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METABOLISM OF 5′-ETHER PRODRUGS OF 1-β-D-ARABINOFURANOSYL-*E*-5-(2-BROMOVINYL)URACIL IN RATS

NORIYUKI ASHIDA, KATSUSHI IJICHI, YOHKO WATANABE and HARUHIKO MACHIDA*
Biology Laboratory, R & D Division, Yamasa Corporation, Choshi 288, Japan

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Abstract—1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU) is a selective antiherpesviral agent that has been shown to be metabolically stable in mice. However, *E*-5-(2-bromovinyl)uracil (BVU) is the major metabolite found after oral dosing in animals other than mice. When BV-araU was given orally to germ-free rats, only small amounts of BVU were found in the plasma, suggesting an important role of enterobacteria in the formation of BVU. Then, the metabolism of BV-araU prodrugs was studied in specific-pathogen free rats to select oral prodrugs of BV-araU with enhanced metabolic stability. 5′-*O*-Ethyl BV-araU (Et-BV-araU) gave about a 2-fold higher BV-araU blood concentration 3 and 6 hr after administration than after oral dosing of BV-araU, while the level of BVU was lower. Other aliphatic alkyl prodrugs also gave a lower level of BVU, but did not give the same elevation in blood concentration of BV-araU as did Et-BV-araU. Dosing of 5′-*O*-acetyl BV-araU resulted in blood concentrations of BV-araU and BVU similar to those after oral administration of BV-araU. 5′-*O*-Aromatic alkyl prodrugs showed poor bioavailability. A nearly 2-fold higher urinary recovery rate was seen for Et-BV-araU than for BV-araU or 5′-*O*-acetyl BV-araU. The conversion of Et-BV-araU to BV-araU was demonstrated *in vitro* using rat liver extract in the presence of co-factors, although the reaction was slow. The 5′-*O*-aliphatic alkyl prodrugs were completely resistant to degradation by enterobacteria, whereas the esters were partially degraded to BVU. Et-BV-araU may be a useful oral prodrug of BV-araU due to its increased metabolic stability and bioavailability.

Key words: alkyl prodrugs of BV-araU, blood concentration, BV-araU, BV-uracil, metabolism of BV-araU, urinary recovery

1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU†, sorivudine) shows potent and selective antiviral activity against herpes simplex virus type 1 (HSV-1) and varicella-zoster virus in cell culture [1–3]. This compound has exhibited antiviral effects against several experimental HSV-1 infections in mice [4–6] and against simian varicella virus infections in monkeys [7, 8]. Oral BV-araU is effective in the treatment of patients with herpes zoster [9, 10], and has been approved for the treatment of zoster in Japan. When tested in mice, BV-araU was well absorbed through the gastrointestinal tract and was much more metabolically stable than its deoxyribonucleoside congener, *E*-5-(2-bromovinyl)-2′-deoxyuridine [11]. However, considerable amounts of *E*-5-(2-bromovinyl)uracil (BVU) have been found in the plasma of rats, monkeys, and humans after oral administration of BV-araU [8, 12, 13], whereas only a minimum amount of BVU was found after i.v. injection of BV-araU in monkeys [8]. BV-araU has been deglycosylated to form BVU by treatment with the contents of rat cecum [12], and the presence of BVU seems to be correlated with the toxicity found in rats given chronic oral treatments with BV-araU [14]. The present study is part of an ongoing effort to find more stable oral prodrugs of BV-araU.

We previously synthesized 5′-*O*-alkyl prodrugs of BV-araU in a search for oral prodrugs of BV-araU resistant to deglycosylation by enterobacteria [15]. Some of the synthesized 5′-*O*-aromatic and 5′-*O*-short-chain aliphatic alkyl derivatives gave high BV-araU plasma concentrations in mice. These aliphatic alkyl prodrugs were stable even in acidic conditions, whereas the corresponding esters were not. However, there was little difference in the blood concentrations of BV-araU and little BVU was found after oral administration in mice of either the 5′-*O*-alkyl prodrugs or the acyl BV-araU congeners. To further evaluate these prodrugs, it is necessary to study their metabolism in animals that may have higher BVU

* Corresponding author: Haruhiko Machida, Ph.D., Biology Laboratory, R & D Division, Yamasa Corporation, 10-1, Araoicho 2-chome, Choshi, Chiba-ken 288, Japan. Tel. (81) 479-22-9835; FAX (81) 479-22-9845.

