Pergamon

Biochemical Pharmacology, Vol. 49, No. 6, pp. 763–766, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00543-5

DEGLYCOSYLATION OF ANTIHERPESVIRAL 5-SUBSTITUTED ARABINOSYLURACIL DERIVATIVES BY RAT LIVER EXTRACT AND ENTEROBACTERIA CELLS

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(*Received* 29 *August* 1994; *accepted* 19 *October* 1994)

Abstract—A number of antiherpesviral 5-substituted derivatives of 1-β-D-arabinofuranosyluracil (araU) were significantly resistant to phosphorolysis by rat liver extract (S-9), but were gradually deglycosylated in a 2% enterobacteria cell suspension. The relative order of the resistance conferred by the different C-5 substituents was: 5-propynyl > 5-(*E*)-2-bromovinyl > 5-(*E*)-2-chlorovinyl > 5-methyl > 5-iodo. The 2′-fluoro derivatives of araU were completely resistant to phosphorolysis by both liver extract and enterobacteria, whereas the corresponding ribofuranosyl and 2′-deoxyribofuranosyl nucleosides were easily phosphorolysed by S-9, and were immediately cleaved in a 1% enterobacteria cell suspension. These findings suggest that antiherpesviral 5-substituted araU analogues can be relatively stable *in vivo*, when injected intravenously, and that degradation of 1-β-D-arabinofuranosyl-5-(*E*-2-bromovinyl)uracil (sorivudine) following oral administration is due primarily to the action of enterobacteria.

Key words: antiherpesvirus; BV-araU; pyrimidine nucleoside analogues; deglycosylation; enterobacteria; pyrimidine phosphorylase

BV-araU‖ (sorivudine) has potent and selective antiviral activity against herpes simplex virus type 1 and varicella-zoster virus in cell culture [1–3]. Oral BV-araU, giving high blood concentrations in humans [4], is effective in the treatment of patients with herpes zoster [5, 6], and was approved for the treatment of zoster in Japan in 1993. BVDU, the deoxyribonucleoside congener of BV-araU, and other 2′-deoxyuridine analogues are easily phosphorolysed to their corresponding bases.

A large amount of the inactive base BVUra was found in the plasma following either i.v. or oral administration of BVDU in mice [7] and i.p. injection in rats [8]. The degradation is attributed to two distinct phosphorylases, thymidine phosphorylase (EC 2.4.2.4) and uridine phosphorylase (EC 2.4.2.3) [9–11]. Such metabolic cleavage of antitumor and antiherpesviral nucleosides strongly affects their biological activity and therapeutic usefulness [8, 11]. Moreover, BVUra increases the 5-fluorouracil concentration of the blood by inhibiting the catabolism of 5-fluorouracil, resulting in a marked enhancement of the action of this anticancer agent [12]. Considerable amounts of BVUra also have been found in the plasma and urine of dogs [13], monkeys [14], and humans [4] following oral administration of BV-araU, although the levels of BVUra detected are much lower than those found after administration of BVDU. In contrast, no deglycosylated metabolite of BV-araU was detectable in the plasma and urine of mice after oral and i.v. administration, when assayed by HPLC [7], and only a minimal amount of BVUra was detected after i.v. administration of BV-araU in monkeys [14].

To determine the metabolic stability of BV-ara-U and other antiherpesviral araU derivatives, we investigated their phosphorolysis by activated rat liver extract, S-9, a 9000 *g* supernatant prepared from the liver of rats pretreated with phenobarbital and 5,6-benzoflavone, and enterobacteria. The phosphorolytic cleavages of some of the corresponding 5-substituted uridine, 2′-deoxyuridine and 2′-fluoro-araU analogues were compared.

MATERIALS AND METHODS

Compounds. The following pyrimidine nucleosides were used: araT, BV-araU, CV-araU, Pyr-araU, I-araU, FMAU, F-BV-araU, thymidine, IDU, BVDU, BV-riboU, and riboT. These nucleosides and the corresponding bases were synthesized in our Chemistry Laboratory. Synthesized compounds were identified by NMR and mass spectrometry, and the purity was determined to be 98% or greater by HPLC.

