

# Short Technical Reports

## Budding Yeast as a Screening Tool for Discovery of Nucleoside Analogs for Use in HSV-1 TK Suicide-Gene Therapy

BioTechniques 27: 772-777 (October 1999)

### ABSTRACT

We present a fast, convenient and inexpensive method that allows the automated, large-scale screening of chemical libraries for compounds that are converted by the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) into inhibitors of cell growth. The method is based on the use of budding yeast (*Saccharomyces cerevisiae*) transformed with the HSV-1 TK gene on a multicopy plasmid. Eight nucleoside analogs (acyclovir, ganciclovir, penciclovir, lobucavir, brivudin, sorivudine, IVDU and ara-T), for which the cytostatic action against mammalian cells expressing the HSV-1 TK gene has been well documented, were studied for their inhibitory effect on the growth of yeast expressing the viral TK. These nucleoside analogs had little or no inhibitory effect on the growth of yeasts transformed with the empty vector, but inhibited to a significant extent the growth of yeast expressing the viral TK. Use of HSV-1 TK-expressing yeast allows quick screening in multi-well plate format for compounds with potential use in HSV-1 TK suicide gene therapy. The method may also be used as a tool to selectively suppress or arrest the growth of one population of yeast out of mixed yeast cell cultures.

### INTRODUCTION

A possible approach for the treatment of cancer is the introduction of the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene into tumor cells, followed by treatment with the acyclic guanosine analog ganciclovir (GCV) (9). The latter molecule is an anti-herpetic compound that depends on the viral TK for its activation to its 5'-monophosphate form (10). GCV monophosphate is then further metabolized to its triphosphate form (GCV-

TP) by cellular kinases. GCV-TP is a potent and selective inhibitor of the HSV DNA polymerase (10). Thus, the reason for the selective inhibition of viral replication by GCV is dual: it depends on (i) the specific activation of GCV to its monophosphate form in virus-infected cells and (ii) the selective inhibition of the viral DNA polymerase by GCV-TP. However, GCV-TP is also a substrate for cellular DNA polymerases, albeit at a higher  $K_i$  (14). Thus, in tumor cells that have been transfected with the viral TK gene, relatively high intracellular concentrations of GCV-TP are being formed that result in a cytostatic effect. Complete regression of rat brain tumors was demonstrated after in situ retrovirus-mediated transformation with the HSV-1 TK gene and subsequent treatment with GCV (9,13). This approach is being evaluated in several clinical trials for the treatment of brain tumors, ovarian cancer and pleural mesothelioma (1,12).

In addition to GCV, a variety of purine and pyrimidine nucleoside analogs have been shown to exert high cytostatic activities against tumor cells (3,6,11) transfected with the TK gene of HSV-1, HSV-2 or varicella-zoster virus (VZV). These include penciclovir (PCV) [(R)-9[(3,4-dihydroxybutyl)guanine]], acyclovir (ACV) [9-(2-hydroxyethoxymethyl)guanine]; lobucavir (LBV) [(R)-9[2,3-bis(hydroxymethyl) cyclobutyl]guanine]; araT [(1-beta-D-arabinofuranosylthymine)], brivudin (BVDU) [(E)-5-(2-bromovinyl)-2'-deoxyuridine], sorivudine (BVaraU) [(E)-5-(2-bromovinyl)-1-beta-D-arabinofuranosyluracil] and IVDU [(E)-5-(2-iodovinyl)-2'-deoxyuridine]. A particular advantage of BVDU is that this compound, besides being (as BVDU-TP) an inhibitor of the cellular (mammalian) DNA polymerase, in its monophosphate form (BVDU-MP), potentially inhibits the cellular thymidylate synthase (4), thus causing a depletion of intracellular dTTP pools and hence inhibition of tumor-cell growth.

Compounds that have been studied for their potential use in a HSV-1 TK suicide-gene-therapy approach have all been identified in mammalian-cell culture systems. However, the search for

compounds that are activated by the viral TK to become inhibitory to the cellular DNA polymerase could be intensified by an efficient, rapid and inexpensive screening method. For these reasons, we have studied whether yeast transfected with the viral TK gene can be used to develop a rapid, safe and inexpensive screening assay for the discovery of novel compounds that may have potential in a HSV-1 TK suicide-gene-therapy approach.