† Abbreviations: BV-araU, 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil; BVU, *E*-5-(2-bromovinyl)uracil; Et-BV-araU, 5′-*O*-ethyl BV-araU; Pr-BV-araU, 5′-*O*-propyl BV-araU; Bu-BV-araU, 5′-*O*-butyl BV-araU; Ac-BV-araU, 5′-*O*-acetyl BV-araU; F-Bn-BV-araU, 5′-*O*-(2-fluorobenzyl) BV-araU; Cl-Bn-BV-araU, 5′-*O*-(2-chlorobenzyl) BV-araU; DiCl-Bn-BV-araU, 5′-*O*-(2,4-dichlorobenzyl) BV-araU; HSV-1, herpes simplex virus type 1; PB, phosphate buffer; and SPF, specific-pathogen free.

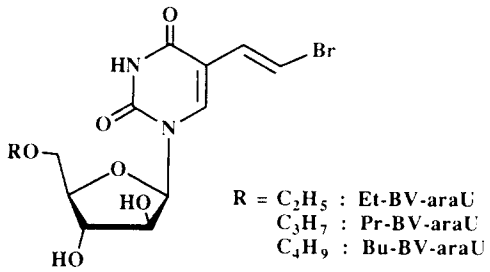

Fig. 1. Structural formulae of 5′-*O*-short-chain aliphatic alkyl prodrugs of BV-araU.

plasma levels than do mice after oral dosing of these prodrugs. The present paper reports on the metabolism of the 5′-*O*-alkyl prodrugs in rats and *in vitro*, the conversion to BV-araU, and the resistance to enterobacteria. We also report on the metabolism of 5′-*O*-acetyl BV-araU (Ac-BV-araU) for comparison.

MATERIALS AND METHODS

Compounds. The following BV-araU prodrugs were used: 5′-*O*-ethyl BV-araU (Et-BV-araU), 5′-*O*-propyl BV-araU (Pr-BV-araU), 5′-*O*-butyl BV-araU (Bu-BV-araU), Ac-BV-araU, 5′-*O*-butyryl BV-araU, 5′-*O*-(2-fluorobenzyl) BV-araU (F-Bn-BV-araU), 5′-*O*-(2-chlorobenzyl) BV-araU (Cl-Bn-BV-araU), 5′-*O*-(2,4-dichlorobenzyl) BV-araU (DiCl-Bn-BV-araU). These were selected on the basis of the high blood levels of BV-araU resulting from their oral administration in mice. The chemical structures of the 5′-*O*-short-chain aliphatic alkyl prodrugs of BV-araU are given in Fig. 1. The synthesis of 5′-ether prodrugs is described elsewhere [15].

Blood concentrations of BV-araU and BVU and their urinary excretion. BV-araU was administered orally by gavage or i.v. to 8-week-old, male, specific-pathogen free (SPF) SD rats, in groups of 4 or 5 animals, at a dose of 100 μmol/kg. For comparison, the same procedure was used on 8-week-old, male, germ-free Wistar rats. All experimental procedures using germ-free rats were performed in sterile conditions. All prodrugs were given orally to the SPF rats. Plasma was obtained from the heart of each rat 3, 6, 12, and 24 hr after administration. The urine was collected from the rats over a 24-hr period. The concentrations of BV-araU, BVU, and unchanged prodrugs in plasma and those of BV-araU and BVU in the urine specimens were determined by HPLC.

In vitro *conversion of prodrugs to BV-araU with rat liver extract.* Solutions of BV-araU and the prodrugs used in the above experiment were prepared at a concentration of 100 μg/mL in 1% dimethyl sulfoxide in 8 mM phosphate buffer (PB). These solutions were mixed with rat liver extract (S-9) (Kikkoman, Noda, Japan) or with an S-9 mixture consisting of S-9 and co-factors for S-9 (Oriental Kobo, Tokyo, Japan), and then incubated at 37° for 6 hr. The co-factors contained a final concentration of 0.25 mM glucose-6-phosphate, 0.2 mM NADPH and 0.2 mM NADH. To stop the reaction and for removal of proteins, an equal volume of cold ethanol was added to a portion of the reaction mixtures. The materials were centrifuged at 1500 *g* for 10 min, and the supernatant was filtered through a 0.45 μm membrane filter. The filtrate was assayed by HPLC.

