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ScienceDirect
Antiviral Research 77 (2008) 77–80
Antiviral Research
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Short communication

Hydroxyurea enhances the activity of acyclovir and cidofovir against herpes simplex virus type 1 resistant strains harboring mutations in the thymidine kinase and/or the DNA polymerase genes

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Received 10 July 2007; accepted 22 August 2007

Abstract

Drug-resistant herpes simplex virus type 1 (HSV-1) recombinant strains harboring mutations in the thymidine kinase and/or the DNA polymerase genes were evaluated for their susceptibility to various antivirals in the presence of 25 μg/ml of hydroxyurea (HyU). The latter compound decreased the 50% inhibitory concentrations of acyclovir by 1.5–3.8-fold and that of cidofovir by 2.7–14.4-fold. However, HyU did not affect the susceptibilities of the various recombinant mutants to foscarnet. Hydroxyurea, a ribonucleotide reductase inhibitor, can increase the activity of nucleoside/nucleotide analogues against drug-resistant viruses.

Keywords: Herpes simplex virus; Hydroxyurea; Acyclovir; Cidofovir; Resistance

The emergence of acyclovir (ACV)-resistant herpes simplex viruses (HSV) with mutations in the thymidine kinase (TK) and/or the viral DNA polymerase (pol) genes is a common problem among immunocompromised patients (Schmit and Boivin, 1999). HSV also encodes a ribonucleotide reductase (RR) that is responsible for the synthesis of deoxyribonucleotide precursors required for DNA elongation. The viral RR is essential for HSV reactivation from latency and for virulence in different animal models of viral infection (Brandt et al., 1991; Idowu et al., 1992; Jacobson et al., 1989; Yamada et al., 1991). Thus, this viral protein represents an alternate therapeutic target in the case of HSV-1 and -2 strains resistant to nucleoside analogues such as ACV. Hydroxyurea (HyU) inhibits the viral and cellular RR, inducing a depletion of the intracellular deoxynucleotide triphosphate (dNTP) pool (Gao et al., 1993; Gao et al., 1994; Johns and Gao, 1998). A synergistic effect between HyU and nucleosidic reverse transcriptase inhibitors has been well documented for human immunodeficiency virus (HIV) type 1 infections (Lori et al., 1994; Lori and Lisziewicz, 1998; Malley et al., 1994; Palmer et al., 1999) but whether such an effect is also seen in the case of ACV-resistant HSV infections has been less extensively studied (Neyts and De Clercq, 1999; Pancheva and Venkova, 2000). Herein, we evaluated the effect of HyU on various anti-herpetic agents i.e. ACV, cidofovir (CDV) and foscarnet (FOS) against several HSV-1 recombinant mutants harboring mutations in the TK and/or the DNA pol genes.

The HSV-1 recombinant strain 17 and all mutant viruses derived from this strain were propagated in Vero cells. The generation of HSV-1 recombinant viruses from a system of overlapping cosmids and plasmids covering the entire viral genome has been previously described (Bestman-Smith and Boivin, 2003; Sergerie and Boivin, 2006). Viral susceptibilities to ACV, CDV and FOS were determined using a plaque reduction assay (PRA) (Safrin et al., 1994). A two-fold difference in 50% inhibitory concentration (IC_{50}) values between the HSV-1 recombinant wild-type (WT) and the mutant viruses was considered significant (Bestman-Smith and Boivin, 2003). The addition of HyU (25 μg/ml, Sigma–Aldrich, Oakville, Ontario, Canada) to other anti-herpetic agents was also assessed by the use of a PRA. All susceptibility experiments were done in duplicate. The cytotoxicity of HyU (up to 500 μg/ml) in combination or not with ACV, FOS or CDV was evaluated using the cell titer 96® AQ_{ueous} non-radioactive cell proliferation assay (Promega, Madison, WI) in non-infected Vero cells after a 3-day exposure.

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doi:10.1016/j.antiviral.2007.08.009

Table 1
Evaluation of hydroxyurea (HyU) cytotoxicity on Vero cells using a cell proliferation assay

HyU concentration (μg/ml)	Absorbance unit[a] (S.E.M.)	Cell viability (%)
0.000	1.012 (±0.019)	100.00
7.812	0.910 (±0.036)	89.92
15.625	0.876 (±0.057)	86.56
31.250	0.701 (±0.047)	69.27
62.500	0.689 (±0.046)	68.08
125.000	0.616 (±0.050)	60.87
250.000	0.556 (±0.055)	54.94
500.000	0.249 (±0.027)	24.60

S.E.M., standard error of mean.
[a] Mean of two independent experiments.

