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SYNTHESIS, BIOTRANSFORMATION, PHARMACOKINETICS, AND ANTIVIRAL PROPERTIES OF 5-ETHYL-5-HALO-6-METHOXY-5,6-DIHYDRO-2′-DEOXYURIDINE DIASTEREOMERS

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Abstract—Diastereomers of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridine were synthesized by the regiospecific addition of XOMe (X = Br, Cl) to the 5,6-olefinic bond of 5-ethyl-2′-deoxyuridine (EDU). 5-(1-Hydroxyethyl)-2′-deoxyuridine (HEDU) was identified as a metabolite of the 5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine diastereomers (BMEDU). The concentration of EDU and 5-ethyluracil (EU) in blood was higher after i.v. administration of the bromo diastereomers (BMEDU) to rats, relative to the concentration of EDU and EU after injection of the chloro (CMEDU) diastereomers. The CMEDU diastereomers were found to be oxidized less extensively to HEDU, were more stable to glycosidic bond cleavage, and were converted more slowly to EDU, than the BMEDU compounds. These BMEDU and CMEDU diastereomers exhibited pharmacokinetics characterized by a biphasic decline in plasma concentration. All diastereomers exhibited a characteristic second maximum blood concentration (C_{max}), which was attributed to reabsorption after biliary excretion. All of these 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds, with the exception of (5*S*,6*S*)-BMEDU, had higher AUC values (ranging from 0.32 to 1.20 $\mu M \cdot hr \cdot mL^{-1}$) and lower plasma clearances (10–36 $mL \cdot min^{-1}$) relative to the AUC values (0.14 $\mu M \cdot hr \cdot mL^{-1}$) and plasma clearance (85 $mL \cdot min^{-1}$) of EDU. These BMEDU and CMEDU diastereomers are more lipophilic (log P = − 0.42 to 0.40 range) than EDU (log P = − 1.09), which should enhance their ability to cross the blood–brain barrier. These 5,6-dihydro compounds showed higher levels (11–22%) of binding to bovine serum albumin than EDU (7%). The BMEDU compounds exhibited equipotent *in vitro* antiviral activity to EDU against HSV-1 and HSV-2, whereas the CMEDU analogs were inactive. The (5*S*,6*R*)-CMEDU diastereomer was equipotent to ganciclovir in the human cytomegalovirus assay.

Key words: 5-ethyl-2′-deoxyuridine; 5,6-dihydro pyrimidine nucleosides; herpes simplex virus; pharmacokinetics; biotransformation

5-Ethyl-2′-deoxyuridine (EDU†, **1**, Fig. 1) exhibits antiviral activity against herpes simplex virus type-1 (HSV-1), type-2 (HSV-2) and vaccinia virus [1–5]. EDU is also effective in the treatment of herpes keratitis in rabbits [6, 7], systemic herpetic infection in mice [8, 9], and cutaneous herpes infections in guinea pigs [10]; it also increases the survival time of HSV-encephalitic mice [8, 9]. Clinical studies have indicated that EDU offers promise for the treatment of ocular herpes [6, 7, 11]. A recent study involving the treatment of patients with recurrent genital herpes using EDU 3% cream indicated reduced viral shedding, reduced signs of herpes, and good drug-tolerance [12].

EDU is a "natural" thymidine analog, which has the same base-pairing properties as thymidine [1]. One significant advantage of EDU is that it is not mutagenic [13]. The potent and selective antiviral activity of EDU is due to its preferential phosphorylation by virus-encoded thymidine kinase (TK) and its preferential incorporation into viral DNA [14, 15]. However, catabolic degradation of EDU by pyrimidine nucleoside phosphorylases results in cleavage of the glycoside bond to release 5-ethyluracil (EU, **5**, Fig. 1), which is inactive against viruses [16] and undergoes *in vivo* biotransformation to 5-(1-hydroxyethyl) uracil (HEU, **6**, Fig. 1). Similar metabolic reactions have also been reported for 5-(2-chloroethyl)-2′-deoxyuridine (CEDU, **2**, Fig. 1), which is a chlorinated analog of EDU [17].

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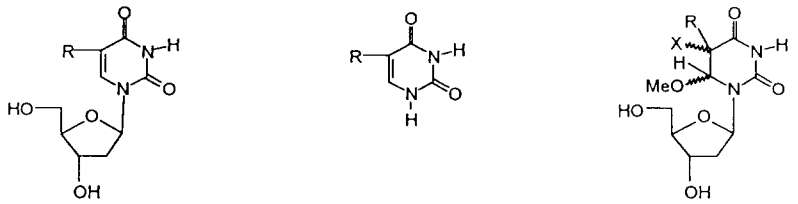
† Abbreviations: EDU, 5-ethyl-2′-deoxyuridine; HEDU, 5-(1-hydroxyethyl)-2′-deoxyuridine; CEDU, 5-(2-chloroethyl)-2′-deoxyuridine; EU, 5-ethyluracil; HEU, 5-(1-hydroxyethyl)uracil; GSH, glutathione; FUDR, 5-fluoro-2′-deoxyuridine; HSV-1, herpes simplex virus Type-1; HSV-2, herpes simplex virus Type-2; TK, thymidine kinase; HCMV, human cytomegalovirus; FU, 5-fluorouracil; HSE, herpes simplex encephalitis; PTLC, preparative thin-layer chromatography; *P*, partition coefficient; CPE, cytopathic effect; HFF, human foreskin fibroblast; (5*R*,6*R*)-BMEDU, (+)-*trans*-(5*R*,6*R*)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine; (5*S*,6*S*)-BMEDU, (−)-*trans*-(5*S*,6*S*)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine; (5*R*,6*R*)-CMEDU, (+)-*trans*-(5*R*,6*R*)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine; (5*S*,6*R*)-CMEDU, (+)-*cis*-(5*S*,6*R*)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine; AUC, area under the blood concentration versus time curve; and BBB, blood–brain barrier.



1, R = Et (EDU)
2, R = CH_2CH_2Cl (CEDU)
3, R = $CH(OH)CH_3$ (HEDU)
4, R = F (FUDR)

5, R = Et (EU)
6, R = $CH(OH)CH_3$ (HEU)
7, R = F (FU)

8, R = F, X = Br
9, R = F, X = Cl
10, R = Me, X = Br

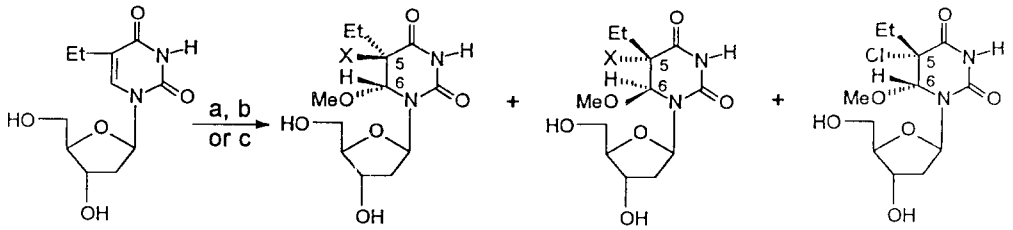
Fig. 1. Structures of EDU and related pyrimidine nucleosides, EU and 5,6-di-substituted pyrimidine nucleosides.

