Identification of the ENT1 Antagonists Dipyridamole and Dilazep as Amplifiers of Oncolytic Herpes Simplex Virus-1 Replication

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Abstract

Oncolytic herpes simplex virus-1 (oHSV) vectors selectively replicate in tumor cells, where they kill through oncolysis while sparing normal cells. One of the drawbacks of oHSV vectors is their limited replication and spread to neighboring cancer cells. Here, we report the outcome of a high-throughput chemical library screen to identify small-molecule compounds that augment the replication of oHSV G47Δ. Of the 2,640-screened bioactive compounds, 6 compounds were identified and subsequently validated for enhanced G47Δ replication. Two of these compounds, dipyridamole and dilazep, interfered with nucleotide metabolism by potently and directly inhibiting the equilibrative nucleoside transporter-1 (ENT1). Replicative amplification promoted by dipyridamole and dilazep were dependent on HSV mutations in ICP6, the large subunit of ribonucleotide reductase. Our results indicate that ENT1 antagonists augment oHSV replication in tumor cells by increasing cellular ribonucleoside activity. Cancer Res; 70(10); 3890-5. ©2010 AACR.

Introduction

Viral vectors genetically engineered for cancer cell–restriction replication represent an attractive strategy for tumor therapy because these viruses can replicate and spread in situ, exhibiting oncolytic activity through direct cytopathic effects (1, 2). We and others have previously shown that appropriately selected pharmaceuticals can synergize with oncolytic herpes simplex virus-1 (oHSV) to increase oncolytic efficacy (3–5). To identify new agents and mechanisms that would increase G47Δ replication in cancer cells, we undertook an unbiased high-throughput screen of known bioactive molecules. We have identified dipyridamole and dilazep as potent enhancers of G47Δ replication, revealing a previously unidentified function for two well-characterized inhibitors of the equilibrative nucleoside transporter-1 (ENT1).

Materials and Methods

**High-throughput screen.** Piloting and primary screen was conducted at the Institute of Chemistry and Cell Biology-Longwood core facility. Z′-factors were used to normalize for plate-to-plate variation (6). PC3 cells were seeded in 384-well cell culture plates; the following day, the compounds were added in duplicate and incubated for 6 hours before infection with G47Δ-GFP [multiplicity of infection (MOI), 0.05]. Forty-eight hours postinfection, PC3 cells were imaged on an automated Image-Xpress inverted fluorescent microscope (Molecular Devices) using two wavelengths, 488 nm to detect G47Δ-GFP infected cells and 350 nm for nuclear DNA bound by Hoechst-33342.

**Viruses.** G47Δ-GFP (G47Δ-BAC) contains a cytomegalovirus promoter–driven enhanced green fluorescent protein (GFP) in place of lacZ in G47Δ (7). R3616 contains 1-kb deletions of both copies of γ34.5 (8). G47Δ was derived from G207 by deleting α47 and the US11 promoter (9). FΔΔ is a strain F–derived recombinant with an ICP6-inactivating lacZ insertion (10).

**Compounds.** Dipyridamole, dialazep, nitrobenzylthioinosine (NBMPR), zaprinast, uridine, thymidine, and adenosine were purchased from Sigma. SB203580, GF109203X, EHNA, AG1517 (PD153035), and 1-methyl-3-isobutylxanthine were purchased from Calbiochem-Novabiochem. Gene expression analysis. Reverse transcription-PCR (RT-PCR) was used to verify human ENT1, RR1, RR2, and GAPDH mRNA levels in tumor cells. For quantitative RT-PCR analysis, PCR was performed using the 7000 Real-Time PCR Sequence Detection System (Applied Biosystems).
Multistep growth assays. Tumor cells were pretreated for 4 to 6 hours with a dose range of compounds followed by virus infection at an MOI of 0.05. Forty-eight hours after infection, virus titers [plaque forming units (pfu)/mL] were determined by standard plaque assay on Vero cells.

Ribonucleotide reductase assay. Ribonucleotide reductase activity was measured using the CDP assay method as previously described (11).

In vivo studies. Du145 cells (5 × 10⁶) were implanted s.c. into the flanks of athymic male mice (National Cancer Institute). Mice were given dipyridamole (dissolved in DMSO and diluted in 0.1 N HCl plus 0.9% NaCl) or dilazep (dissolved in H₂O and diluted in PBS) i.p. (40 mg/kg/injection) for 14 consecutive days starting 2 days before the first intratumoral injection of G47Δ (2 × 10⁶ pfu).

