid coding therefor, for the manufacture of a composition for the killing of human T lymphocytes in vitro, ex vivo or in vivo.

4. A pharmaceutical composition for the treatment of human cell proliferative diseases comprising an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide or a nucleic acid coding therefor.

5. The pharmaceutical composition of claim 4 further comprising a nucleoside analog.

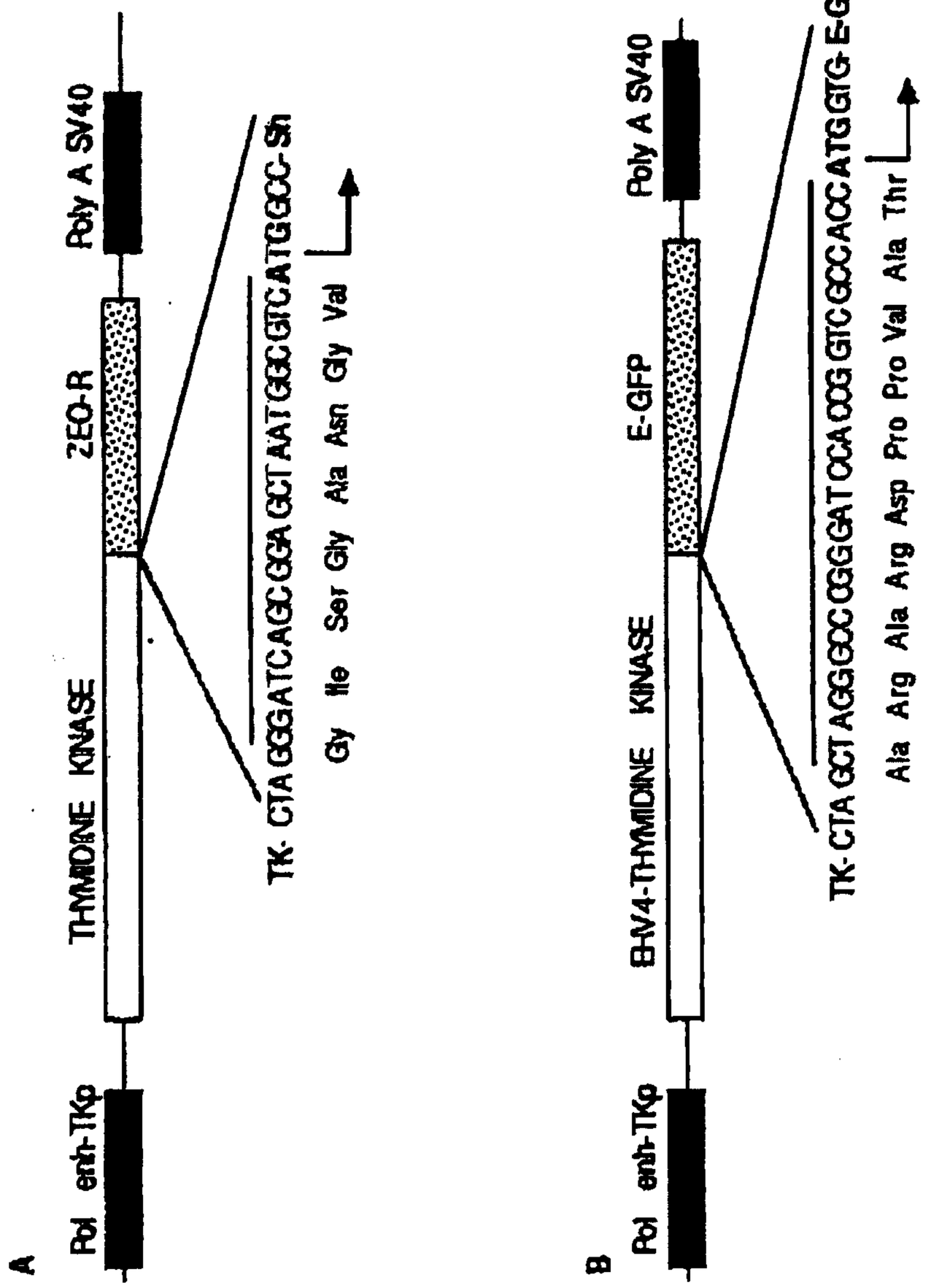
6. The use of claim 1 or 2, wherein said Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide comprises wild-type EHV4-TK or a fragment or variant thereof.