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‖ Abbreviations: BV-araU, 1-β-D-arabinofuranosyl-5-(*E*-2-bromovinyl)uracil; araT, 1-β-D-arabinofuranosylthymine; araU, 1-β-D-arabinofuranosyluracil; CV-araU, 1-β-D-arabinofuranosyl-5-(*E*-2-chlorovinyl)uracil; Pry-araU, 1-β-D-arabinofuranosyl-5-(1-propynyl)uracil; I-araU, 1-β-D-arabinofuranosyl-5-iodouracil; FMAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil; F-BV-araU, 5-(*E*-2-bromovinyl)-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil; BVDU, 5-(*E*-2-bromovinyl)-2′-deoxyuridine; IDU, idoxuridine; riboT, 1-β-D-ribofuranosylthymine; BV-riboU, 5-(*E*-2-bromovinyl)uridine; BVUra, 5-(*E*-2-bromovinyl)uracil; and TEAA, triethylammonium acetate buffer, pH 7.0.

Phosphorolytic reactions by rat liver extract and enterobacteria cells. Stock solutions of test compounds were prepared at a concentration of 10 mM in dimethyl sulfoxide. For the determination of phosphorolysis by rat liver extract, 40 μL of the stock solution was mixed with 0.4 mL of S-9 (Kikkoman, Noda, Japan) and 3.56 mL of 10 mM phosphate-buffered saline, and then incubated at 37° for 4 hr. Portions of the reaction mixture were taken at different times and boiled for 3 min in order to stop the reaction, and proteins were removed by centrifugation at 1500 *g* for 10 min. The supernatant was filtered through a 0.45-μm membrane filter. For degradation by enterobacteria, a cell suspension of *Klebsiella pneumoniae* (ATCC 8329), an enterobacterium with particularly strong pyrimidine phosphorylase activity, was prepared in 55 mM phosphate buffer (pH 7.0), as described previously [15]. A portion of the stock solution of a test compound was combined with *K. pneumoniae* cell suspension (1.1% or 2.2%) and dimethyl sulfoxide at a ratio of 1:18:1. The mixture was incubated at 37° for 4 hr. At different intervals, 0.5 mL of the reaction mixture was removed and filtered through a 0.45-μm membrane filter to remove the bacterial cells.

HPLC analysis. Each material obtained from the reaction mixture was assayed by HPLC using a Hitachi L-6000 System and an Inertsil ODS-2 column (4.6 mm i.d. × 250 mm; GL Sciences Inc., Tokyo). Ten microlitres of each filtered sample of reaction mixture was injected onto the column, and eluted at a flow rate of 1 mL/min. The following mobile phases were used to detect various pyrimidine nucleosides and their corresponding bases; riboT and thymine; 50 mM TEAA, thymidine, araT, and thymine; 50 mM TEAA for 20 min followed by 1% acetonitrile in 50 mM TEAA, FMAU and thymine; a linear gradient from 50 mM TEAA to 5% acetonitrile in 50 mM TEAA over 20 min, Pry-araU and 5-propynyluracil; IDU, I-araU, and 5-iodouracil; 5% acetonitrile in 50 mM TEAA, BVDU and BVUra; a linear gradient from 5% acetonitrile to 15% acetonitrile in 50 mM TEAA over 20 min, BV-araU, BV-riboU, and BVUra; CV-araU and 5-chlorovinyluracil; 15% acetonitrile in 50 mM TEAA, F-BV-araU, and BVUra; 20% acetonitrile in 50 mM TEAA. The eluate was monitored with a UV-detector set at 265 nm except for detection of 5-iodouracil compounds for which the detector was set at 288 nm. Test compounds and the corresponding bases were identified from the retention time of the authentic compounds in each HPLC condition. The concentration of compounds was estimated from the standard curves obtained by using authentic compounds. The detection limit for all compounds in the HPLC analysis was about 1 μM in the reaction mixture. The rate of deglycosylation in the reaction mixture was expressed as the percentage of phosphorolysis on a molar basis: the concentration of each specific base relative to the total concentrations of base and unchanged nucleoside. Each experiment was repeated to confirm the reproducibility of the test.