### MATERIALS AND METHODS

#### Reagents

We purchased acyclovir (Zovirax<sup>®</sup>) from Glaxo Wellcome (Aalst, Belgium); ganciclovir (Cymevene<sup>®</sup>) from Sarva Syntex (Palo Alto, CA, USA). Lobucavir was kindly provided by Dr. R. Colonna (Bristol Myers Squibb, Wallingford, CT, USA); penciclovir was provided by SmithKline Beecham (London, England, UK); BVDU was from the Rega Institute (Leuven, Belgium); BVaraU was provided by H. Machida (Yamasa Shoyu, Coshi, Japan). IVDU was synthesized by E. Knaus and L. Wiebe (University of Alberta, Alberta, Canada) and araT was purchased from Sigma (Bornem, Belgium).

#### Cloning and Expression of HSV-1 TK

HSV-1 TK cDNA was generated by polymerase chain reaction (PCR) using the following oligonucleotides: 5'-GAGGAATTCATGGCTTCGTACCCCGGCCATC-3' and 5'-CTCGTCGACAGGTTAGCCTCCCCATCTCCCG-3', subcloned in the pGEM-T<sup>®</sup> vector (Promega, Leiden, The Netherlands) and subsequently cloned in the *EcoRI* and *SaI* sites of the yeast multicopy expression vector PYX232<sup>®</sup> (R&D Systems, Minneapolis, MN, USA). Wild-type yeast (W303 strain) was transformed with either the empty or the TK-encoding PYX232 vector using the method described by Chen et al. (8). Transformants were selected on minimal medium without tryptophan. HSV-1 TK activity was measured in yeast-cell extracts as described previously for mammalian cells (5,7).

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## Yeast Culture and Growth Curves

Transformed yeasts were grown in minimal media lacking tryptophan and containing 2% glucose as the carbon source. Growth curves were made using cultures of 100–350  $\mu\text{L}$  in 100-well plates in a Bioscreen™ C apparatus (Labsystems, Zellik, Belgium). For this purpose, stationary-phase cultures were diluted to an  $\text{OD}_{600}$  of 0.1 (photometer length of 1 cm) in the presence or absence of drugs. Growth was followed over a 12–24 h period with a 30-s shaking period every minute and  $\text{OD}_{600}$  measurements every 30 min.

## RESULTS AND DISCUSSION

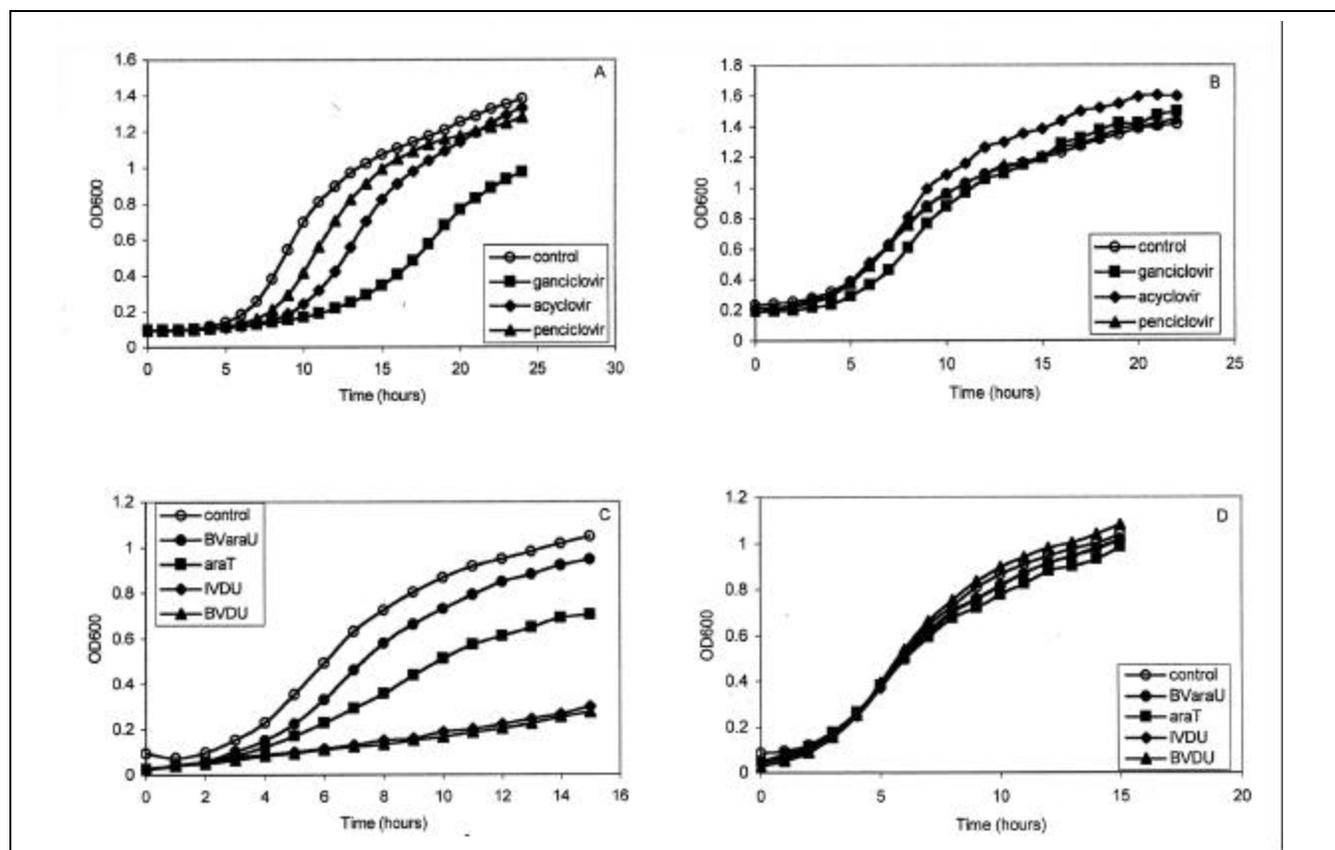
Wild-type yeast cells were transformed with a multicopy HSV1 TK expression vector or with the empty vector (control yeasts) as described in the Materials and Methods section. Express-