7. The use of claim 1 or 2, wherein said nucleic acid encoding an Equine herpes virus type-4 (EHV4) thymidine kinase polypeptide comprises a nucleic acid encoding wild-type EHV4-TK or a nucleic acid which hybridizes therewith and codes for a biologically active EHV4-TK polypeptide.

8. The use according to any one of claims 1, 2, 6, 7, wherein said nucleic acid is contained in a vector.

9. The use according to claim 8, wherein said vector is a recombinant virus.

10. The use according to any one of claims 1, 2, 6-9, wherein said nucleoside analog is GCV, ACV or BVDU, preferably GCV.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THYMIDINE KINASE
2EO-R
Poly A SV40
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Gy Ile Ser Gly Ala Asn Gly Val
B
Rol enh-TKp
ENV4-THYMIDINE KINASE
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FIGURE 1

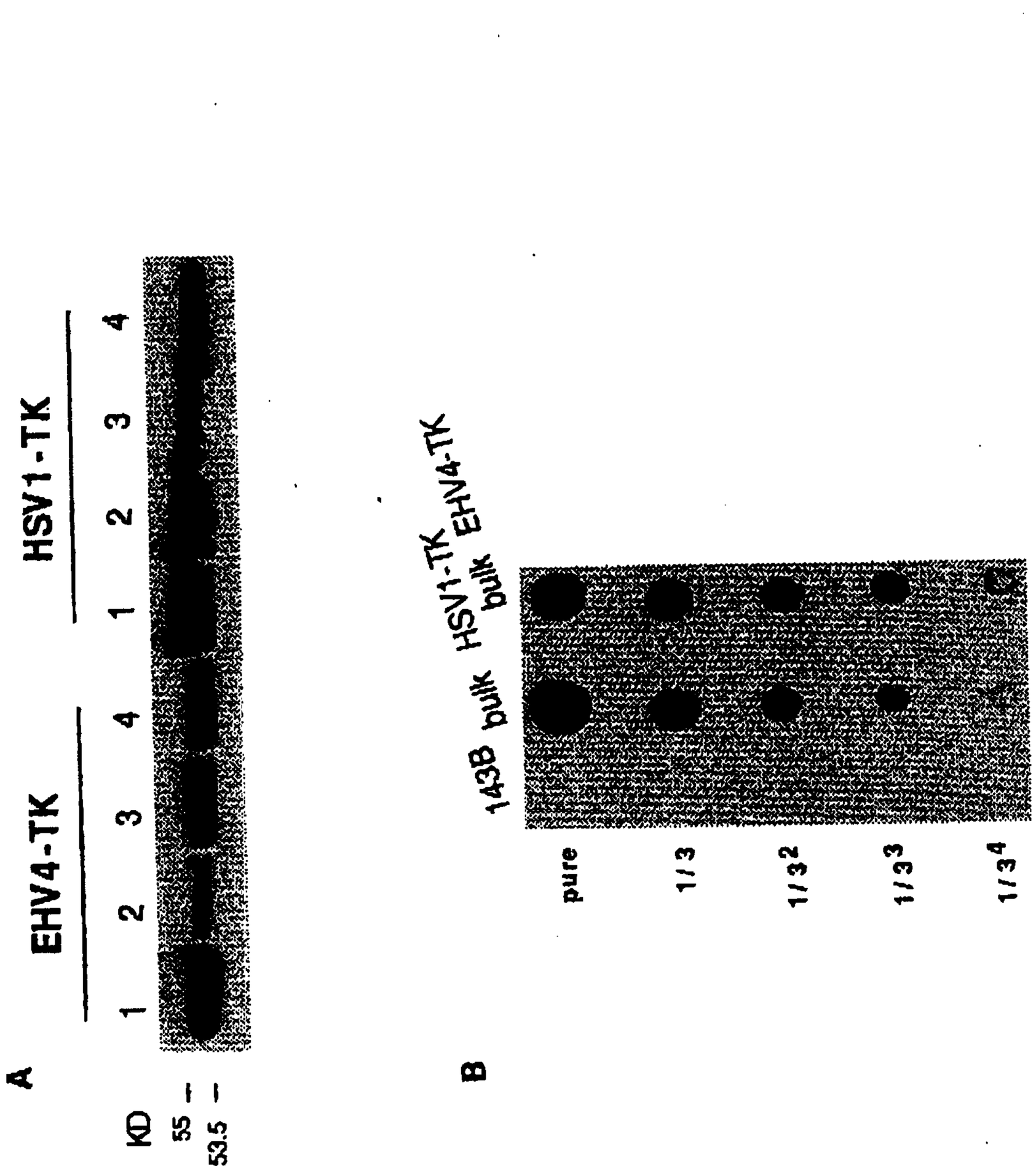

A
EHV4-TK
1
2
3
4
HSV1-TK
1
2
3
4
KD
55
53.5
B
143B bulk HSV1-TK EHV4-TK bulk bulk
pure
1/3
1/3²
1/3³
1/3⁴