Degradation by enterobacteria cells. Solutions of test compounds were prepared in 20% dimethyl sulfoxide at a concentration of 0.2 mM. Aliquots of 1 mL were mixed with 1 mL of a 20% *Klebsiella pneumoniae* cell suspension prepared in 10 mM PB, pH 7.0. This strain had been selected from type cultures of enterobacteria known to produce high levels of pyrimidine phosphorylase activity, as determined at our institute (Yamauchi H, Yamaguchi T, Kumagai M and Machida H, unpublished data). For the reaction without cells, the mixture was prepared in the same way except that the bacterial cells were omitted. The mixture was incubated at 37° for 24 hr, and then centrifuged at 13,000 *g* for 5 min to spin down the bacterial cells. The supernatant was filtered through a 0.45 μm membrane filter, and assayed by HPLC to estimate degradation of the test compounds to BV-araU and BVU. 5′-*O*-Benzyl prodrugs were not tested due to their low solubility.

HPLC analysis. A Hitachi L-6000 System and an Inertsil ODS-2 column (250 × 4.6 mm i.d., GL Sciences, Tokyo, Japan) were used for all HPLC analyses. To determine the concentration of BV-araU and BVU in rat plasma, 10 μL of each filtered plasma sample was injected onto the column and the eluate was monitored with a UV-detector set at 265 nm. The samples were eluted with 20.25% acetonitrile in 0.1 M acetate buffer. For detection of unchanged Et-BV-araU and Ac-BV-araU, and Pr-BV-araU, Bu-BV-araU, and benzyl prodrugs, we used 36% acetonitrile and 45% acetonitrile in 0.1 M acetate buffer, respectively, as the mobile phase.

The amounts of BV-araU and BVU in the urine were measured in the same manner, under the following conditions: 10 μL of each diluted and filtered urine sample was injected onto the column and eluted with a linear gradient from 10 mM PB, pH 7.0, to 10% acetonitrile in 10 mM PB over 20 min. This was followed by 10% acetonitrile in 10 mM PB with the UV-detector set at 290 nm.

The following procedure was used for the analyses of reaction mixtures with S-9 in the presence or absence of co-factors, and the incubation mixture with bacterial cells: 20 μL of each sample was injected onto the column and the sample was eluted with a linear gradient from 10% acetonitrile in 50 mM triethylammonium acetate buffer, pH 7.0, to 50% acetonitrile over 30 min, followed by 50% acetonitrile in 50 mM triethylammonium acetate buffer. The eluate was monitored with a UV-detector set at 265 nm.

RESULTS

Blood concentrations of BV-araU and BVU, and urinary excretion in SPF and germ-free rats after dosing of BV-araU. To elucidate the role of