5,6-Dihydropyrimidine nucleosides have attracted attention as potential anticancer and antiviral agents, since these dihydro compounds play an important role in nucleic acid metabolism and frequently appear in the sequence of t-RNA [18]. 5-Fluoro-5-halo - 6 - methoxy - 5,6 - dihydro - 2' - deoxyuridine diastereomers (**8–9**, Fig. 1) have been investigated as prodrugs [19] to 5-fluoro-2'-deoxyuridine (FUDR, **4**, Fig. 1). The 5-halo diastereomers (**8–9**) possessed a number of desirable properties; for example, the 5-bromo (**8**) but not the 5-chloro (**9**) diastereomer undergoes regeneration of the 5,6-double bond upon *in vitro* incubation with glutathione (GSH) to give FUDR. The 5-halo diastereomers (**8–9**), unlike FUDR, were stable to pyrimidine phosphorylases. There was a correlation between the capacity of the diastereomers (**8**) to regenerate the 5,6-double bond *in vitro* to form FUDR and 5-fluorouracil (FU), and the antileukemic effect observed. Furthermore, the plasma half-life of FUDR in cancer patients treated with the (+)-diastereomer of **8** was 10-fold longer than that observed using FUDR. Other studies have shown that 5-bromo-6-methoxy-5,6-dihydrothymidine diastereomers (**10**, Fig. 1) act as competitive substrates of TK with respect to thymidine [20, 21]. These beneficial properties of the dihydropyrimidines **8–10** prompted us to investigate 5-halo-6-methoxy-5,6-dihydro analogs of EDU, which are expected to possess greater *in vivo* stability towards pyrimidine phosphorylases than EDU. The increased lipophilic character of these dihydro derivatives should enhance their ability to cross the blood–brain barrier (BBB), which is essential for the treatment of cephalic viral infections such as herpes simplex encephalitis (HSE). We now report the synthesis, biotransformation, pharmacokinetics and antiviral properties of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2'-deoxyuridine diastereomers (**11–15**, Scheme 1).

MATERIALS AND METHODS

Chemistry

Melting points were determined with a Buchi capillary apparatus and were uncorrected. Nuclear magnetic resonance spectra (^1H NMR, ^{13}C NMR) were determined on a Bruker AM-300 spectrometer using Me_4Si as an internal standard (^1H NMR). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O. ^{13}C NMR spectra were acquired using the J modulated spin echo technique where methyl and methine carbon resonances appear as positive peaks and methylene and quaternary carbons appear as negative peaks. Preparative thin-layer chromatography (PTLC) was performed using Whatman PLK5F plates, 1.0 mm in thickness, and silica gel column chromatography was carried out using Merck 7734 silica gel (100–200 μm particle size). EDU (**1**) and EU (**5**) were purchased from the Sigma Chemical Co. (St. Louis,



1 (5R,6R)-**11** (X = Br) (5S,6S)-**12** (X = Br)
(5R,6R)-**13** (X = Cl) (5S,6S)-**14** (X = Cl) (5S,6R)-**15**

Scheme 1. Reaction conditions for synthesis of 5,6-di-substituted EDU derivatives: (a) Br_2, MeOH, 25° (products **11–12**); (b) *N*-chlorosuccinimide, MeOH, HOAc, 25° (Method A, products **13** and **15**); and (c) Cl_2, MeOH, 25° (Method B, products **13**, **14** and **15**).

MO). HEDU (**3**, Fig. 1) and HEU (**6**) were prepared using literature procedures [22].

*(+)-*Trans*-(5R,6R)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**11**) and (−)-*trans*-(5S,6S)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**12**)*

A freshly prepared solution of methyl hypobromite was added dropwise to a solution of EDU (0.1 g, 0.39 mmol) in methanol (10 mL) at 25° with stirring until the yellow color persisted. The reaction was allowed to proceed with stirring at 25° for 15 min prior to neutralization with methanolic sodium hydroxide. Removal of the solvent *in vacuo* and purification of the residue obtained by separation on a silica gel column using $CHCl_3$-MeOH (95:5, v/v) as eluent afforded **12** (25 mg, 16%): m.p. 119–121° dec.; R_f 0.43 ($CHCl_3$-MeOH, 9:1, v/v); $[\alpha]_D^{25} = -54.65°$ (c 0.86, MeOH); ^{1}H NMR (CD_3OD) δ 1.10 (t, $J = 7.0$ Hz, 3H, $CH_2C\underline{H}_3$), 1.98 (sextet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 1H, $C\overline{H}'\underline{H}''CH_3$), 2.12 (ddd, $J_{2',2''} = 14.0$, d, $J_{1',2''} = 7.2$, $J_{2'',3'} = 2.9$ Hz, 1H, H-2''), 2.24 (sextet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 1H, $C\underline{H}'H''CH_3$), 2.68 (quintet, $J_{2',2''} = 14.0$, $J_{1',2'} = J_{2',3'} = 7.2$ Hz, 1H, H-2'), 3.58 (s, 3H, $O\overline{C}H_3$), 3.69 (dd, $J_{5',5''} = 11.6$, $J_{4',5''} = 4.0$ Hz, 1H, H-5''), 3.75 (dd, $J_{5',5''} = 11.6$, $J_{4',5'} = 3.5$ Hz, 1H, H-5'), 3.90 (ddd, $J_{4',5''} = 4.0$, $J_{4',5'} = 3.5$, $J_{3',4'} = 2.8$ Hz, 1H, H-4'), 4.42 (ddd, $J_{2',3'} = 7.2$, $J_{2'',3'} = 2.9$, $J_{3',4'} = 2.8$ Hz, 1H, H-3'), 4.98 (s, 1H, H-6), 5.58 (t, $J_{1',2'} = J_{1',2''} = 7.2$ Hz, 1H, H-1'); ^{13}C NMR (CD_3OD) δ 8.65 ($CH_2\underline{C}H_3$), 28.06 ($\underline{C}H_2CH_3$), 38.58 (C-2'), 57.29 ($O\overline{C}H_3$), 61.07 (C-5), 63.91 (C-5'), 73.08 (C-3'), 88.62 (C-4'), 91.80 (C-6), 92.43 (C-1'), 152.40 (C-2 $\underline{C}$ = O), 169.74 (C-4 $\underline{C}$ = O). Anal. Calcd. for $C_{12}H_{19}BrN_2O_6 \cdot 1/4H_2O$: C, 38.77; H, 5.28; N, 7.53. Found: C, 38.53; H, 4.92; N, 7.40.