FIGURE 2

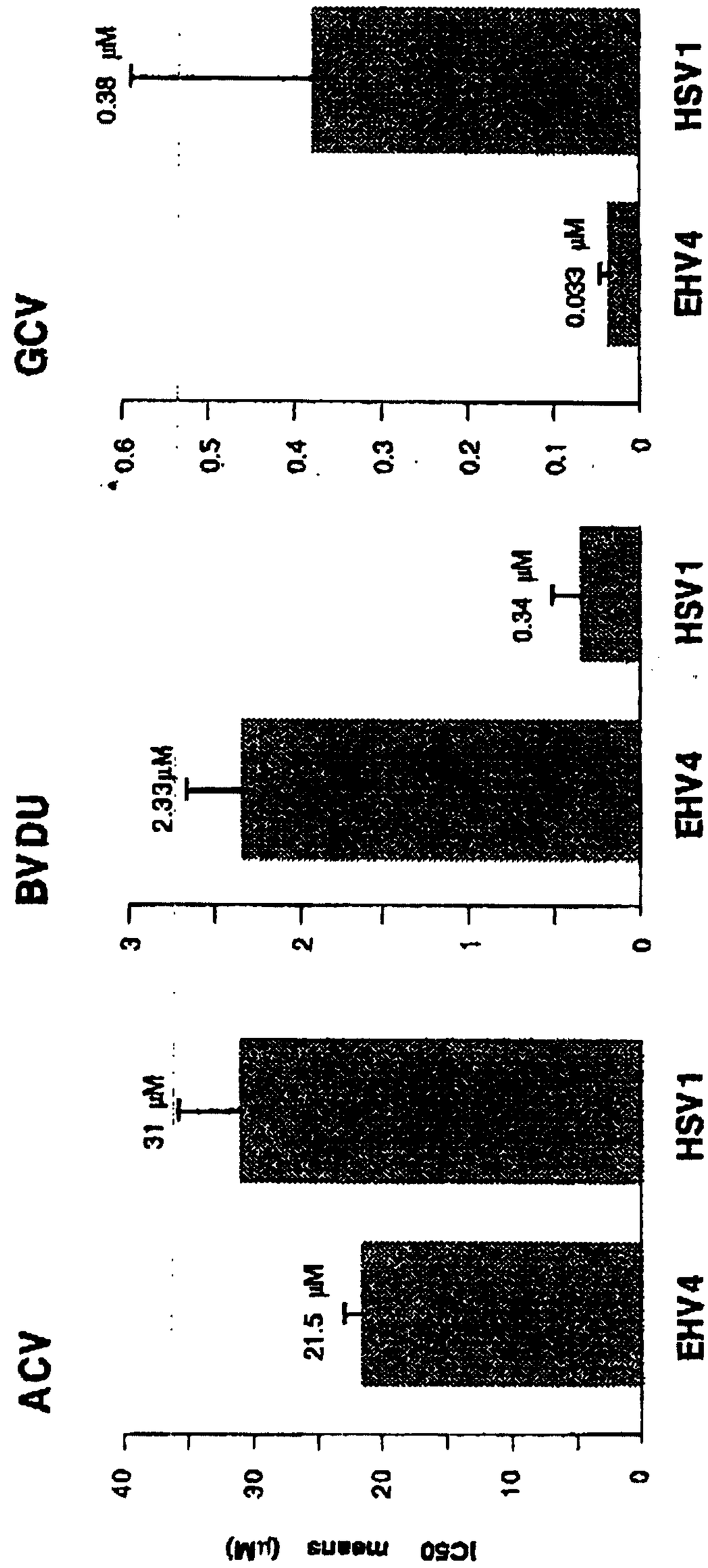

GCV
0.38 µM
HSV1
0.033 µM
EHV4
0.6
0.5
0.4
0.3
0.2
0.1
0
BVDU
0.34 µM
HSV1
2.33µM
EHV4
3
2
1
0
ACV
31 µM
HSV1
21.5 µM
EHV4
40
30
20
10
0
IC50 means (µM)