ion of functional TK activity was demonstrated by measuring the TK activity in extracts of wild-type and transformed yeast (data not shown). In a first series of experiments, we studied the effect of a single concentration (1 mg/mL) of ACV, GCV, PCV, LBV, BVDU, BVaraU, IVDU or araT on the growth of these yeast cells. None of the compounds, with the exception of LBV, which caused 25% inhibition, had an inhibitory effect on the growth of the control yeasts, but all eight drugs inhibited the growth of yeasts expressing the HSV-1 TK (Figure 1, panels A–D). When the  $\text{OD}_{600}$  of the cultures without nucleoside analogs was 0.5, growth inhibition amounted to 59% by PCV, 82% by ACV, 88% by GCV, 66% by LBV (or 41% when the inhibition of the control culture was taken into account), 93% by BVDU, 44% by BVaraU, 81% by IVDU and 57% by araT.

The relative potency of the different compounds on the growth of the yeast-

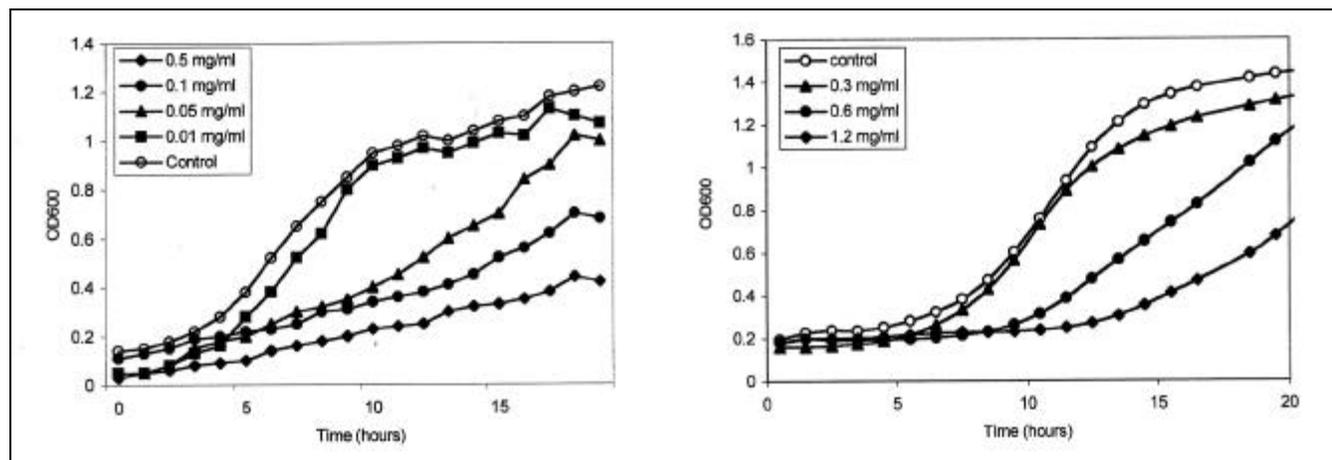
expressing TK was as follows: BVDU = IVDU > araT = GCV > LBV > ACV > PCV = BVaraU. The pyrimidine nucleoside analogs BVDU and IVDU proved to be clearly more effective than the purine nucleoside analogs GCV, LBV, ACV and PCV. Unlike BVDU and IVDU, however, the pyrimidine nucleoside analog BVaraU elicited only limited cytostatic effect in TK-expressing yeast. A more or less similar series has been described in mammalian FM3A cells expressing the HSV-1 TK: BVDU = IVDU > GCV > ACV = PCV > BVaraU (2,3).

