Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 195-201

A CYTOTOXIC EFFECT ASSOCIATED WITH 9-(1,3-DIHYDROXY-2-PROPOXYMETHYL)-GUANINE IS OBSERVED DURING THE SELECTION FOR DRUG RESISTANT HUMAN CELLS CONTAINING A SINGLE HERPESVIRUS THYMIDINE KINASE GENE

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Received October 17, 1985

SUMMARY: A cytotoxic effect associated with 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG) was discovered while searching for spontaneous mutations in a single copy, integrated HSV-1 thymidine kinase (TK) gene in the human 143 TK^- cell line. It was found that spontaneous $DHPG^R$ mutations could not be selected while other anti-TK drugs resulted in selectable mutation frequencies of 10^{-4} to 10^{-3}. When 143 TK^- cells were mixed with these HSV-1 TK^+ cells and subjected to DHPG, a 90% to 100% decrease in recoverable TK^- colonies was observed. In addition, the media from the HSV-1 TK^+ cells metabolizing DHPG was shown to inhibit the growth of the TK^- cells.

The recent approach to developing a suitable treatment for Herpesvirus infections is the use of nucleoside analogs which are selectively used by the Herpesvirus thymidine kinase and DNA polymerase (1,2,3,4). The standard method to test the activity of these compounds is to measure the inhibition of viral plaque formation after 24 to 48 hours of exposure to the analog (5,6,7). For cytotoxicity, uninfected cells are exposed to the analog for a period of 24 to 72 hours and the inhibition of division is measured (5,6,7). These methods demonstrate very well the selective action of these nucleoside analogs. However, they can not examine the long-term effects of these compounds and their metabolites on uninfected cells proximal to infected cells. We have developed an *in vitro* system which can be used to study the effects of these antiviral compounds on thymidine kinase deficient (TK^-) cells growing adjacent to cells

Abbreviations: Acyclovir (ACV), 9-[2-hydroxy-1-(hydroxymethyl)-ethoxymethyl]-guanine; DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; Trifluorothymidine (TFT), 5-trifluromethyl-2'-deoxyuridine; DMEM, Dulbecco's Modified Eagles Medium; HAT, Hypoxanthine Amethopterin Thymidine; TK, Thymidine Kinase.

0006-291X/85 $1.50

containing a single integrated copy of the HSV-1 **TK** gene. Originally, this system was designed to isolate and characterize spontaneous mutations in the **TK** gene. While screening for drug resistant clones, a dramatic cytotoxic effect on the **TK**$^-$ cells was associated with the addition of 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG), as determined by the lack of recovery of drug-resistant mutants. This was not seen with the nucleoside analogs acyclovir (ACV) and trifluorothymidine (TFT).

METHODS

Construction of the plasmid pSV2*ned*KT: The plasmid pSV2*neo* (8) was linearized by hydrolysis with *Bam* H1 (Boehringer Mannheim Canada) and ligated to the HSV-1 **TK** *Bam* H1 fragment (from pHSV-1, kindly provided by Dr. G. Shore). The ligated DNA was used to transform *Escherichia coli* DH1. *Ampicillin* resistant colonies were selected and screened for the presence of the resultant plasmid pSV2*ned*KT (9.1 kb).

Transfection of 143 **TK**$^-$ cells: Culture plates (100mm) containing $3X10^5$ 143 **TK**$^-$ cells (kindly provided by Dr. J.A. Hassell) were treated with 100 ng of pSV2*ned*KT per plate by the calcium phosphate precipitation method of Graham and Van der Eb (9) as modified by Wigler et al. (10). **TK**$^+$ colonies were isolated after growth in HAT (0.1 mM Hypoxanthine, 0.4 μM Amethopterin, 16 μM Thymidine) media (11) and tested for resistance to 400 μg/ml G418 (Gibco Canada).

Analysis of **TK**$^+$, **G418**^{R} clones: High molecular weight DNA was prepared (12) and analyzed by Southern Blotting (13) using GeneScreen Membrane filters to determine the copy number and orientation of the plasmids. Prehybridization and hybridization procedures were performed according to the manufacturer's instructions using 50% formamide and 10% dextran sulphate at 42°C. A clone containing a single integrated copy of the plasmid was kept for further use (the KT cell line).