Organ culture assay. G47Δ titers were assessed in the presence or absence of dipyridamole or dilazep in prostate organ cultures as previously described (12).

Statistical analysis. For cell susceptibility assays and in vivo efficacy studies, Student’s t test (two-tailed) was used to analyze significance between two treatment groups using GraphPad Prism v.4.

Results and Discussion

We screened 2,640 compounds of known bioactives derived from three pharmacologically active libraries (NINDS,
Biomed, and Prestwick1-collection). PC3 prostate cancer cells were pretreated with compounds and subsequently infected with G47Δ-expressing GFP at a MOI of 0.05 for an additional 48 hours (Supplementary Fig. S1). Compounds that amplified G47Δ-GFP (as measured by GFP+ cells) at least 3 SD above the overall plate average were considered strong "hits." Scatter plot analysis showed that 15 (0.57%) of the library compounds reflected potential "amplifiers" of viral spread (Fig. 1A). Many of the small-molecule compounds that fulfilled these criteria were antimetabolites: two antifolates (pyrimethamine and methotrexate) and two fluoropyrimidines [fluorodeoxouridine (FdUrd) and carmofur; Table 1]. FdUrd has been reported to enhance the spread of oncolytic G207 (13), a HSV vector related to G47Δ; therefore, its identification validated the effectiveness of the chemical library screen. Dipyridamole and dilazep have been clinically used as vasodilators and fall into another pharmacologic group, termed ENT1 inhibitors (14). We focused our efforts on dipyridamole and dilazep because they represent a class of compounds that have not been studied in the context of virotherapy and they could be readily translated to clinical trial. Multi-step growth curve assays (herein referred to as plaque assays) were performed in PC3 cells to validate the amplifying-promoting activities of these compounds (Fig. 1B). Pretreatment of PC3 cells with dipyridamole or dilazep resulted in a dose-dependent increase in G47Δ production 48 hours postinfection. Fluorescent imaging of G47Δ-GFP–infected PC3 cells showed that dipyridamole and dilazep increased GFP+ cell numbers (Fig. 1B).

The effects of dipyridamole or dilazep on oHSV replication were also tested in other human tumor cell lines (Fig. 1C). HCT-116, Du145, and HT-1080 were particularly sensitive to the effects of dipyridamole and dilazep on G47HCT-116, Du145, and HT-1080 were particularly sensitive to- were also tested in other human tumor cell lines (Fig. 1C). To determine whether one or more of the deletion mutations within G47Δ confers augmented viral replication by di- pyridamole or dilazep, we tested HSV-1 viruses FΔ (ICP6Δ), R3616 (γ34.5Δ), and G207 (γ34.5Δ, ICP6Δ), and wild-type strain F in the presence or absence of dipyridamole or dilazep. Plaque assays consistently revealed that oHSVs lacking ICP6, a gene that encodes the large subunit of ribonucleotide reductase, were associated with augmented viral replication by dipyridamole and dilazep (Fig. 2A). Furthermore, quantitative RT-PCR showed that viral transcripts representing immediate early (ICP4), early (UL23), or late (UL44) genes were enhanced by dipyridamole and dilazep from viruses lacking ICP6 (G47Δ and FΔ6), whereas minimal changes were seen in transcripts from ICP6-intact strain F (Fig. 2B).

Dipyridamole and dilazep are also known to function as phosphodiesterase (PDE) antagonists as well as protein kinase (PK) inhibitors (14, 15). Therefore, we tested representative inhibitors of PDE and PK activity using virus yield assays and fluorescent imaging (Fig. 2C; Supplementary Fig. S4). Inhibitors of PDE (Zaprinast, IBMX, and EHNA) or PK (SB203580, GF109203x, and AG1517) had minimal effects on increasing G47Δ replication relative to virus alone. Furthermore, increasing adenosine, uridine, or thymidine concentrations had no effect. This is in contrast to the action of NBMPR, a potent ENT1 antagonist (16), which increased G47Δ virus replication to a similar degree as dipyridamole or dilazep (Fig. 2C). We next examined the effects on G47Δ replication of a panel of dipyridamole analogues that have been previously shown to exhibit a range of binding affinities toward ENT1 based on structure-activity relationship studies (17). A number of dipyridamole derivatives reported to exhibit strong binding affinities toward ENT1 (compounds 2, 4, 11, 15, 52, 58, and 64) also promoted notable increases in G47Δ replication in PC3 cells (Supplementary Fig. S5). These data show that dipyridamole and dilazep likely act on ENT1 to augment the replication of ICP6-deficient HSV vectors.