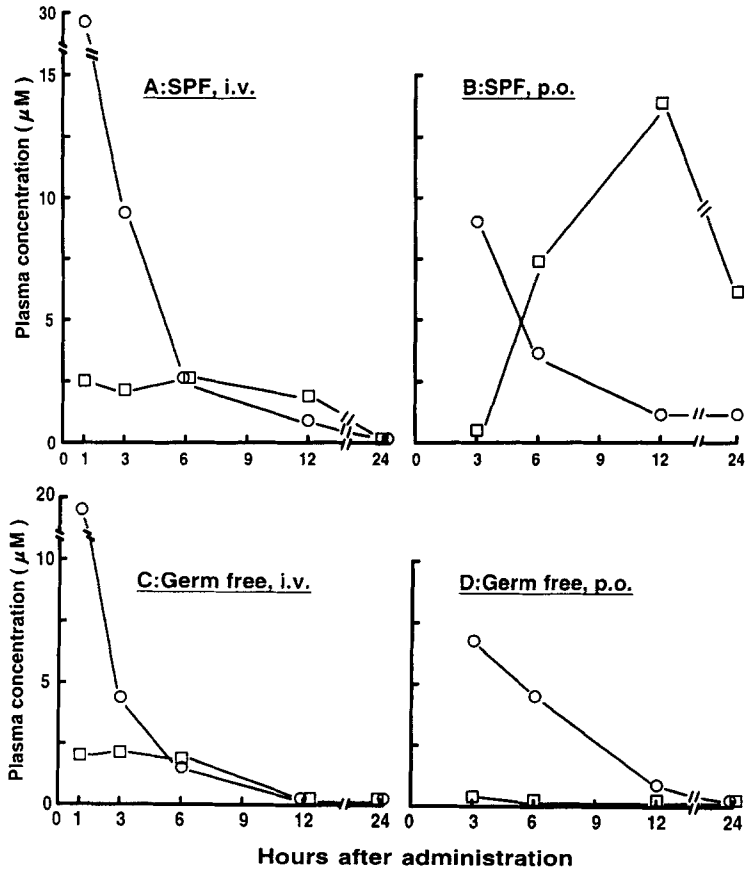

Fig. 2. Blood concentrations of BV-araU and BV-uracil (BVU) in the SPF and germ-free rats after administration of BV-araU. BV-araU was administered i.v. (A and C) or orally (B and D) in SPF male SD rats (A and B) or germ-free male Wistar rats (C and D), at a dose of 100 μmol/kg. Blood concentrations of BV-araU (○) and BVU (□) were determined by HPLC. Each point represents the mean of 4 or 5 rats.

enterobacteria in the formation of BVU after oral administration of BV-araU, blood concentrations of BV-araU and BVU were compared between SPF and germ-free rats. Figure 2A shows that a low level of BVU was detected in the blood of SPF rats at 3–12 hr after i.v. administration. Considerable amounts of BVU were found in the plasma at 6–24 hr after oral administration, and eventually exceeded the concentration of BV-araU (Fig. 2B). In contrast, the blood level of BVU after oral dosing in the germ-free rats was below the detection limit by HPLC analysis (1 μM), with the exception of one of the four rats, which showed a blood level of 1.5 μM at 3 hr after dosing (Fig. 2D). Plasma levels of BVU resulting from i.v. administration in germ-free rats remained low for 6 hr (Fig. 2C), as in the SPF rats. The amount of BVU recovered from urine after 24 hr of collection from germ-free rats given BV-araU orally was 0.15 μmol per animal, which was the same as that from the rats receiving BV-araU via i.v. injection.

Blood concentrations of BV-araU, BVU, and unchanged prodrugs in SPF rats after oral dosing of prodrugs. Figure 3 shows the blood concentrations of BV-araU, BVU, and unchanged prodrugs 3–24 hr after oral administration of prodrugs in SPF rats. Administration of Et-BV-araU resulted in high blood concentrations of BV-araU but low levels of BVU (Fig. 3A). The blood levels at 3 and 6 hr after administration were about 2-fold higher than those after oral administration of BV-araU. The blood level of BVU was similar to the level after i.v. administration of BV-araU. Unchanged Et-BV-araU was detected in the plasma for 12 hr after administration. When Pr-BV-araU was administered, the formation of BVU was reduced compared with after oral dosing of BV-araU (Fig. 3B). However, it did not give the same elevation in blood concentration of BV-araU as did Et-BV-araU. Similar results were seen for Bu-BV-araU with the exception that levels of BV-araU and unchanged prodrug at 3 hr were lower than after dosing with Pr-BV-araU (data not shown). The changes of blood levels of BV-araU and BVU after dosing with Ac-BV-araU were very similar to those resulting from oral dosing with BV-araU. No unchanged Ac-BV-