FIGURE 3

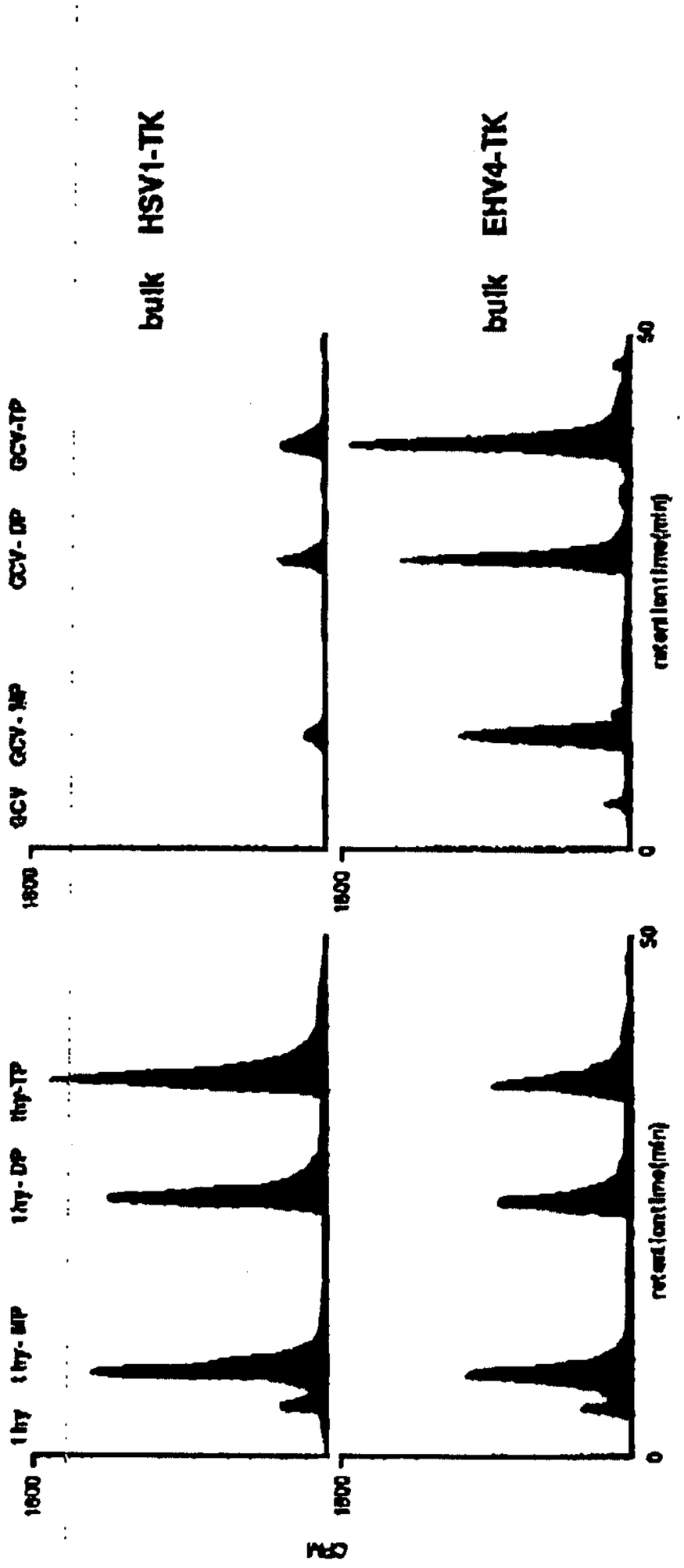
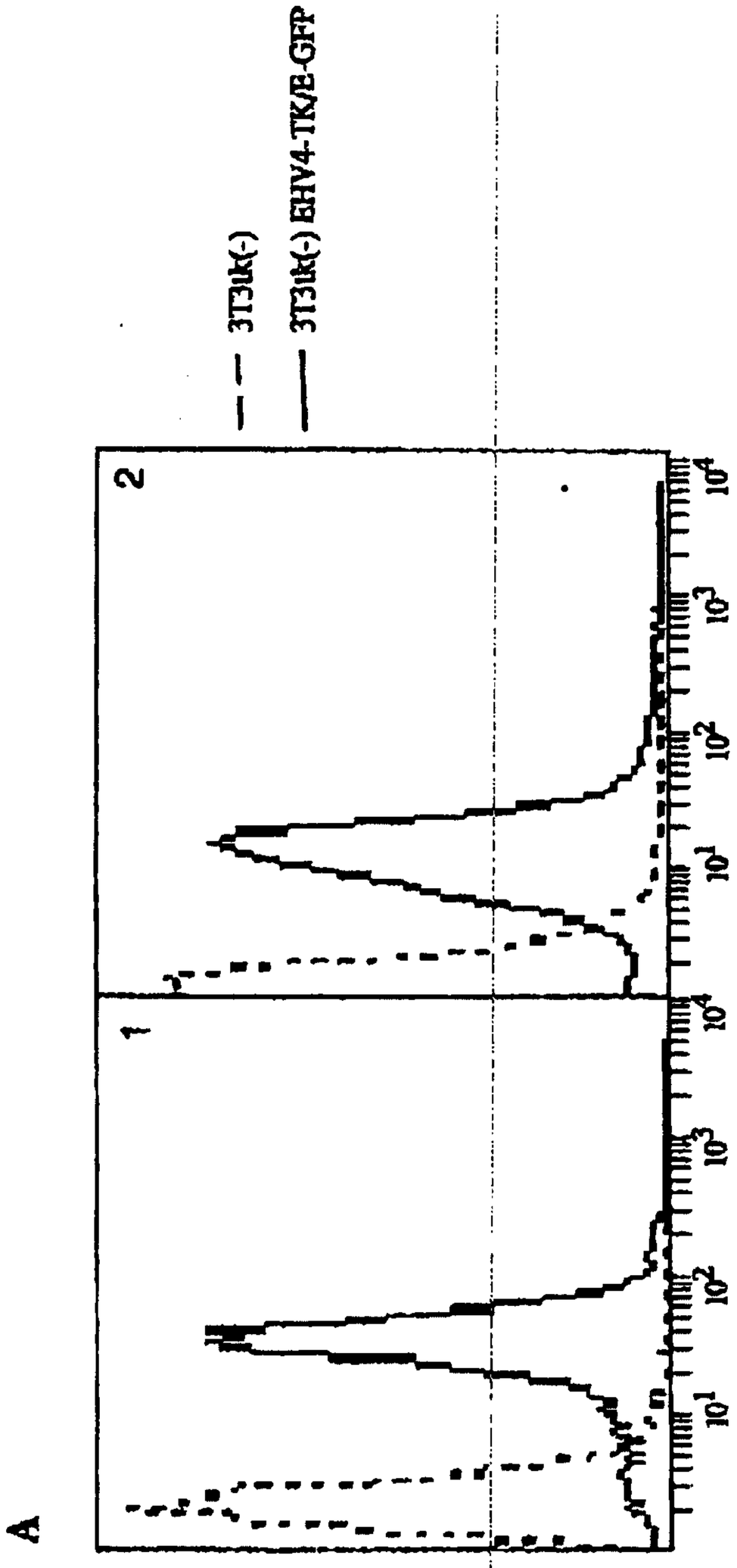

bulk HSV1-TK
bulk EHV4-TK
GCV
GCV-MP
GCV-DP
GCV-TP
thy
thy-MP
thy-DP
thy-TP
1800
0
50
retention time(min)