Further elution with the same solvent yielded **11** (92 mg, 64%): m.p. 115–118° dec.; R_f 0.30 ($CHCl_3$-MeOH, 9:1, v/v); $[\alpha]_D^{25} = +48.38°$ (c 0.68, MeOH); ^{1}H NMR (CD_3OD) δ 1.10 (t, $J = 7.0$ Hz, 3H, $CH_2C\underline{H}_3$), 2.02 (sextet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 1H, $C\overline{H}'\underline{H}''CH_3$), 2.10 (ddd, $J_{2',2''} = 13.8$, $J_{1',2''} = 6.6$, $J_{2'',3'} = 3.3$ Hz, 1H, H-2''), 2.24 (sextet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 1H, $C\underline{H}'H''CH_3$), 2.36 (quintet, $J_{2',2''} = 13.8$, $J_{1',2'} = J_{2',3'} = 6.6$ Hz, 1H, H-2'), 3.72 (dd, $J_{5',5''} = 12.9$, $J_{4',5''} = 3.5$ Hz, 1H, H-5''), 3.78 (dd, $J_{5',5''} = 12.9$, $J_{4',5'} = 3.1$ Hz, 1H, H-5'), 3.90 (ddd, $J_{4',5''} = 3.5$, $J_{4',5'} = J_{3',4'} = 3.1$ Hz, 1H, H-4'), 4.37 (ddd, $J_{2',3'} = 6.6$, $J_{2'',3'} = 3.3$, $J_{3',4'} = 3.1$ Hz, 1H, H-3'), 5.36 (s, 1H, H-6), 6.15 (t, $J_{1',2'} = J_{1',2''} = 6.6$ Hz, 1H, H-1'); ^{13}C NMR (CD_3OD) δ 8.74 ($CH_2\underline{C}H_3$), 28.15 ($\underline{C}H_2CH_3$), 40.79 (C-2'), 58.07 ($O\overline{C}H_3$), 61.52 (C-5), 62.75 (C-5'), 72.12 (C-3'), 85.08 ($\overline{C}$-6), 86.26 (C-1'), 88.17 (C-4'), 152.78 (C-2 $\underline{C}$ = O), 169.92 (C-4 $\underline{C}$ = O). Anal. Calcd. for $C_{12}H_{19}BrN_2O_6$: C, 39.24; H, 5.21; N, 7.63. Found: C, 39.63; H, 5.34; N, 7.58.

*(+)-*Trans*-(5R,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**13**), (−)-*trans*-(5S,6S)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**14**), and (+)-*cis*-(5S,6R)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridine (**15**)*

Method A (**13**, **15**). *N*-Chlorosuccinimide (60 mg, 0.45 mmol) was added to a solution of EDU (0.105 g, 0.41 mmol) in methanol (10 mL) and glacial acetic acid (0.15 mL) at 25° with stirring. The reaction was allowed to proceed for 8 hr, an additional aliquot of *N*-chlorosuccinimide (0.20 g, 1.5 mmol) in glacial acetic acid (0.6 mL) was then added, and the reaction was allowed to continue for another 24 hr at 25° with stirring. The reaction mixture was neutralized with methanolic sodium hydroxide (pH ≈ 6), the solvent was removed *in vacuo*, and the residue obtained was separated by PTLC using chloroform–methanol (90:10, v/v) as development solvent. The desired product band was extracted to yield **13** (35 mg, 26%): m.p. 140–142° (sublimes); R_f 0.39 ($CHCl_3$-MeOH, 9:1, v/v); $[\alpha]_D^{25} = +40.0°$ (c 0.2, MeOH); ^{1}H NMR (CD_3OD) δ 1.08 (t, $J = 7.0$ Hz, $CH_2C\underline{H}_3$), 2.02 (sextet, $J_{gem} = 14.4$, $J_{vic} = 7.0$ Hz, 1H, $CH'\underline{H}''CH_3$), 2.10 (ddd, $J_{2',2''} = 13.2$, $J_{1',2''} = 6.1$, $J_{2'',3'} = 3.1$ Hz, 1H, H-2''), 2.22 (sextet, $J_{gem} = 14.4$, $J_{vic} = 7.0$ Hz, 1H, $C\underline{H}'H''CH_3$), 2.30 (ddd, $J_{2',2''} = 13.2$, $J_{1',2'} = 7.6$, $J_{2',3'} = 6.9$ Hz, 1H, H-2'), 3.72 (dd, $J_{gem} = 11.2$, $J_{4',5''} = 3.5$ Hz, 1H, H-5''), 3.76 (dd, $J_{gem} = 11.2$, $J_{4',5'} = 3.2$ Hz, 1H, H-5'), 3.88–3.92 (m, 1H, H-4'), 4.33–4.38 (m, 1H, H-3'), 5.30 (s, 1H, H-6), 6.18 (dd, $J_{1',2'} = 7.6$, $J_{1',2''} = 6.1$ Hz, 1H, H-1'); ^{13}C NMR (CD_3OD) δ 7.45 ($CH_2\underline{C}H_3$), 27.78 ($\underline{C}H_2CH_3$), 40.67 (C-2'), 57.89 ($O\overline{C}H_3$), 62.74 (C-5'), 66.73 (C-5), 72.18 (C-3'), 84.79 (C-6), 86.20 (C-1'), 88.15 (C-4'), 152.72 (C-2 $\underline{C}$ = O), 169.38 (C-4 $\underline{C}$ = O). Anal. Calcd. for $C_{12}H_{19}ClN_2O_6$: C, 44.65; H, 5.93; N, 8.68. Found: C, 44.92; H, 6.17; N, 8.50.

Extraction of the product band having R_f 0.37 ($CHCl_3$-MeOH, 9:1, v/v) afforded **15** (25 mg, 19%): m.p. 140–142° (sublimes); $[\alpha]_D^{25} = +17.33°$ (c 0.3, MeOH); ^{1}H NMR (CD_3OD) δ 1.06 (t, $J = 7.0$ Hz, 3H, $CH_2C\underline{H}_3$), 2.0 (octet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 2H, $C\underline{H}_2C\overline{H}_3$), 2.11 (ddd, $J_{2',2''} = 14.2$, $J_{1',2''} = 5.7$, $J_{2'',3'} = 2.8$ Hz, 1H, H-2''), 2.24 (ddd, $J_{2',2''} = 14.2$, $J_{1',2'} = 8.4$, $J_{2',3'} = 6.1$ Hz, 1H, H-2'), 3.55 (s, 3H, $O\underline{C}H_3$), 3.70 (dd, $J_{gem} = 11.4$, $J_{4',5''} = 4.1$ Hz, 1H, H-5''), 3.76 (dd, $J_{gem} = 11.4$, $J_{4',5'} = 3.5$ Hz, 1H, H-5'), 3.91 (ddd, $J_{4',5''} = 4.1$, $J_{4',5'} = 3.5$, $J_{3',4'} = 2.9$ Hz, 1H, H-4'), 4.34 (ddd, $J_{2',3'} = 6.1$, $J_{3',4'} = 2.9$, $J_{2'',3'} = 2.8$ Hz, 1H, H-3'), 5.06 (s, 1H, H-6), 6.02 (dd, $J_{1',2'} = 8.4$, $J_{1',2''} = 5.7$ Hz, 1H, H-1'); ^{13}C NMR (CD_3OD) δ 8.74 ($CH_2\underline{C}H_3$), 31.95 ($\underline{C}H_2CH_3$), 40.61 (C-2'), 58.97 ($O\underline{C}H_3$), 63.16 (C-5'), 72.42 (C-3'), 74.72 (C-5), 87.01 (C-1'), 88.29 (C-4'), 89.48 (C-6), 152.87 (C-2 $\underline{C}$ = O), 169.14 (C-4 $\underline{C}$ = O). Anal. Calcd. for $C_{12}H_{19}ClN_2O_6$: C, 44.65; H, 5.93; N, 8.68. Found: C, 44.45; H, 5.96; N, 8.27.