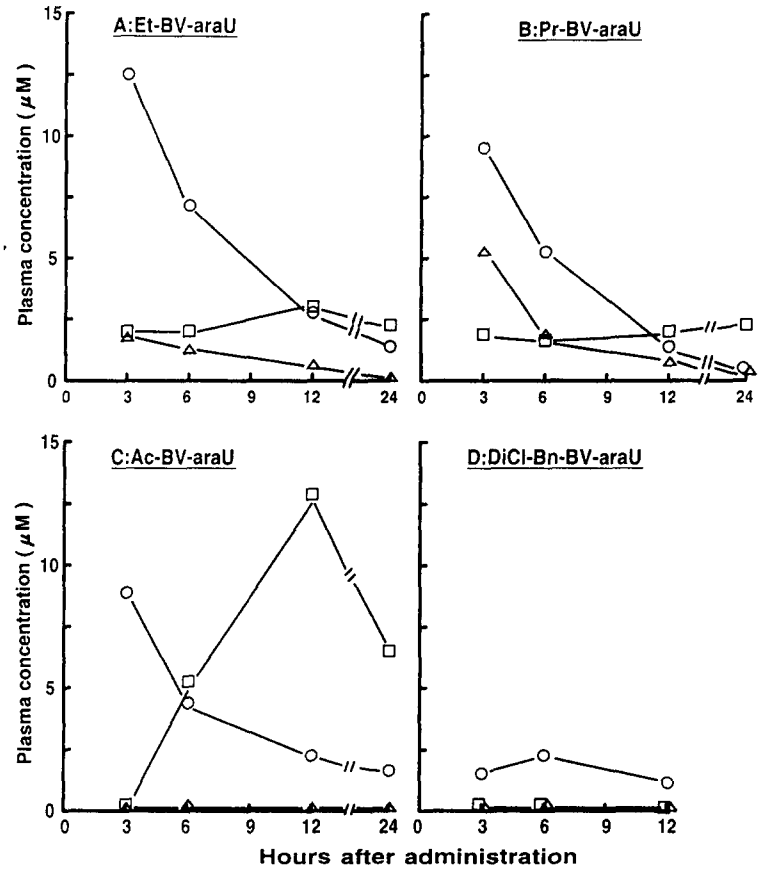

Fig. 3. Blood concentrations of BV-araU, BVU, and unchanged prodrugs after oral administration of prodrugs. Et-BV-araU (A), Pr-BV-araU (B), Ac-BV-araU (C), and DiCl-Bn-BV-araU (D) were administered orally in SPF male SD rats at a dose of 100 μmol/kg. Blood concentrations of BV-araU (○), BVU (□), and unchanged prodrugs (△) were determined by HPLC. Each point represents the mean of 4 or 5 rats. The blood concentration 24 hr after administration of DiCl-Bn-BV-araU was not followed.

araU was detected during the experimental period (Fig. 3C). Administration of DiCl-Bn-BV-araU resulted in low levels of blood BV-araU (Fig. 3D). The same was true when rats were given F-Bn-BV-araU and Cl-Bn-BV-araU (data not shown). None of the benzyl derivatives were detected as unchanged form in the plasma after oral administration.

Urinary excretion of BV-araU and BVU. Nearly 2-fold higher urinary recovery of BV-araU was found for Et-BV-araU than or oral BV-araU (Fig. 4). On the other hand, the amount recovered as BVU was smaller than that detected after oral administration of BV-araU such that the ratio of BVU to BV-araU found in the urine samples was lower than after oral administration. The recovery of BV-araU decreased with chain-length of the alkyl group, but the recovery even for Bu-BV-araU was slightly higher than that for oral BV-araU. The amount of BV-araU recovered after administration of Ac-BV-araU was somewhat lower than that after oral BV-araU. Only a small amount of BV-araU was recovered after oral administration of Cl-Bn-BV-araU or DiCl-Bn-BV-araU. The urinary recovery for F-Bn-BV-araU was equal to that for Cl-Bn-BV-araU (data not shown).

Conversion of prodrugs to BV-araU in vitro. Et-BV-araU was gradually converted to BV-araU in an S-9 mixture, in the presence of co-factors (Fig. 5), but failed to convert in the absence of co-factors. In contrast, Ac-BV-araU was converted rapidly (30 min) to BV-araU in both the presence and absence of co-factors. This was also the case for 5′-*O*-butyryl BV-araU (data not shown). A very small amount (0.2 to 0.8%) of BVU was detected during the incubation of BV-araU and the acyl prodrugs for 1 and 3 hr.