IC_{50} values were analyzed using a Student's *t*-test for independent samples. All statistical analyses were carried out with the SPSS software 11.0 (SPSS Inc., Chicago, Illinois).

Plaque formation was not altered by the addition of up to 50 μg/ml of HyU (data not shown). When this concentration of HyU was exceeded, the count of plaques became difficult due to cell toxicity. At a concentration of 500 μg/ml, HyU reduced the viability of confluent Vero cells by 75%, whereas cell viability was reduced by about 13% at a concentration of 15 μg/ml (Table 1). The dose of 25 μg/ml of HyU was selected for further experiments considering its reported synergistic effect with various antiviral compounds (Neyts and De Clercq, 1999) and its relatively low cell toxicity (19%) when interpolated from the cell proliferation assay curve.

Six well-characterized HSV-1 recombinant viruses (WT, two TK mutants, two DNA pol mutants and one dual TK-DNA pol mutant) (Bestman-Smith and Boivin, 2003; Schmit and Boivin, 1999; Sergerie and Boivin, 2006) were tested for their susceptibilities to ACV, FOS and CDV in combination or not with HyU. Drug IC_{50} values for the recombinant viruses are reported in Table 2. All recombinant mutants were resistant to ACV with the double TK (C467del, deletion of a cytosine at position 467 resulting in a truncated protein)-DNA pol (D907V) mutant being the most resistant (80-fold increase in IC_{50} value compared to WT). The TK recombinant mutants K62N and C467del were also highly resistant to ACV (51.7- and 57.7-fold increases in IC_{50} values, respectively). The DNA pol recombinant viruses L778M and D907V were less resistant to ACV (3.7- and 2.0-fold increases in IC_{50} values). Only the DNA pol recombinant virus L778M was resistant to CDV (2.0-fold increase in IC_{50} value). As expected, the TK mutant viruses K62N and C467del were susceptible to FOS (0.7- and 0.8-fold increases in IC_{50}

Table 2
Susceptibilities of recombinant HSV-1 viruses to (A) acyclovir (ACV); (B) cidofovir (CDV); and (C) foscarnet (FOS) in combination or not with 25 μg/ml of hydroxyurea (HyU)

(A)

Mutations		ACV IC_{50} (μg/ml)	Fold increase over WT	ACV + HyU IC_{50} (μg/ml)	Fold increase over WT	Ratio ACV/ACV + HyU
TK	DNA pol					
–	–	0.03 ± 0.01	1.0	0.02 ± 0.01	1.0	1.5
–	L778M	0.11 ± 0.01	3.7	0.03 ± 0.01	1.5	3.8
–	D907V	0.06 ± 0.00	2.0	0.02 ± 0.01	1.0	3.0
K62N	–	1.55 ± 0.37	51.7	0.56 ± 0.13	28.0	2.8
C467del	–	1.73 ± 0.35	57.7	1.03 ± 0.12	51.5	1.8
C467del	D907V	2.40 ± 0.23	80.0	0.74 ± 0.01	37.0	3.2

(B)

Mutations		CDV IC_{50} (μg/ml)	Fold increase over WT	CDV + HyU IC_{50} (μg/ml)	Fold increase over WT	Ratio CDV/CDV + HyU
TK	DNA pol					
–	–	0.36 ± 0.03	1.0	0.11 ± 0.02	1.0	3.3
–	L778M	0.72 ± 0.03	2.0	0.05 ± 0.00	0.5	14.4
–	D907V	0.31 ± 0.02	0.9	0.06 ± 0.01	0.5	5.2
K62N	–	0.23 ± 0.00	0.6	0.07 ± 0.01	0.6	3.3
C467del	–	0.27 ± 0.06	0.8	0.10 ± 0.00	0.9	2.7
C467del	D907V	0.31 ± 0.04	0.9	0.05 ± 0.00	0.5	6.2

(C)

Mutations		FOS IC_{50} (μg/ml)	Fold increase over WT	FOS + HyU IC_{50} (μg/ml)	Fold increase over WT	Ratio FOS/FOS + HyU
TK	DNA pol					
–	–	16.73 ± 2.84	1.0	21.31 ± 0.76	1.0	0.8
–	L778M	40.17 ± 1.40	2.4	32.34 ± 1.65	1.1	1.2
–	D907V	43.88 ± 0.39	2.6	43.61 ± 1.88	2.1	1.0
K62N	–	12.25 ± 1.06	0.7	15.16 ± 0.05	0.7	0.8
C467del	–	13.55 ± 1.82	0.8	21.00 ± 1.33	1.0	0.7
C467del	D907V	40.52 ± 4.39	2.4	36.11 ± 2.74	1.7	1.1

TK: thymidine kinase; pol: polymerase. *Note*: results represent means of two independent experiments.

values, respectively) whereas the DNA pol mutants L778M and D907V as well as the dual TK/DNA pol mutant C467del/D907V were resistant to FOS (2.4-, 2.6- and 2.4-fold increases in IC_{50} values, respectively).

