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DEOXYRIBOSYL EXCHANGE REACTIONS LEADING TO THE *IN VIVO* GENERATION AND REGENERATION OF THE ANTIVIRAL AGENTS (*E*)-5-(2-BROMOVINYL)-2′-DEOXYURIDINE, 5-ETHYL-2′-DEOXYURIDINE AND 5-(2-CHLOROETHYL)-2′-DEOXYURIDINE

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Abstract—In the rat, the highly potent anti-herpes drug (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdUrd) is rapidly converted to its base (*E*)-5-(2-bromovinyl)uracil (BVUra) through the action of pyrimidine nucleoside phosphorylases. However, BVdUrd can be regenerated or even generated *de novo* from BVUra by a pentosyl transfer reaction upon the administration of 2′-deoxythymidine (dThd), 2′-deoxyuridine (dUrd) or 5-ethyl-2′-deoxyuridine (EtdUrd). The antiherpetic drugs EtdUrd and 5-(2-chloroethyl)-2′-deoxyuridine (ClEtdUrd) can also be regenerated or generated *de novo* from their respective bases 5-ethyluracil (EtUra) and 5-(2-chloroethyl)uracil (ClEtUra), by a pentosyl transfer mediated by the administration of dThd or dUrd as deoxyribosyl donor. The generation or regeneration of BVdUrd, EtdUrd and ClEtdUrd from their bases (BVUra, EtUra and ClEtUra, respectively) is readily achieved because the latter have long half-lifes. Thus, the active anti-herpes drugs can be (re)generated repeatedly after a single administration of these nucleosides or their bases, followed by repeated administrations of dUrd.

5-Substituted 2′-deoxyuridine (dUrd) analogues are efficient substrates for pyrimidine nucleoside phosphorylases (PNPases) [1–3]. These PNPases degrade the dUrd analogues to their free pyridimidine bases. The *in vivo* degradation of those dUrd analogues that are endowed with clinical potentials, i.e. antiviral and antineoplastic properties, may obviously affect the therapeutic usefulness of the drugs. Various attempts have been made to limit the *in vivo* degradation of these compounds, i.e. by the concomitant use of inhibitors of PNPases [4–6] or by chemical modification of the dUrd analogues so as to increase their resistance to PNPases [7, 8].

For the highly potent anti-herpes agent (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdUrd) [9] we have recently designed a novel procedure to regenerate *in vivo* the active compound BVdUrd from its inactive base (*E*)-5-(2-bromovinyl)uracil (BVUra) [10]. This procedure is based on a pentosyl exchange reaction between a pyrimidine deoxynucleoside [such as thymidine (dThd), 5-iodo-dUrd (IdUrd), 5-fluoro-dUrd (FdUrd) or 5-trifluoromethyl-dUrd (CF_3dUrd)] and BVUra. BVUra is available for such deoxyribosyl transfer since it has a long half-life in the plasma, apparently because it is not a substrate for the first enzyme of the degradative pathway of pyrimidines, i.e. dihydrothymine (H_2Thy) dehydrogenase [11]. Furthermore, this pentosyl transfer can be repeated several times upon repeated injections of dThd following a single administration of BVdUrd or BVUra [6].

However, pyrimidine deoxyribonucleosides such as dThd, are by themselves capable of reversing the antiviral activity of other 5-substituted dUrd analogues, i.e. BVdUrd, 5-vinyl-dUrd (VdUrd), 5-ethyl-dUrd (EtdUrd), 5-propyl-dUrd and 5-allyl-dUrd [12–15]. The present study was therefore focused on the generation or regeneration of BVdUrd from BVUra with as deoxyribosyl donor a pyrimidine deoxynucleoside with no reversing effect on the antiviral activity of BVdUrd, such as dUrd, or being itself active as an antiviral agent, such as EtdUrd. The latter is a fairly potent and selective inhibitor of herpes simplex virus and vaccinia virus [12, 16, 17]. As demonstrated here, EtdUrd is rapidly degraded *in vivo* to its base 5-ethyluracil (EtUra) but can be regenerated from EtUra by a pentosyl exchange reaction with dThd or dUrd as deoxyribosyl donor. Like BVUra, EtUra is not a substrate for H_2Thy dehydrogenase [18]. Consequently, it persists for a prolonged time in the bloodstream, and can, therefore, serve as a substrate for the deoxyribosyl exchange reaction. 5-(2-chloroethyl)-dUrd (ClEtdUrd), a new EtdUrd analogue [19] with potent and selective antiherpes activities [19, 20], can be regenerated from its base 5-(2-chloroethyl)uracil (ClEtUra) by a similar process as EtdUrd.