Degradation of prodrugs by enterobacteria cells. Table 1 shows degradation of drugs by enterobacteria (*K. pneumoniae*). BV-araU was easily deglycosylated in the highly concentrated suspension of enterobacteria cells. About half of the BV-araU was converted to BVU after 4 hr of incubation. However, no degradation of 5′-*O*-short-chain aliphatic alkyl prodrugs of BV-araU was seen after 24 hr of incubation. Large portions of Ac-BV-araU and 5′-*O*-butyryl BV-araU were degraded to BVU. These portions were larger than those hydrolyzed to BV-araU without bacteria cells but containing the same concentration of DMSO (non-enzymatic hydrolysis).

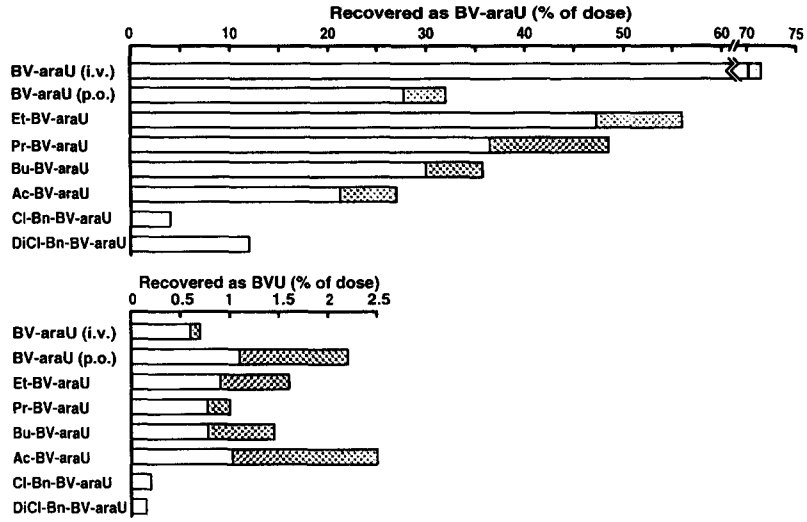

Fig. 4. Urinary recovery of BV-araU prodrugs. SPF SD male rats were given BV-araU or a prodrug orally, or BV-araU i.v., at a dose of 100 μmol/kg. Urine was collected for 0–12 hr (open bars) and for 12–24 hr (shaded bars) and was analyzed by HPLC. Urinary recovery as BV-araU and BVU is expressed separately as a percent of the dose. Each value represents the mean of 4 or 5 rats. The urine from rats given the benzyl prodrugs was collected only for 0–12 hr.

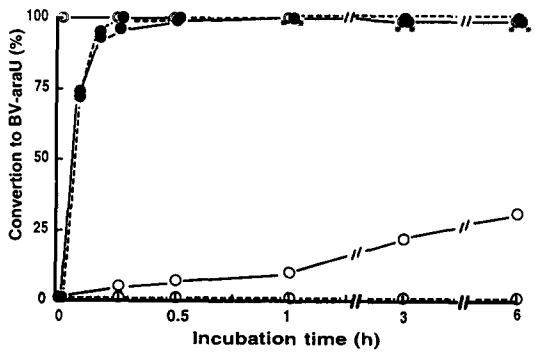

Fig. 5. Conversion of prodrugs to BV-araU with rat liver extract. Et-BV-araU (○), Ac-BV-araU (●), and BV-araU (◎) were incubated with S-9 at 37° in the presence (S-9 mixture; solid lines) or absence (S-9 only; broken lines) of co-factors, and were analyzed by HPLC. Conversion to BV-araU is presented as the molar percent of BV-araU in the reaction mixture. Each point represents the mean of two separate experiments. Key: (*) indicates the presence of a small amount of BVU (0.2 to 0.8%) in the reaction mixture with co-factors.

These ester prodrugs could be hydrolyzed to BV-araU both spontaneously under the test conditions and by the action of bacteria cells.