RESULTS

Deglycosylation by rat liver extract. As shown in Fig. 1A, araU analogues were fairly resistant to phosphorolysis by rat liver extract. Even araT, the least resistant among the araU analogues tested, was cleaved only 3% after 4 hr of incubation. Substitution of halogenovinyl or propynyl for the methyl group at the C-5 position of araT increased the resistance. Approximately 1% of BV-araU was degraded to BVUra after 24 hr of incubation with rat liver extract (data not shown). Although the differences were very small, the relative order of resistance to deglycosylation by rat liver extract for the different 5-substituted araU analogues was: Pry-araU > BV-araU ≅ CV-araU > araT. The amount of 5-iodouracil detected following incubation of I-araU with rat liver extract was also very small (1.8% of the amount of nucleoside used was detected as 5-iodouracil following 4 hr of incubation). However, the rate of phosphorolysis of I-araU could not be determined, because the recovery of I-araU from the reaction mixture decreased markedly with time during the incubation. No corresponding base was found after 4 hr of incubation of Pry-araU. FMAU and F-BV-araU, the 2′-fluoro-araU analogues, were completely resistant to deglycosylation by rat liver extract (data not shown). On the other hand, the corresponding 2′-deoxyuridine and uridine derivatives were very susceptible to phosphorolysis by rat liver extract (Fig. 1B). Substitution at the C-5 position of thymidine with iodine increased the rate of phosphorolysis, while substitution with *E*-2-bromovinyl had no effect on the rate of degradation.

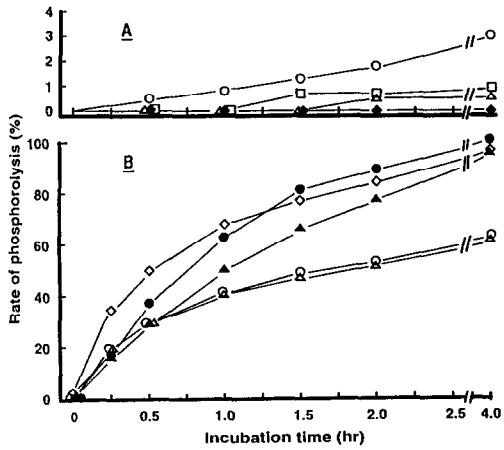

Fig. 1. Deglycosylation of antiviral araU analogues and the corresponding uridine and 2′-deoxyuridine derivatives by rat liver extract (S-9). Drugs were incubated with S-9 at a final concentration of 0.1 mM at 37°. The rate of phosphorolysis (%) is presented as conversion of the nucleoside tested to the corresponding base, determined by HPLC, on a molar basis. Each point shows the mean of two determinations. Key: (A) araT (○), BV-araU (△), CV-araU (□), and Pry-araU (◆); (B) thymidine (○), BVDU (△), IDU (◇), riboT (●), and BV-riboU (▲).

Deglycosylation by enterobacteria cells. Thymidine, riboT, and IDU were almost completely cleaved in a 1% *K. pneumoniae* cell suspension within 15 min (Fig. 2A). Thymidine, in particular, was completely

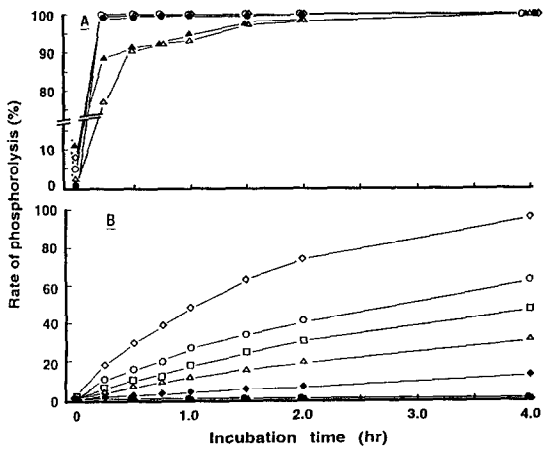

Fig. 2. Deglycosylation of araU, 2′-deoxyuridine, uridine, and 2′-fluoro-araU derivatives by enterobacteria cells. Compounds were incubated in a 1% (A) or 2% (B) *K. pneumoniae* cell suspension at a final concentration of 0.5 mM at 37°. The rate of phosphorolysis (%) is presented as conversion of the nucleoside tested to the corresponding base, determined by HPLC, on a molar basis. Each point shows the mean of two determinations. Key: (A) thymidine (○), IDU (◇), BVDU (△), riboT (●), and BV-riboU (▲); (B) araT (○), I-araU (◇), BV-araU (△), CV-araU (□), Pry-araU (◆), FMAU (●), and F-BV-araU (▲). The asterisks (*) indicate bases detected in the reaction mixtures at 0 time. See also text.

cleaved after as little as 5 min of incubation (data shown). Despite the fact that for the 0-time assay the reaction mixture was prepared on ice, 2–11% of the total amount of compounds tested could be detected as the specific bases due to the rapid phosphorolysis. All 2′-deoxyuridine and uridine derivatives tested were completely cleaved after 4 hr of incubation, although the initial rate of phosphorolysis of BVDU and BV-riboU was slightly slower than that of the thymine nucleosides. When the phosphorolysis occurred this rapidly, no marked difference in the rate of phosphorolysis of 2′-deoxyuridine and uridine derivatives, with the same base, could be detected. In contrast, araT and BV-araU were relatively resistant to degradation in the 1% *K. pneumoniae* cell suspension (data not shown).