Compounds: The DHPG (kindly provided by Dr. K.K. Ogilvie) and ACV (Burroughs Wellcome Inc.) solutions were made up according to Smith *et al.* (7). TFT (Sigma) was dissolved in DMEM. For each compound, the concentration range at which the analog was not cytotoxic to the 143 **TK**$^-$ cells (after 2-3 weeks), but still able to inhibit the growth of the KT cells, was determined. TFT was used at 3.4 μM, DHPG at 3.9 μM, and ACV at 111 μM.

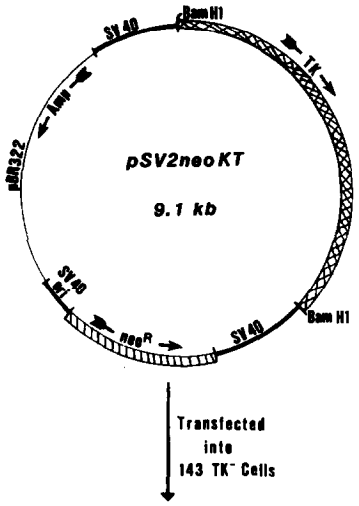
Selection of drug resistant mutants: KT cells were removed from HAT media containing 400 μg/ml G418 and plated in media containing only G418 for 3 days to allow spontaneous mutations to occur and be expressed in the HSV-1 **TK** gene. They were then replated at $1X10^4$ to $1X10^5$ cells per plate (100mm) in media containing one of the nucleoside analogs and G418. The medium was changed every three days until the appearance of colonies. The plates were then fixed and stained with giemsa stain (14).

Selection of **TK**$^-$ colonies in a mixed population of cells: Approximately $5X10^2$ to $1X10^3$ 143 **TK**$^-$ cells were plated with $1X10^5$ to $3X10^5$ KT cells. The selection was the same as above except the G418 was ommitted.

Testing the toxicity of media from cells under selection: Every 24 hours, media from plates initially containing $1X10^5$ KT cells growing in the analogs were transferred to plates originally containing $1X10^4$ 143 TK^- cells. Fresh plates of KT cells were set up on the fourth day to continue for a total period of 6 days. The number of 143 TK^- colonies remaining at the end of this period was used to determine the cytotoxicity of byproducts produced from the KT cells in the presence of the nucleoside analogs.

RESULTS

The plasmid pSV2*neo*KT (Fig. 1) was transfected into the 143 TK^- cell line and TK^+, $G418^R$ clones were selected. By Southern blot analysis, one clone (the KT cell line) was identified as having a single integrated copy of the plasmid pSV2*neo*KT and the orientation of the plasmid was determined (data not shown). A detailed map of the integrated plasmid is shown in Fig. 1.



Integrated pSV2neoKT

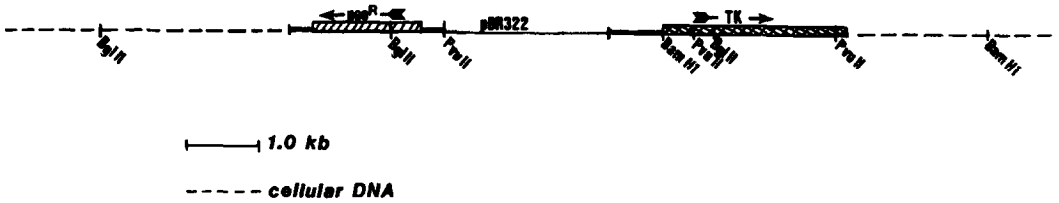


Figure 1: Plasmid map of pSV2*neo*KT. The vector contains coding sequences for the HSV-1 thymidine kinase and the *neomycin* gene which confers resistance to G418. The integrated form of pSV2*neo*KT in the 143 TK^- cells is shown at the bottom.

The cytotoxicity associated with DHPG was discovered during attempts to select and study the molecular basis of spontaneous mutations in the chromosomally-located HSV-1 **TK** gene. Using TFT or ACV as a selective agent, the frequency of resistant (**TK**$^-$) colonies was about 10^{-4} for TFT and 10^{-3} for ACV. Under identical conditions, the frequency of **DHPG**^{R} colonies was zero. These results were reproduced several times. Virtually all **TFT**^{R} mutants tested were found to grow in DHPG, thus demonstrating that the **TK**$^-$ mutants in the KT cell line would grow normally under the DHPG selective conditions. The higher frequency of **ACV**^{R} clones (as compared to **TFT**^{R}) may be due to weaker potency of ACV. About 57% of the **ACV**^{R} clones were found to be resistant to TFT.