DISCUSSION

Several studies have described the ester prodrugs of antiherpesviral nucleosides, such as acyclovir, penciclovir, and 6-methoxypurine arabinoside [16–19]. The introduction of aliphatic alkyl groups into the terminal hydroxy groups of a ganciclovir has also been reported to improve pharmacokinetic properties and *in vivo* antiherpesviral potency [20]. These prodrugs were developed to improve oral absorption and solubility in water or organic solvents. By contrast, the oral prodrugs of BV-araU have not been actively developed because BV-araU shows good bioavailability and because no major metabolite had been detected in the plasma or urine of mice after oral or i.v. administration [11]. Additionally, BV-araU is not deglycosylated to BVU in rat liver extract in the absence of co-factors.* Recently, however, BVU has been detected in the plasma of animals other than mice after oral administration of BV-araU [8, 12, 13]. The present results indicate that BV-araU is easily deglycosylated to BVU when given orally in SPF rats. However, little BVU was detected in the plasma of germ-free rats, and oral or i.v. administration gave similar levels of BVU in the urine of germ-free rats. These results indicate that the formation of BVU is predominantly due to the action of enterobacteria. A very small amount of BVU was formed by incubation of BV-araU in the presence of co-factors. This is consistent with the finding that a very small amount of BVU was detected in the plasma of both germ-free and SPF rats after i.v. administration of BV-araU. It is likely that BV-araU is also deglycosylated to BVU in rat liver, although in smaller amounts than are formed by the action of enterobacteria.

We previously introduced alkyl groups into the 5' position of the arabinose moiety of BV-araU in

* Yamaguchi T, Kumagai M, Sakata S, Ikeda T, Ashida N and Machida H, Comparison of antiviral effects and enzymatic deglycosylation of BV-araU and related compounds. *Annual meeting of The Pharmaceutical Society of Japan*, 1990.

Table 1. Resistance of BV-araU prodrugs to degradation by enterobacteria

Compound	Formation of BV-araU and BV-uracil (molar %)			
	With cells		Without cells*	
	BV-araU	BVU	BV-araU	BVU
BV-araU	3.3†	96.7	100†	0
Et-BV-araU	0	0	0	0
Pr-BV-araU	0	0	0	0
Bu-BV-araU	0	0	0	0
Ac-BV-araU	3.2	11.8	5.8	0
5′-*O*-Butyryl BV-araU	12.5	32.0	3.3	0

The amounts of BV-araU and BV-uracil (BVU) were determined by HPLC after 24 hr of incubation with *K. pneumoniae* cells at 37°.

* Without *K. pneumoniae* cells in the presence of DMSO.

† Amounts of residual BV-araU.

order to prevent degradation by enterobacteria [15]. We evaluated 5′-*O*-short-chain aliphatic alkyl and some benzyl BV-araU derivatives *in vitro* and *in vivo*, which had given high blood concentrations of BV-araU in mice [15]. The 5′-*O*-benzyl BV-araU derivatives did not elevate the plasma level of BV-araU in rats and were not detected in the rat plasma as unchanged forms. These phenomena may be attributed to poor absorption through the gastrointestinal tract due to low water-solubility, low susceptibility to the enzyme(s) which converts the prodrugs to BV-araU, or both. These results conflict with the findings of Yamashita *et al.* [21], who reported that the 5′-*O*- or 3′-*O*-benzyl derivatives of 2′-deoxy-5-(trifluoromethyl)uridine and 2′-deoxy-5-fluorouridine enhance the antitimor activity of the parent compounds.

In the present study, 5′-*O*-short-chain aliphatic alkyl prodrugs of BV-araU were detected in the plasma as an unchanged form and gave higher blood BV-araU levels and urinary recovery than oral dosing of BV-araU in the SPF rats. Et-BV-araU gave the highest blood BV-araU level and urinary recovery. These ethers were completely resistant to degradation by enterobacteria, while some of the 5′-*O*-acyl BV-araU was degraded to BVU. The levels of BVU found in the plasma and urine after administration of the ether prodrugs were lower than those after oral administration of BV-araU or Ac-BV-araU. Conversion of Et-BV-araU to BV-araU was demonstrated *in vitro* using an S-9 mixture. These findings suggest that Et-BV-araU, given orally, can partially prevent the degradation to BVU *in vivo*. The increased metabolic stability and bioavailability of Et-BV-araU may make it a useful oral prodrug of BV-araU.

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