Ribbonucleotide reductase, which is composed of two subunits, RR1 and RR2, plays a critical role in the synthesis of DNA by reducing ribonucleotides to their corresponding deoxyribonucleotides, thereby providing an essential reservoir of precursors for DNA synthesis and repair (18). As dipyridamole, dilazep, and NBMPR inhibit nucleoside import/export through binding to ENT1 (16), we hypothesized that the actions of these compounds could possibly affect RR1 and RR2 mRNA levels. RT-PCR analysis indicated that RR1 and RR2 mRNA levels were indistinguishable from dipyridamole- or dilazep-treated PC3 cells compared with untreated controls (Supplementary Fig. S6). Next, we investigated whether treatment with either dipyridamole or dilazep results in increased ribonucleotide reductase activity.

### Table 1. List of bioactives that significantly increased G47Δ titers

<table>
<thead>
<tr>
<th>Class/name</th>
<th>Mode of action</th>
<th>z-Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimetabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Inhibits folic acid metabolism</td>
<td>3.3</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>Inhibits folic acid synthesis</td>
<td>3.6</td>
</tr>
<tr>
<td>Carmofur</td>
<td>Inhibits thymidylate synthase</td>
<td>3.1</td>
</tr>
<tr>
<td>Flouxuridine</td>
<td>Inhibits thymidylate synthase</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Vasodilators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipyridamole†</td>
<td>Inhibits ENTs</td>
<td>3.6</td>
</tr>
<tr>
<td>Dilazep</td>
<td>Inhibits ENTs</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*z-Score value indicates SD above the plate mean for each readout value.

†Identified in all three libraries with z-score ≥3.0.
Figure 2D shows that by 6 hours after treatment with dipyr-idamole or dilazep, ribonucleotide reductase activity increased by 30% and 36%, respectively, over baseline levels in PC3 cells. By contrast, ribonucleotide reductase activity in PANC-1 cells was not increased with treatment of either compound and, moreover, was minimally detected (Fig. 2D). This observation strongly parallels the data derived in Fig. 1B, illustrating that neither drug augmented G47Δ in PANC-1 cells.

Last, we evaluated the in vivo antitumor efficacy of G47Δ in combination with either dipyr-idamole or dilazep in s.c. Du145 tumors. By day 45 after tumor implantation, a statistically significant decrease in tumor volume was observed between control (882 ± 86 mm³, n = 7) and all of the treatment groups (Fig. 3A). As compared with mock, treatment with G47Δ resulted in a notable reduction in tumor size (625 ± 76 mm³; n = 7), whereas the combination of G47Δ with dipyr-idamole (426 ± 37 mm³; n = 6) or dilazep (377 ± 56 mm³; n = 6) resulted in a statistically significant tumor regression compared with G47Δ alone. Neither compound by itself reduced or delayed tumor growth (data not shown). At day 45, statistically significant differences in tumor weights were also observed between untreated control and G47Δ treatment groups, as well as for G47Δ alone and in combination with dipyr-idamole or dilazep (Fig. 3B). Recently, we reported on the use of prostate organ cultures derived from radical prostatectomies to assess oHSV target specificity and replication competence (13). We exploited this system to address whether dipyr-idamole and dilazep enhances G47Δ replication in primary human prostate cancer specimens. These results show that treatment of organ cultures with dipyr-idamole or dilazep over a 3-day period increased G47Δ titers by 2- to 4-fold over the no-treatment control (Fig. 3C).

Using an unbiased chemical library screen, we have identified a novel application for the ENT1 antagonists, dipyr-idamole and dilazep (and NBMPR), namely to “amplify” the replication of G47Δ in cancer cells. Our results reveal that these ENT1 antagonists augment HSV vectors specifically lacking ICP6, which is the viral homologue of the human RR1 gene, and that ENT1 antagonists may predispose some cancer cells to augmented oHSV replication by increasing...
cellular ribonucleotide reductase activity. From a translational perspective, dipyridamole has been used to potentiate the effects of chemotherapeutic agents in phase II clinical trials in solid tumors (19). Although the therapeutic dosing for these studies was less than what was used in the present study, dipyridamole is well tolerated at higher doses with minimal adverse reactions in animals (20). These data suggest that the combination of dipyridamole and oncolytic herpes virus-1 neurovirulence to human glioma derives from a high-throughput screen; and Melissa Marinelli for laboratory assistance.

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Disclosures of Potential Conflicts of Interest

R.L. Martuza and S.D. Rabkin are consultants to MediGene Ag, which has a license from Georgetown University for G207. The other authors disclosed no potential conflicts of interest.

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