In order to rule out the possibility that the lack of **DHPG**^{R} colonies was due to residual thymidine kinase activity in the spontaneously mutated cells, we performed a mixing experiment and introduced 143 **TK**$^-$ cells into the selection system (Table 1). The 143 **TK**$^-$ cells, when grown separately, did not show reduced growth at this concentration of DHPG. However, when they were mixed with the KT cells, there was a 90% to 100% decrease in the recovery of colonies as compared to the TFT selection. The differences in the number of colonies recovered from each trial is due to the different number of 143 **TK**$^-$ seeded at the beginning of each experiment. For the DHPG selections, it is also influenced by the number of KT cells which are metabolizing the drug. The number of 143 **TK**$^-$ colonies recovered at the end of the experiment decreased with an increased number of KT cells under DHPG selection. In the third trial, an additional set of

TABLE 1. Inhibition of 143 **TK**$^-$ growth in a mixed population of cells

Trial Number	Initial Number of KT cells with drug	Average Number of **TK**$^-$ colonies		
		No drug[a] in media	TFT in media	DHPG in media
1	$3X10^5$	ND[b]	693	11
2	$3X10^5$	ND	254	0
3	$1X10^5$	526	437	54

[a]KT cells were not added
[b]Not Determined

TABLE 2. Inhibition of 143 TK$^-$ growth by the medium transferred from KT Cells

Type of cells in the medium to be transferred	Drug in the medium to be transferred	Average number of TK$^-$ colonies	Percent inhibition
KT	none	1633	-
KT	TFT	1562	4%
KT	ACV	1372	16%
KT	DHPG	635	61%
143 TK$^-$	DHPG	1347	17%

plates were seeded with only 143 TK$^-$ cells to determine the number of viable cells mixed with the KT cells. This was done to show that TFT does not have a significant toxic effect under these conditions.

Our results could be accounted for by a toxic product (such as the phosphorylated form of DHPG) being produced by the KT cells in the presence of DHPG and its subsequent release into the media resulting in TK$^-$ cell growth inhibition. To test this hypothesis, media containing one of the analogs was removed daily from plates with growing KT cells and transferred to plates containing the 143 TK$^-$ cells. The results indicate that the media containing DHPG from the KT cells does contain a toxic product reducing the growth of TK$^-$ colonies by about 60% (Table 2). The toxicity of the media could only be demonstrated when the media was transferred daily. Longer transfer periods (3 days) resulted in very little growth inhibiton of the TK$^-$ cells.

DISCUSSION

This study suggests that a toxic agent is produced from DHPG by cells containing the HSV-1 TK gene which inhibits the growth of TK$^-$ cells. There are several possibilities as to how this product is formed. One possibility is that it is a breakdown product. For example, TFT has been shown to undergo hydrolysis resulting in a toxic product, 5-carboxy-2'-deoxyuridine. This compound is toxic to uninfected cells which do not have an active viral thymidine kinase and its

toxicity is not dependent on phosphorylation (15,16). However, DHPG toxicity is only seen when cells with an active herpes thymidine kinase are present.

A more likely possibility for the toxicity associated with DHPG is that a phosphorylated form of DHPG is released into the media by the KT cells and taken up by the 143 TK^- cells. Cheng *et al.* have shown that the mono-, di-, and tri- phosphate derivatives of DHPG are present in virally infected cells (2). The viral thymidine kinase is responsible for the first phosphorylation and the subsequent phosphorylations are carried out by cellular kinases. Consequently, any of these three forms could be cytotoxic if they are taken up by the 143 TK^- cells. We have also found that the stability of the toxic product decreases with time, further implicating a metastable product such as the phosphorylated DHPG as the toxic product.

Our results suggest that the KT cell line could be useful for studying the effects of antiherpes drugs whose mode of action requires an active herpes thymidine kinase, mimicking the situation of "infected" and neighbouring "uninfected" cells. This system is not designed to replace the conventional methods which are very important for determining the selective action of these compounds. As an additional test, however, it can be used to examine the effects of these compounds in a mixed population of cells.

ACKNOWLEDGEMENTS

We would like to thank Dr. G. Shore and Dr. J.A. Hassell for plasmids and cell lines, and Dr. K.K. Ogilvie for useful discussions and his gift of DHPG.

This work was supported by a Strategic Grant (G0907) from the Natural Sciences and Engineering Research Council and by a grant from the National Cancer Institute of Canada. D.R.G. was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council, and M.S.D. is a Scholar of the Medical Research Council of Canada.

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