A clear difference was seen in the rate of phosphorolysis of araU analogues when tested in a 2% *K. pneumoniae* cell suspension with different substituents at the C-5 position (Fig. 2B). The relative rates of phosphorolysis were calculated by dividing the rate of phosphorolysis of araU analogues by the rate of generation of BVUra at the 30 min incubation. The relative rates for I-araU, araT, CV-araU, BV-araU, and Pry-araU were 4.8, 2.5, 1.6, 1.0, and 0.3, respectively. Replacement of the C-5 methyl group of araT with iodine caused a marked increase in the rate of deglycosylation, whereas substitution with halogenovinyl or propynyl lowered the rate of phosphorolysis. FMAU and F-BV-araU were completely resistant to phosphorolytic cleavage under these conditions.

DISCUSSION

Our study demonstrates that antiherpesviral 5-substituted araU derivatives are generally resistant to phosphorolysis by rat liver extract, in contrast to the corresponding ribo- and deoxyribofuranosyl nucleoside analogues. However, in enterobacteria cell suspensions, araU analogues were phosphorolysed. Some differences were seen in the rate of phosphorolysis of araU analogues with different substituents at the C-5 position. The relative order of resistance to phosphorolysis for different 5-substituted araT analogues was: propynyl > *E*-2-bromovinyl > *E*-2-chlorovinyl > methyl > iodo. Also of interest, a large amount of 5-propynyluracil was recovered from the urine of humans after oral administration of Pry-araU*, a novel oral anti-varicella-zoster virus drug [16]. Thus, enterobacteria and mammalian pyrimidine phosphorylases both determine the metabolic fate of antiherpetic pyrimidine nucleosides, especially when drugs are administered orally.

Since incubation of BV-araU with the contents of rat cecum resulted in the formation of BVUra, the phosphorolytic cleavage of BV-araU, unlike BVDU, was suggested to be caused by the action of enterobacteria [17]. As shown in the present study, only a very small amount of BVUra was formed by incubation of BV-araU with rat liver extract, whereas a significantly greater amount of BV-araU was degraded to BVUra during incubation with an enterobacteria cell suspension. In our previous pharmacokinetic studies, very little BVUra was detected in the plasma of germ-free rats after oral administration of BV-araU, but considerable amounts of BVUra were found in specific pathogen-free rats [15]. These *in vitro* and *in vivo* observations are very consistent, and indicate that the formation of BVUra after oral administration of BV-araU in conventional animals and humans is due primarily to the effects of enterobacteria. This conclusion is supported by the observation that little degradation of BV-araU to BVUra can be detected following i.v. administration in humans.† Taken together, i.v. administration of BV-araU, and other araU analogues, can minimize the formation of the corresponding inactive base and could significantly improve the therapeutic efficacy of these compounds.

REFERENCES

1. Machida H, Sakata S, Kuninaka A and Yoshino H, Antiherpesviral and anticellular effects of 1-β-D-arabinofuranosyl - *E* - 5 - (2 - halogenovinyl)uracils. *Antimicrob Agents Chemother* **20**: 47–52, 1981.

* Peck RW, Wootton R, Weatherley BC and Posner J, The single dose pharmacokinetics and metabolism of 882C87, a potent anti-VZV agent with a long half-life. *7th International Conference on Antiviral Research*, Charleston, SC, USA, 1994.

† Olsen S, Stewart M, Devault A, Weaver J, Sugerman A, Tu J, Hedden B, Shostack G, Reilly-Gauvin K and Sherman J, Ascending multiple-dose pharmacokinetic study of intravenous SQ32,756 (BV-araU). *31st Interscience Conference on Antimicrobial Agents and Chemotherapy*, New Orleans, LA, USA, 1991.