Method B (**13**, **14**, **15**). Chlorine gas was bubbled slowly into a suspension of EDU (0.256 g, 1 mmol) in methanol (60 mL) at 25° with stirring until the pale yellow color of the resulting solution persisted. The pH of the solution was adjusted to 6.5 using a solution of methanolic sodium hydroxide and the mixture was filtered. Removal of the solvent from the filtrate *in vacuo* and separation of the residue obtained by elution from a silica gel column using chloroform–methanol (95:5) as eluent afforded **14** as a viscous oil (32 mg, 10%): R_f 0.44 ($CHCl_3$-MeOH, 9:1, v/v); $[\alpha]_D^{25} = -38.33°$ (c 0.18, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.08 (t, $J = 7.0$ Hz, 3H, $CH_2C\underline{H}_3$), 2.03 (sextet, $J_{gem} = 14.0$, $J_{vic} = 7.0$ Hz, 1H, $C\overline{H}'\underline{H}''CH_3$), 2.09 (ddd, $J_{2',2''} = 12.9$, $J_{1',2'} = 7.2$, $J_{2'',3'} = 2.9$ Hz, 1H, H-2''), 2.20 (sextet, $J_{gem} = 14.0$,

J_{vic} = 7.0 Hz, 1H, CH′H″CH$_3$), 2.64 (quintet, $J_{2',2''}$ = 12.9, $J_{1',2'} = J_{2',3'}$ = 7.2 Hz, 1H, H-2′), 3.56 (s, 3H, OCH$_3$), 3.68 (dd, J_{gem} = 13.0, $J_{4',5''}$ = 4.4 Hz, 1H, H-5″), 3.76 (dd, J_{gem} = 13.0, $J_{4',5'}$ = 3.3 Hz, 1H, H-5′), 3.90 (ddd, $J_{4',5''}$ = 4.4, $J_{4',5'}$ = 3.3, $J_{3',4'}$ = 2.8 Hz, 1H, H-4′), 4.42 (ddd, $J_{2',3'}$ = 7.2, $J_{2'',3'}$ = 2.9, $J_{3',4'}$ = 2.8 Hz, 1H, H-3′), 4.94 (s, 1H, H-6), 5.62 (t, $J_{1',2'} = J_{1',2''}$ = 7.2 Hz, 1H, H-1′); ^{13}C NMR (CD$_3$OD) δ 7.39 (CH$_2$CH$_3$), 27.22 (CH$_2$CH$_3$), 38.63 (C-2′), 57.28 (OCH$_3$), 63.90 (C-5′), 66.43 (C-5), 73.07 (C-3′), 88.56 (C-4′), 91.32 (C-6), 92.08 (C-1′), 152.42 (C-2 C = O). Anal. Calcd. for $C_{12}H_{19}ClN_2O_6$: C, 44.65; H, 5.93; N, 8.68. Found: C, 44.42; H, 5.96; N, 8.60. Compound **14** was not studied *in vitro* or *in vivo*.

Continuous elution with the same solvent yielded **13** (0.15 g, 46%) and **15** (90 mg, 28%), respectively. Products **13** and **15** were identical (m.p., ^{1}H NMR) with the sample products prepared by method A.

Biological studies

Male Sprague–Dawley rats, 200–260 g in weight, were purchased from the University of Alberta Animal Services Facility. Either four or five animals were used in each experiment. All studies were done according to the Canadian Council on Animal Care guidelines, with review and approval by the University of Alberta Health Sciences Animal Care Committee.

The biotransformation and pharmacokinetics of four 5,6-dihydro compounds, (+)-*trans*-(5*R*,6*R*)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine [**11**, (5*R*,6*R*)-BMEDU], (−)-*trans*-(5*S*,6*S*)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine [**12**, (5*S*,6*S*)-BMEDU], (+)-*trans*-(5*R*,6*R*)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine [**13**, (5*R*,6*R*)-CMEDU] and (+)-*cis*-(5*S*,6*R*)-5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine [**15**, (5*S*,6*R*)-CMEDU] were investigated in rats having an implanted jugular vein catheter. The test compound [**11**, **12**, **13**, **15** or EDU (**1**)] was injected (100 μL) into the jugular vein using a dose of 0.7 mmol/kg dissolved in DMSO–water (50:50, v/v). Blood samples (200–400 μL) were collected up to 7 hr post-injection of the test compound, and the catheter was washed by injection of a volume of heparinized normal saline, equal to the volume of the blood sample, into the jugular vein catheter. Each blood sample collected was extracted by shaking the whole blood sample with methanol (2 mL) in a mechanical shaker for 1 hr. This mixture was centrifuged for 10 min at 950 *g*, and the supernatant fraction was then filtered through a Sep-Pak (C18, Waters Millipore) cartridge. Each Sep-Pak cartridge was preconditioned by washing with methanol (3 mL) and then water (2 mL). The filtrate from the supernatant was dried under a stream of nitrogen gas, and the residue obtained was dissolved in methanol (100 μL).

A 10-μL aliquot of this solution was then subjected to quantitative HPLC analysis using an HPLC system comprised of a Waters Baseline 810 computer program running on a 486/33 MHz computer, Waters model 501 pumps, a Waters model U6K injector and a Waters model 486 variable wavelength absorbance detector. All separations and quantitative analyses were carried out on a Waters Radial-Pak C18 reverse phase cartridge column (10 μm, 8 mm × 10 cm) at 25° using a gradient of acetonitrile (0% for the first 6 min → 15% for the next 11 min → 0% for the rest of the HPLC run) in water (v/v) during a 25-min time interval, with a flow rate gradient of 1.5 mL/min for the first 6 min → 2.5 mL/min for the next 19 min during the separation, with UV detection at 230 nm. The identity of each compound present in the sample was determined by comparison of its retention time to that of an authentic sample. In some instances, the presence of a particular compound was confirmed by spiking an aliquot of the blood sample extract with an authentic sample prior to further HPLC analysis. The concentration of each compound in blood, as a function of time, was plotted using the Sigma-plot program (Jandel Scientific). The pharmacokinetic parameters were calculated using the Lagran program.

Percent protein binding (%PB)

The percent protein binding of these 5,6-dihydro compounds **11**, **12**, **13**, **15** or EDU (**1**) to BSA (clinical reagent grade, 98%+, ICN Biochemical Inc., Mississauga, Ontario) was determined by adding different volumes (ranging from 20 to 300 μL) of a 1×10^{-3} M freshly prepared solution of the test compound in DMSO–water (30:70, v/v) to 0.25 mL of a 1% solution of BSA, previously equilibrated at 37°. This mixture was agitated using a mechanical shaker for 4 hr at the same temperature, and then the mixture was filtered through a 0.45 μm HV-Type filter (Millipore). The filtrate was analyzed by HPLC using the procedure described above. The mobile phase used for quantitation of the samples was isocratic water–acetonitrile (80:20, v/v). The percent of compound bound was calculated from the concentration of free compound (C_f) in the filtrate using the following equation:

$$\%PB = [(C_t - C_f)/C_t] \times 100$$

where C_t is the concentration of the EDU or the 5,6-dihydro compound added to the protein solution.