The addition of 25 μg/ml of HyU decreased the ACV IC_{50} values by a factor of 1.5 for the WT recombinant HSV-1 strain ($p > 0.05$), 1.8–2.8 for the TK mutants ($p = 0.007$), 3.0–3.8 for the DNA pol mutants ($p = 0.019$) and 3.2 for the dual TK/DNA pol mutant ($p = 0.009$) (Table 2A). The effect of HyU on CDV susceptibilities was somewhat more important with a reduction in the IC_{50} values by a factor of 3.3 for the WT recombinant HSV-1 strain ($p = 0.009$), 2.7–3.3 for the TK mutants ($p = 0.001$), 5.2–14.4 for the DNA pol mutants ($p = 0.032$) and 6.2 for the dual TK/DNA pol mutant ($p = 0.013$) (Table 2B). Susceptibilities of all WT and mutant viruses to FOS remained virtually unchanged following the addition of HyU ($p > 0.05$) (Table 2C).

ACV is a guanosine nucleoside analogue that is phosphorylated by the HSV-encoded TK to the 5′-monophosphate form upon its entry into infected cell. Cellular kinases then convert this metabolite to the biological active form, ACV triphosphate. The latter is a selective inhibitor of the viral DNA pol and acts as a competitor of the natural substrate (dGTP) for incorporation into the elongating DNA chain (De Clercq, 1995). CDV is a nucleotide analogue that bypasses the initial phosphorylation step catalyzed by the viral TK (De Clercq et al., 1986; De Clercq et al., 1987). This drug is phosphorylated within the cell by cellular kinases to its diphosphate active form, which is also a competitive inhibitor of the viral DNA pol (Xiong et al., 1996; Xiong et al., 1997). Those antiviral compounds were then tested against our recombinant viruses in combination or not with 25 μg/ml of HyU, which represented a good compromise between antiviral activity and cytotoxicity. This concentration of HyU corresponds to approximately 330 μM, which is below clinically achievable peak concentrations estimated at 1000 μM with doses utilized during cancer therapy (Belt et al., 1980). In HIV-infected patients receiving 500 mg b.i.d. of HyU, a peak serum concentration of 135 μM was reported (Villani et al., 1996). Subsequently, a daily dose of 600 mg orally proved to have the best therapeutic ratio in combination with didanosine and stavudine in HIV-infected subjects (Lori et al., 2004). Further animal studies are thus needed to determine the most appropriate dose of HyU in combination with nucleoside analogues to treat HSV infection. An additive effect between HyU and those drugs against our HSV mutants was expected since the former diminishes the intracellular dNTP pool. This mechanism was well demonstrated by counteracting the effect of HyU on DNA synthesis following dNTP addition (Lagergren and Reichard, 1987). A decrease in dNTP pool would then favor the incorporation of phosphorylated drugs during viral replication. Such synergic effect between HyU and ACV has been previously reported for a single TK-deficient HSV-1 strain (Neyts and De Clercq, 1999) but, to our knowledge, this is the first report showing consistent additive effects between HyU and ACV or CDV on various TK and DNA pol HSV mutants. The precise mechanism by which HyU increases the susceptibility of DNA pol mutants to nucleoside/nucleotide analogues remains to be investigated. FOS is a pyrophosphate analogue that directly inhibits the viral DNA pol without prior viral or cellular phosphorylation (Chrisp and Clissold, 1991). In agreement with this non-competitive mechanism, we found no additive effect between HyU and FOS for all tested HSV-1 recombinant viruses. These data suggest that the results obtained with the combination of nucleoside/nucleotide analogues and HyU do not stem from HyU-related toxicity. Moreover, when Vero cells were treated with nucleoside analogues at concentrations of 100 or 20 μg/ml together with different concentrations of HyU, the viability of the cultures did not deviate much from that of the cultures that had been treated with HyU only (Neyts and De Clercq, 1999). Considering the limited antiviral options available for treatment of ACV-resistant HSV infection (i.e. FOS and CDV) and their important toxicity, the data obtained with HyU may be of clinical relevance although further animal and human studies are required.

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