FIGURE 4


A
1
2
3T3tk(-)
3T3tk(-) EHV4-TK/E-GFP
10^{-1}
1
10^{1}
10^{2}
10^{3}
10^{4}

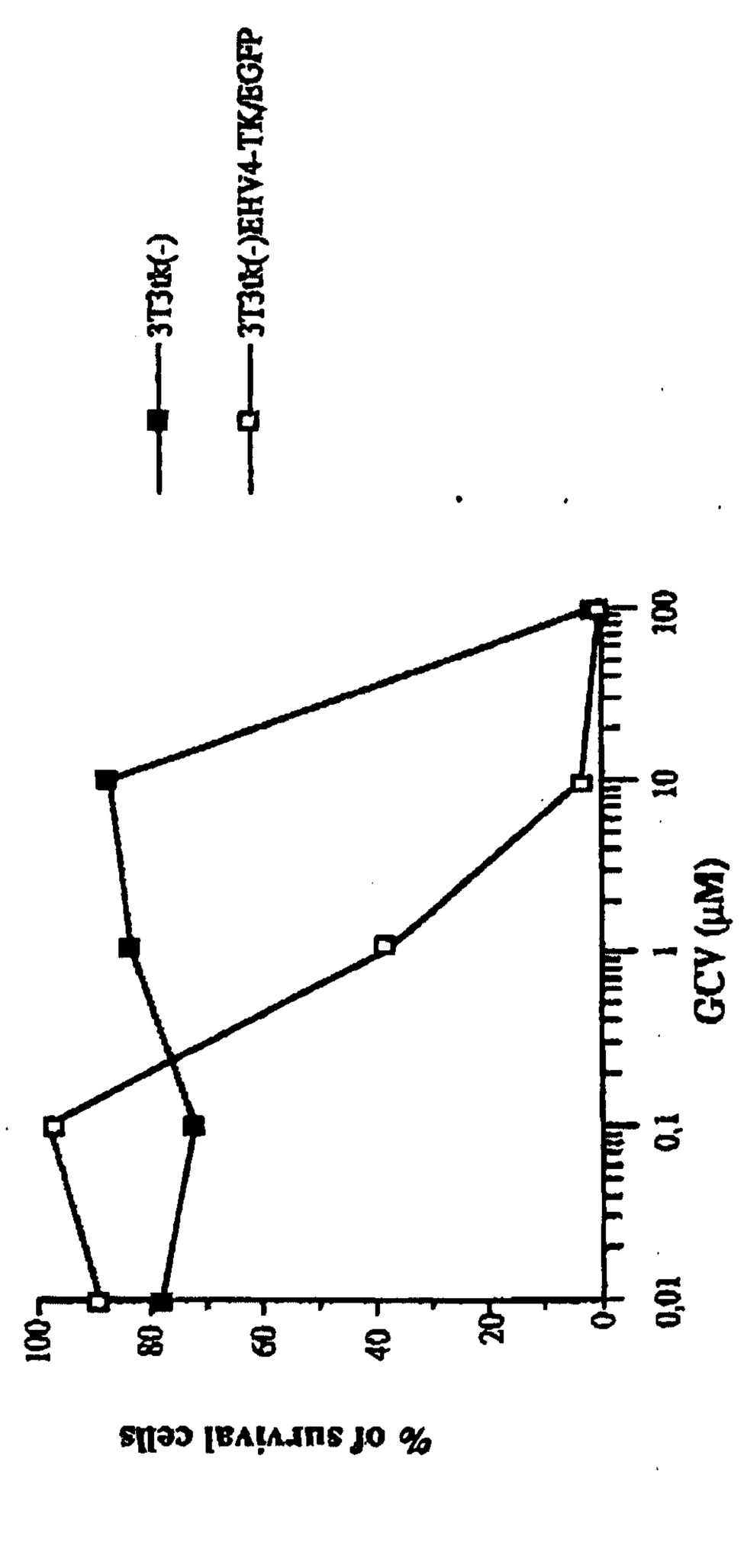

B
3T3tk(-)
3T3tk(-)EHV4-TK/EGFP
% of survival cells
100
80
60
40
20
0
0,01
0,1
1
10
100
GCV (µM)

FIGURE 5