Partition coefficients (P)

The nucleoside test compound was partitioned between equal volumes of presaturated 1-octanol and water. The concentration of the test compound in the water phase, before and after 1-octanol partitioning, was determined using the HPLC procedure [23] for compound **15**, or the UV spectrophotometer procedure [24] for compounds **11–13** and EDU, reported previously. Partition coefficients (*P*) were calculated as ratios of concentration in the octanol to concentration in the water phase.

Antiviral activity assays

In vitro cytopathic effect (CPE) inhibition assays for HSV-1, HSV-2 and human cytomegalovirus (HCMV) were performed under the NIH Antiviral Research Branch testing program using human foreskin fibroblast (HFF) cells as described earlier [25].

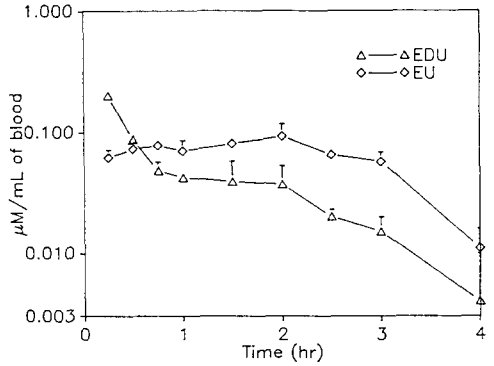


Fig. 2. Concentration of EDU and EU in blood (μM/mL) after i.v. injection of EDU into rats. Values are means ± SD (N = 4).

RESULTS

Chemistry

Reaction of EDU with molecular bromine in methanol at 25° afforded the (+)-*trans*-(5*R*,6*R*)-(**11**) and (−)-*trans*-(5*S*,6*S*)-5-bromo-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine (**12**) diastereomers in 64 and 16% yields, respectively (Scheme 1). A similar reaction (Method A) of EDU with *N*-chlorosuccinimide in methanol in the presence of glacial acetic acid gave the (+)-*trans*-(5*R*,6*R*)-(**13**) (26%) and (+)-*cis*-(5*S*,6*R*)- (**15**) (19%) diastereomers of 5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine. The reaction of EDU with molecular chlorine in methanol (Method B) yielded the (+)-*trans*-(5*R*,6*R*)- (**13**) (46%), (−)-*trans*-(5*S*,6*S*)- (**14**) (10%) and (+)-*cis*-(5*S*,6*R*)- (**15**) (28%) diastereomers of 5-chloro-5-ethyl-6-methoxy-5,6-dihydro-2′-deoxyuridine. These 5-ethyl-5-halo-6-methoxy-5,6-dihydro derivatives (**11–15**) of EDU most likely arise via the initial formation of a 5,6-halonium ion intermediate which is susceptible to regiospecific nucleophilic attack by methanol at the sterically less hindered C-6 position. The configuration of compounds **11–15** at the C-5 and C-6 positions was assigned by comparing the optical rotation and ^{1}H NMR spectral data with that of similar compounds, for which the absolute configuration is known, such as 5-bromo-6-methoxy-5,6-dihydrothymidine [20] and 5-bromo-6-hydroxy-5,6-dihydrothymidine [26] diastereomers. The most

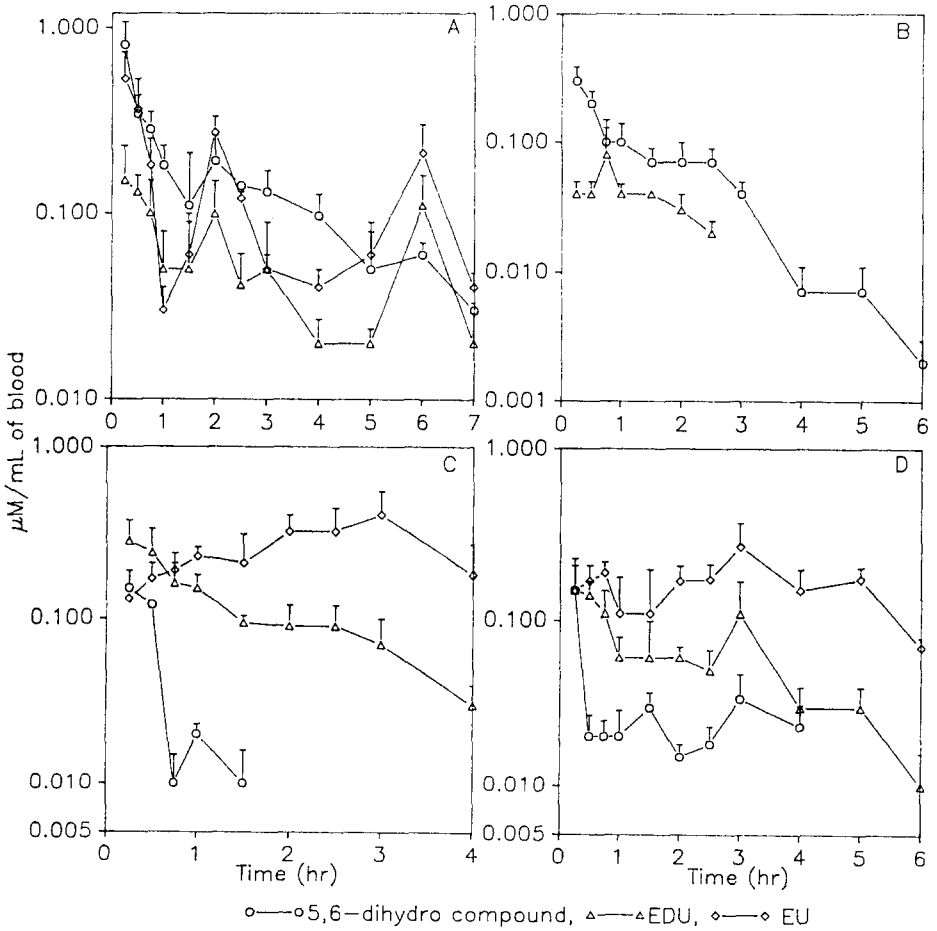


Fig. 3. Concentration of 5,6-dihydro compounds, EDU and EU in blood (μM · mL^{-1}) after i.v. injection of 5,6-dihydro compounds. (A) (5*R*,6*R*)-CMEDU; (B) (5*S*,6*R*)-CMEDU; (C) (5*S*,6*S*)-BMEDU; and (D) (5*R*,6*R*)-BMEDU. Values are means ± SD (N = 4 or 5).