From the above results, it is clear that high levels of TK expression in yeasts (driven by the strong triose-phosphate-isomerase promoter on a multicopy plasmid) lead to sensitivity of these yeasts to nucleoside analogs that depend for their activation on the viral TK. This inhibitory effect is dose-dependent as illustrated for BVDU and ACV (Figure 2). The inhibition of cell



**Figure 1. Nucleoside analogs inhibit the growth of HSV-1 TK expressing yeast.** Wild-type yeast cells were transformed with either an expression vector encoding HSV-1 TK (panels A and C) or the empty vector (panels B and D). Cultures were incubated at 30°C in a bioscreen apparatus in the absence or presence of 1 mg/mL of the indicated nucleoside analogues. Illustrated data represent the mean of triplicate experiments. Standard errors of the means for all time points are lower than 5%.

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**Figure 2. Dose-dependent inhibition of the growth of HSV-1 TK-expressing yeast by BVDU and ACV.** Wild-type yeast cells were transformed with an expression vector encoding HSV-1 TK. Cultures were incubated at 30°C in a bioscreen apparatus in the presence of the indicated concentrations of BVDU (panel A) or ACV (panel B). Illustrated data represent the mean of triplicate experiments. Standard errors of the means for all time points are lower than 5%.

growth is expected to be caused by inhibition of the cellular DNA polymerase by the triphosphorylated forms of the nucleoside analogs. In mammalian cells, it has been described that BVDU may also lead to a depletion of

the intracellular dTTP pools caused by the inhibition of cellular thymidylate synthase by BVDU-MP. It remains to be established, however, whether this would also be the case in yeast.

Because they have a rigid cell wall, some molecules may theoretically not be taken up by yeast as efficiently as by mammalian cells, which could lead to false-negatives. In addition, DNA polymerases and enzymes of nucleoside and nucleotide metabolism from mammalian cells and yeast may have some different characteristics. However, all compounds studied thus far have selectively inhibited the growth of HSV-1 TK-expressing yeasts, which makes it rather unlikely that the use of this system would not pick up molecules with a potentially interesting profile. Yeast cultures of 100  $\mu$ L are routinely used for screening purposes, but even smaller volumes to limit consumption of compounds.

Inhibiting the growth of TK-expressing yeasts by nucleoside analogues might be used to specifically inhibit the growth of yeasts out of mixed yeast cell cultures. Also, in promoter studies, the expression of HSV-1 TK behind the promoter of interest will rapidly reveal under which conditions this promoter is active (growth inhibition by nucleoside analogs) or silent (no effect of the addition of nucleoside analogs). Moreover, the method presented will most likely also function in HSV-1 TK gene-transformed *Candida*

or other yeasts, facilitating studies of biology and promoter regulation in these organisms.

In conclusion, the method presented offers several advantages: (i) it is rapid (determination of growth inhibition can be performed in less than 12 h), (ii) inexpensive; (iii) safe; and (iv) it can be fully automated to allow large-scale screening.

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*S.W. and J.N. are Postdoctoral Fellows of the Fund for Scientific Research (FWO)-Flanders (Belgium). B.D. is a recipient of an IWT fellowship from the "Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie". The authors wish to thank R.*

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Received 23 April 1999; accepted 6 July 1999.

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