2. Machida H and Sakata S, *In vitro* and *in vivo* antiviral activity of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU) and related compounds. *Antiviral Res* **4**: 135–141, 1984.
3. Machida H, Comparison of susceptibilities of varicella-zoster virus and herpes simplex viruses to nucleoside analogs. *Antimicrob Agents Chemother* **29**: 524–526, 1986.
4. Ogiwara T, Mikami H, Nakamaru M, Ohtsuka A and Kumabara Y, Phase I clinical study of YN-72 (BV-araU, brovavir). *Jpn Pharmacol Ther* **18**: 507–523, 1990.
5. Niimura M, A double-blind clinical study in patients with herpes zoster to establish YN-72 (brovavir) dose. In: *Immunobiology and Prophylaxis of Human Herpesvirus Infections* (Eds. Lopez C, Mori R, Roizman B and Whitley RJ), pp. 267–275. Plenum Press, New York, 1990.
6. Hiraoka A, Masaoka T, Nagai K, Horiuchi A, Kanamaru A, Niimura M, Hamada T and Takahashi M, Clinical effect of BV-araU on varicella-zoster virus infection in immunocompromised patients with hematological malignancies. *J Antimicrob Chemother* **27**: 361–367, 1991.
7. Machida H, Sakata S and Saito K, Bromovinyl-arabinosyluracil (BV-araU): Antiviral activity and pharmacology. In: *Herpes Viruses and Virus Chemotherapy* (Eds. Kono R and Nakajima A), pp. 63–66. Excerpta Medica, Amsterdam, 1985.
8. Desgranges C, Razaka G, Drouillet F, Bricaud H, Herdewijn P and De Clercq E, Regeneration of the antiviral drug (*E*)-5-(2-bromovinyl)-2′-deoxyuridine *in vivo*. *Nucleic Acids Res* **12**: 2081–2090, 1984.
9. Desgranges C, Razaka G, Rabaud M, Bricaud H, Balzarini J and De Clercq E, Phosphorolysis of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and other 5-substituted-2′-deoxyuridines by purified human thymidine phosphorylase and intact blood platelets. *Biochem Pharmacol* **32**: 3583–3590, 1983.
10. Nakayama C, Wataya Y, Meyer RB and Santi DV Jr, Thymidine phosphorylase. Substrate specificity for 5-substituted 2′-deoxyuridines. *J Med Chem* **23**: 962–964, 1980.
11. Woodman PW, Sarrif AM and Heidelberger C, Specificity of pyrimidine nucleoside phosphorylases and the phosphorolysis of 5-fluoro-2′-deoxyuridine. *Cancer Res* **40**: 507–511, 1980.
12. Desgranges C, Razaka G, De Clercq E, Herdewijn P, Balzarini J, Drouillet F and Bricaud H, Effect of (*E*)-5-(2-bromovinyl)uracil on the catabolism and antitumor activity of 5-fluorouracil in rats and leukemic mice. *Cancer Res* **46**: 1094–1101, 1986
13. Sakamoto H, Yokoyama N, Nishimoto T, Ohata K, Kohno S, Murai K and Tatsumi H, Studies on the metabolic fate of brovavir (YN-72). V. Absorption, distribution, metabolism and excretion in dogs. *Iyakuhin Kenkyu* **21**: 410–420, 1990.
14. Soike K, Huang J-L, Tu J-I, Stouffer B, Mitroka JG, Swerdel M, Olsen S, Bonner DP, Toumari AV and Field AK, Oral bioavailability and anti-simian varicella virus efficacy of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU) in monkeys. *J Infect Dis* **165**: 732–736, 1992.
15. Ashida N, Ijichi K, Watanabe Y and Machida H, Metabolism of 5′-ether prodrugs of 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil in rats. *Biochem Pharmacol* **46**: 2201–2207, 1993.
16. Rahim SG, Trevidi N, Selway J, Darby G, Collins P, Powell KL and Purifoy DJM, 5-Alkynyl pyrimidine nucleosides as potent selective inhibitors of varicella-zoster virus. *Antiviral Chem Chemother* **3**: 293–297, 1992.
17. Nishimoto T, Yokoyama N, Sakamoto H, Ohata K, Kohno S, Murai K and Tatsumi H, Studies on the metabolic fate of brovavir (YN-72). II. Metabolism in rats. *Iyakuhin Kenkyu* **21**: 378–389, 1990.