Table 1. *In vitro* antiviral activity, partition coefficients, percent protein binding, AUC, and plasma clearance of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridines

Compound	EC_{50}*(μM) HSV-1 (E-377)†	HSV-2 (MS)†	HCMV (AD 169)†	P‡	log P	%PB	AUC (μM · hr · mL $^{-1}$)	Clearance (mL · min $^{-1}$)
(5*R*,6*R*)-BMEDU	0.29	0.53	>50	1.9	0.28	19 ± 6.6§	0.34 0.51‖	35
(5*S*,6*S*)-BMEDU	0.67	0.13	0.28	2.5	0.40	14 ± 2.5	0.07 0.36‖	168
(5*R*,6*R*)-CMEDU	>50	>50	>50	2.5	0.40	22 ± 5.3	1.20 0.41‖	10
(5*S*,6*R*)-CMEDU	>50	>50	0.04	0.38	−0.42	11 ± 1.3	0.32 0.15‖	36
EDU	0.29	0.38	0.06	0.081	−1.09	7 ± 2.2	0.14	85
Acyclovir	0.03	0.03	ND¶	0.0013**	−2.88**	ND	ND	ND
Ganciclovir	ND	ND	<0.03	0.0085**	−2.07**	ND	ND	ND

* The drug concentration required to reduce the viral cytopathic effect in infected monolayers to 50% of untreated uninfected controls.

† The strain of virus that was used for antiviral testing.

‡ Partition coefficient (*P*) = concentration in 1-octanol/concentration in water.

§ Mean ± SD (N = 3).

‖ AUC of EDU (**1**) after i.v. injection of the 5,6-dihydro derivative.

¶ ND = not determined.

** Data from Ref. 27.

distinct differences in chemical shift positions in the ^{1}H NMR spectra of these diastereomers occur at the H-1′, H-2′ and H-2″ protons in the sugar moiety and the H-6 proton of the base. The methylene protons of the C-5 ethyl substituent are chemically non-equivalent due to the chiral centre at C-5. The diastereomers **11** and **12**, and **13**, **14** and **15**, are stable products that were separated by silica gel or column chromatography.

Biological studies

The concentrations of the 5,6-dihydro compounds (5*R*,6*R*)-BMEDU (**11**), (5*S*,6*S*)-BMEDU (**12**), (5*R*,6*R*)-CMEDU (**13**), (5*S*,6*R*)-CMEDU (**15**), EDU (**1**) and the metabolites EDU and EU in rat blood were determined as a function of time after injection, and the results are summarized in Figs. 2 and 3. The 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds **11**, **12**, **13**, and **15**, but especially the bromo diastereomers **11** and **12**, have a very short distribution half-life. However, the 5-chloro-6-methoxy diastereomers, particularly (5*R*,6*R*)-CMEDU, had a very long blood residence time, up to 7 hr post-injection. The concentration of the 5-bromo-6-methoxy diastereomers (**11**, **12**) in blood was very similar to that of EDU at 15 min post-injection. Rapid regeneration of the 5,6-olefinic bond for the 5-bromo-6-methoxy-5,6-dihydro diastereomers gives rise to a high blood concentration of EDU. The pharmacokinetic parameters of these 5,6-dihydro compounds and EDU are summarized in Table 1. The AUC values of these 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds, with the exception of (5*S*,6*S*)-BMEDU, were higher (ranging from 0.32 to 1.20 μM · hr · mL^{-1}) relative to that of EDU (0.14 μM · hr · mL^{-1}). These 5,6-dihydro compounds, except for (5*S*,6*S*)-BMEDU, also showed lower plasma clearance (10–36 mL · min $^{-1}$) than EDU (85 mL · min $^{-1}$).

(5*S*,6*S*)-BMEDU exhibited the shortest blood residence time, among the compounds investigated, in spite of the fact that it afforded a very high concentration of EDU in blood (Fig. 3). These 5,6-dihydro diastereomers showed different pharmacokinetics and *in vivo* biotransformations, which indicates that the nature of the C-5 halogen atom and the configuration at the C-5 and C-6 positions exert a strong influence on metabolism. Although EDU and EU were the two major metabolites of these 5,6-dihydro compounds, EU was not detected in blood following the administration of (5*S*,6*R*)-CMEDU.

These 5,6-dihydro compounds showed higher levels (11–22%) of binding to BSA than EDU (7%). It also appeared that (5*R*,6*R*) diastereomers showed higher protein binding (19% for **11** and 22% for **13**) than the corresponding (5*S*,6*S*) and (5*S*,6*R*) compounds.

The 5-ethyl-5-halo-6-methoxy-5,6-dihydro diastereomers investigated were much more lipophilic (*P* = 0.38–2.5 range) than EDU (*P* = 0.081).

(5*S*,6*S*)-BMEDU and (5*R*,6*R*)-BMEDU exhibited antiviral activity against HSV-1 and HSV-2 that was comparable to that of EDU. Although (5*R*,6*R*)-CMEDU and (5*S*,6*R*)-CMEDU were both inactive against HSV-1 and HSV-2, (5*S*,6*R*)-CMEDU exhibited antiviral activity against HCMV that was comparable to that of ganciclovir (Table 1).

DISCUSSION

EDU undergoes rapid metabolism to the inactive

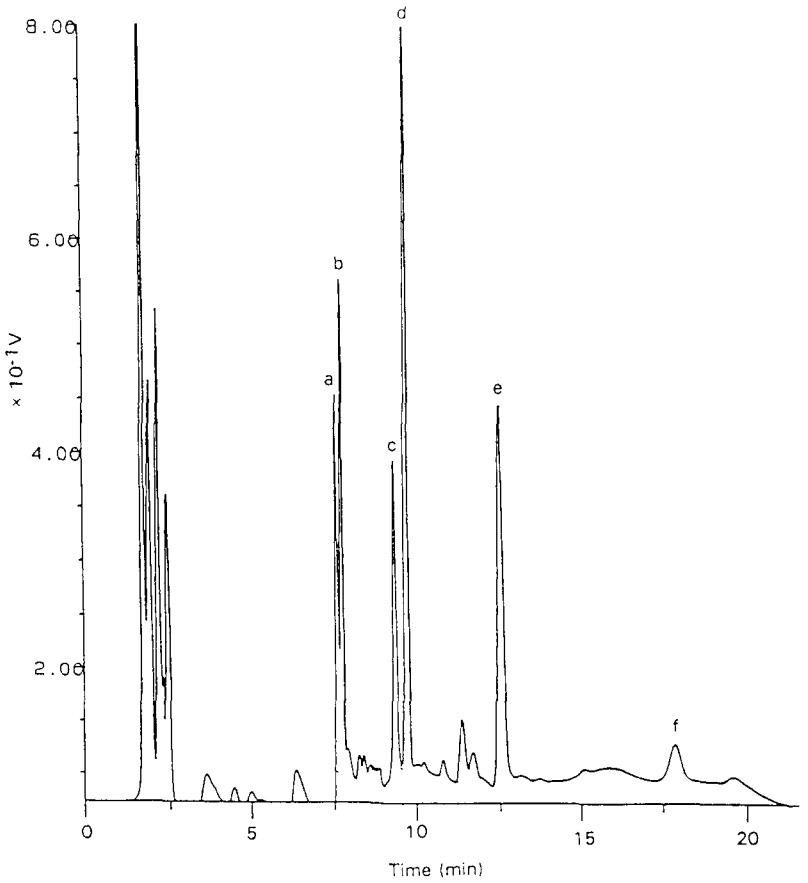
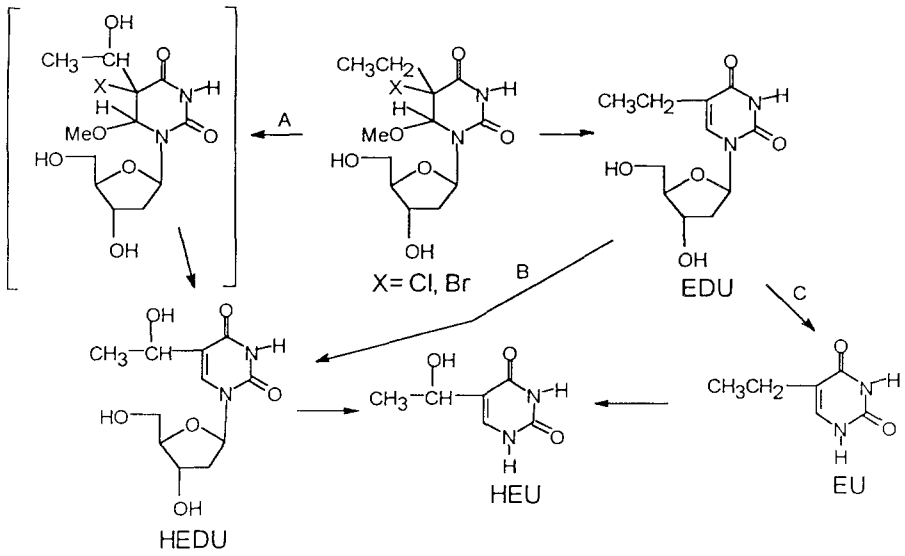


Fig. 4. HPLC analysis of rat blood extract after injection of BMEDU. Retention time (min): HEDU (a, 7.56), HEU (b, 7.75), EU (c, 9.35), EDU (d, 9.73), internal standard (e, 12.54), and BMEDU (f, 17.88).

EU in the presence of pyrimidine phosphorylases. One approach to prevent this undesirable biotransformation is through a prodrug such as a 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridine, that is not a substrate for the phosphorylase. There is a precedent for this concept, since it has been reported that 5-bromo-5-fluoro-6-methoxy-5,6-dihydro-2′-deoxyuridine acts as a slow releaser of FUDR *in vivo* [19]. It was also shown that this FUDR prodrug was not cleaved by either *Escherichia coli* nucleosidase or a nucleoside phosphorylase prepared from Ehrlich ascites cells, which readily degraded FUDR to FU. Other studies have shown that 6-alkoxy-5-bromo-5,6-dihydrothymidines are inhibitors of TK [20, 28]. The results from experiments with 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridines, as described earlier in this paper, show that these 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridines should be useful prodrugs to EDU, with a longer blood residence time. These 5,6-dihydro prodrugs could undergo *in vivo* regeneration of the 5,6-olefinic bond to produce the active EDU. It is expected that this rate of regeneration should be dependent upon the nature of the C-5 halogen substituent. The 5,6-dihydro compounds are expected to be more lipophilic than EDU, which should enhance their ability to cross the BBB and entry into the brain. A high brain uptake of an antiviral drug, which provides a therapeutically effective brain concentration, is essential for the treatment of cephalic infections such as HSE.

The identification of HEU (**6**) in the urine of rats dosed with EDU indicated that hydroxylation of the 5-ethyl substituent is also a route of EDU metabolism [29–31]. Separation of parent, base and hydroxy metabolites by HPLC usually requires specific conditions that were developed for this purpose [32]. Since there are significant differences in the solubility and partition characteristics of the 5,6-dihydro compounds used in this study, relative to EDU, it was necessary to develop a gradient elution HPLC method to separate the parent 5,6-dihydro compound and its metabolites. The HPLC conditions described elsewhere in this paper provided a good resolution of the 5,6-dihydro compounds from EDU, EU and their hydroxylated metabolites (Fig. 4).

A putative metabolic pathway for the biotransformations observed for the 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds investigated is presented in

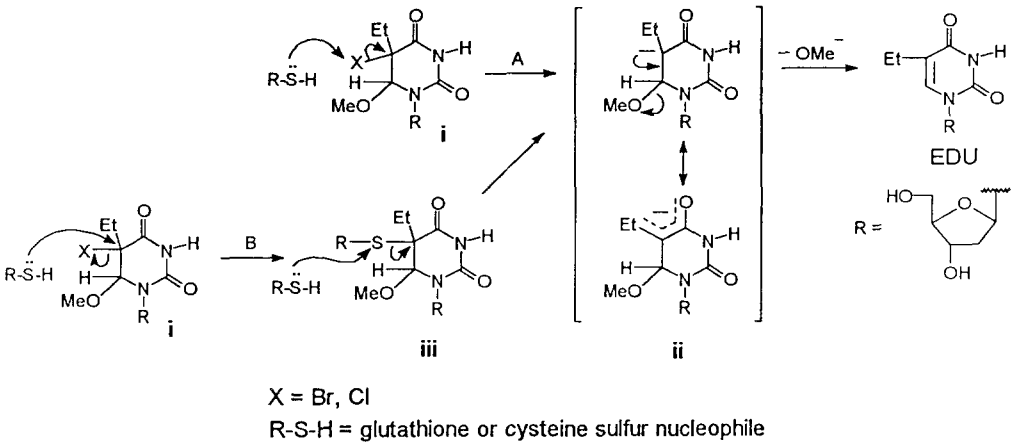


Scheme 2. Putative metabolic transformation of 5-ethyl-5-halo-6-methoxy-2′-deoxyuridines *in vivo*. (Path A) formation of HEDU and HEU via a 5-halo-6-methoxy intermediate; (Path B) formation of HEDU and HEU via EDU (the presence of HEDU in blood after injection of EDU into rats was neither detected in these experiments nor reported previously); and (Path C) formation of HEU via EU.

Scheme 2. The formation of HEDU, as a metabolite of EDU in urine or blood, has not been reported previously, presumably due to fast *in vivo* cleavage of the glycosidic bond. In contrast, in this study HEDU was detected, but not quantitated, in the extract of blood samples following injection of the bromo compounds (5*R*,6*R*)-BMEDU and (5*S*,6*S*)-BMEDU (Fig. 4). Since the quantity of hydroxyl metabolites present in blood samples following injection of the chloro compounds (5*R*,6*R*)-CMEDU and (5*S*,6*R*)-CMEDU was very low, it is likely that HEDU arises directly from the parent 5-ethyl-5-halo - 6 - methoxy - 5,6 - dihydro - 2′ - deoxyuridine (Scheme 2, pathway A). Although EDU and EU were present in significant levels in blood samples following injection of the two bromo BMEDU diastereomers, the concentration of these metabolites was much lower for the two chloro CMEDU diastereomers. These results indicate that the chloro CMEDU diastereomers, especially (5*R*,6*R*)-CMEDU, are less susceptible to *in vivo* metabolism. EU was not detected as a metabolite of (5*S*,6*R*)-CMEDU. Clearly the *in vivo* stability of these 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds is dependent upon the nature of the halogen substituent, with the chloro being more stable than the bromo analogs. The higher concentrations of the chloro CMEDU diastereomers present in blood indicate that they are more stable, are oxidized very slowly to HEDU, are stable to pyrimidine phosphorylase, and are converted slowly to EDU.

Plausible mechanisms for the conversion of 5-ethyl - 5 - halo - 6 - methoxy - 5,6 - dihydro - 2′ - deoxyuridine to EDU are shown in Scheme 3. It is likely that dehalogenation and elimination to generate EDU occur by reaction with GSH, or a related tissue nucleophile such as cysteine, which are widely distributed in body fluids and tissues. There is precedent for this mechanism since it has been shown that *in vitro* incubation of 5-bromo-5-fluoro - 6 - methoxy - 5,6 - dihydro - 2′ - deoxyuridine diastereomers with GSH regenerates the 5,6-olefinic bond to give FUDR [19]. The reaction with GSH (RSH) could occur by two mechanisms. Elimination of the C-5 halo substituent (X) through a nucleophilic attack by RSH would give the carbanion or enolate anion (ii) (Scheme 3, pathway A). Alternatively, an S_N2 displacement of X by RSH to give (iii) (Scheme 3, pathway B) and a subsequent reaction with GSH would also yield the carbanion (ii). Elimination of methoxide anion from the carbanion intermediate (ii) would regenerate the 5,6-olefinic bond to afford EDU. Nucleophilic attack on halogen at C-5 and sulfur attack at sulfur bonded to C-5 in 5,6-dihydrouracils have been reported previously [33, 34].

The results illustrated in Fig. 3 indicate that these 5 - ethyl - 5 - halo - 6 - methoxy - 5,6 - dihydro compounds exhibit a biphasic decline in the plasma concentrations. The terminal excretion phase was characterized by a low concentration in plasma for an extended time, especially for the two CMEDU diastereomers, following i.v. injection. Furthermore, these 5,6-dihydro compounds show a second C_{max} in blood. This second blood C_{max}, which was observed for parent 5,6-dihydro compounds and their metabolites, could be due to reabsorption of these 5,6-dihydro compounds. Among these 5-ethyl-5-halo - 6 - methoxy - 5,6 - dihydro - 2′ - deoxyuridines, (5*S*,6*S*)-BMEDU showed the lowest AUC ($0.07\ \mu M \cdot hr \cdot mL^{-1}$) and the highest clearance ($168\ mL \cdot min^{-1}$) which indicates its very short blood



Scheme 3. Proposed mechanisms for the *in vivo* conversion of 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridines to 5-ethyl-2′-deoxyuridine. (Path A) elimination under the influence of a nucleophile; and (Path B) nucleophilic substitution of R-S-H for X, followed by elimination of R-S- under the influence of a second nucleophile.

residence time. On the other extreme, (5*R*,6*R*)-CMEDU had the highest AUC (1.20 μM · hr · mL^{-1}) and the lowest clearance (10 mL · min^{-1}) which indicates that this compound has a longer blood residence time. All of these 5-ethyl-5-halo-6-methoxy-5,6-dihydro compounds, with the exception of (5*S*,6*S*)-BMEDU, had higher AUC values (ranging from 0.32 to 1.20 μM · hr · mL^{-1}) and lower clearance rates (10–36 mL · min^{-1}) than EDU (0.14 μM · hr · mL^{-1} and 85 mL · min^{-1}, respectively).

The concentration of EU in blood after injection of EDU reached its peak 2 hr post-injection and then declined. In contrast to EDU, the concentration of EU after injection of the 5,6-dihydro compounds fluctuated. These fluctuations may be explained by the fact that reabsorption of these compounds through an enterohepatic mechanism would increase the blood concentration of EDU, which is subsequently cleaved to EU.

The BMEDU and CMEDU diastereomers were much more lipophilic than EDU. For example, the 5-ethyl-5-halo-6-methoxy-5,6-dihydro-2′-deoxyuridines exhibited high octanol-water coefficients (P = 0.38 to 2.5 range) relative to EDU (P = 0.081) (Table 1). These compounds also showed higher levels (11–22%) of binding to BSA than EDU (7%). The (5*R*,6*R*)-BMEDU and (5*R*,6*R*)-CMEDU diastereomers showed the highest protein binding to BSA (Table 1), which indicates the crucial role that the configuration of the halogen and methoxy groups at C-5 and C-6 exert on the percent of protein binding of these compounds.

The enhanced lipophilicity of these 5,6-dihydro compounds may enable them to enter cells more readily by diffusion. The high blood concentration of the CMEDU diastereomers, in conjunction with their enhanced lipophilicity, suggests that these compounds should provide a higher concentration in the brain than EDU. This postulate is based on the observation that brain capillary permeability is often related to the octanol-water partition coefficients (P) and molecular weights of the compounds. Increasing the lipophilicity of compounds with a molecular weight of less than 400 has been reported to improve brain permeability [35]. Furthermore, the parabolic relationship between log P values and brain extractability for a group of ^{11}C-labeled compounds suggests an optimal log P range of 0.9 to 2.5 for radiopharmaceuticals designed to cross the BBB by virtue of their lipid solubility [36].

The (5*R*,6*R*)- (**11**) and (5*S*,6*S*)- (**12**) diastereomers of BMEDU exhibited equipotent *in vitro* antiviral activity to that of the reference drug EDU against both HSV-1 and HSV-2. In contrast, the (5*R*,6*R*)- (**13**), and (5*S*,6*R*)- (**15**) diastereomers of CMEDU were both inactive against HSV-1 and HSV-2. These results suggest that the (5*R*,6*R*)- (**11**) and (5*S*,6*S*)- (**12**) diastereomers of BMEDU, which are much more lipophilic (log P = 0.28 to 0.40) than EDU (log P = −1.09), would be more useful for the treatment of HSE than EDU.

In the HCMV antiviral assay, (5*S*,6*S*)-BMEDU exhibited 1/10 of the potency of ganciclovir, whereas (5*S*,6*R*)-CMEDU was approximately equipotent to ganciclovir. These latter results indicate that the nature of the halogen atom at C-5 and the configuration at C-5 and/or C-6 positions are determinants of HSV-1 and HSV-2 antiviral activities: [(5*S*,6*S*)-BMEDU and (5*R*,6*R*)-BMEDU ≫ (5*R*,6*R*)-CMEDU and (5*S*,6*R*)-CMEDU], and for HCMV, [(5*S*,6*S*)-BMEDU ≫ (5*R*,6*R*)-BMEDU; (5*S*,6*R*)-CMEDU ≫ (5*R*,6*R*)-CMEDU]. The fact that (5*S*,6*R*)-CMEDU was equipotent to ganciclovir against HCMV suggests that it could serve as a useful lead compound for the development of an improved anti-HCMV drug, which is urgently required for antiviral chemotherapy.

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