Preliminary findings have indicated that dUrd and EtdUrd, but not dThd, when combined with BVdUrd, potentiate the protective activity of the

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latter in mice infected intraperitoneally with herpes simplex virus type 1, i.e. the mortality rate of the infected mice was decreased from 90% to 40% if a single oral dose of 100 mg BVDU/kg per day was followed by three intraperitoneal doses (at 3-hr intervals) of 50 mg dUrd or EtdUrd (E. De Clercq unpublished observations). This means that based on the regeneration procedure, BVdUrd may acquire a greater antiviral efficacy *in vivo*.

MATERIALS AND METHODS

Compounds. dThd and dUrd were obtained from Sigma (St Louis, MO). VdUrd, BVdUrd and the closely related (*E*)-5-(2-chlorovinyl)-dUrd (ClVdUrd) and (*E*)-5-(2-iodovinyl)-dUrd (IVdUrd) were synthesized as previously described [9, 21]. BVUra was prepared according to Barr *et al.* [22]. EtdUrd and EtUra were provided by Robugen GmbH (Esslingen, F.R.G.) and ClEtdUrd was obtained from Sandoz Forschungsinstitut (Vienna, Austria).

Pharmacokinetics in the rat. The nucleosides and their bases were administered intraperitoneally (i.p.) to adult male Wistar rats weighing approximately 400–500 g, according to a schedule described in the legends to the figures. The methods for preparation of the plasma samples and quantitation of the nucleosides and bases by HPLC have been described previously [2, 10].

Gradients for separation of the nucleosides and their bases by HPLC. Mono- or biphasic linear gradients of 10 mM potassium phosphate (pH 5.5) (buffer A) and methanol–potassium phosphate buffer (80:20) were used as followed: (i) gradient 1, from 100 to 89% of buffer A within 13 min for the separation of Ura (4.3 min) and dUrd (9.4 min); (ii) gradient 2, from 100 to 89% of buffer A within 13 min, then to 40% of buffer A in 30 min for separation of Ura, dUrd, BVUra (26.9 min) and BVdUrd (28.9 min); (iii) gradient 3, from 100 to 61% of buffer A within 17 min for the separation of Thy (6.3 min), dUrd (6.9 min), dThd (10.4 min), EtUra (11.2 min), ClEtUra (12.8 min), EtdUrd (13.8 min) and ClEtdUrd (15.9 min); (iv) gradient 4, from 100 to 61%, then to 40% of buffer A, in 17 and 30 min, respectively, for the separation of BVUra (21.5 min) and BVdUrd (24.1 min).

In vitro *phosphorolysis of dUrd analogues.* Thymidine phosphorylase (dThd Pase) was extracted from human blood platelets [23] and had a specific activity of 10 U/mg of protein, one unit of enzyme activity corresponding to the conversion of 1 μmol of dThd/min at 37° and pH 6, with 0.1 mM dThd and 50 mM potassium phosphate as substrates. Uridine phosphorylase (Urd Pase) was partially purified by DEAE-cellulose chromatography according to Krenitsky *et al.* [24] and had a specific activity of 0.03 U/mg with dThd as substrate. The phosphorolysis of dUrd and 5-substituted dUrd analogues was studied by a continuous spectrophotometric assay as previously described [2], except that the concentration of the potassium phosphate buffer was 50 mM and its pH 6. The reaction mixture consisted of 0.1 mM nucleoside in the buffer mentioned above and enzyme at a final concentration of 0.3 μg/ml (dThd Pase) or 18 μg/ml (Urd Pase). The absorbance change was monitored at that wavelength where the difference between the deoxynucleoside and its base was maximal: dUrd ($\Delta\varepsilon = 2300$ at 270 nm), dThd ($\Delta\varepsilon = 2340$ at 275 nm), EtdUrd ($\Delta\varepsilon = 2440$ at 272 nm), ClEtdUrd ($\Delta\varepsilon = 2450$ at 274 nm), VdUrd ($\Delta\varepsilon = 2050$ at 300 nm), ClVdUrd ($\Delta\varepsilon = 2250$ at 303 nm), BVdUrd ($\Delta\varepsilon = 2400$ at 304 nm), and IVdUrd ($\Delta\varepsilon = 2000$ at 309 nm), $\Delta\varepsilon$ being expressed in M^{-1} cm^{-1}. The linear part of the curve of ΔA (absorbance increment) versus time was used to determine the initial velocities.

Table 1. Effect of dThd and dUrd on the antiviral activity of BVdUrd and EtdUrd in primary rabbit kidney (PRK) and human embryonic skin (E_6SM) cell cultures

Compounds		Minimal inhibitory concentration* (nmol/ml) herpes simplex virus-1 (KOS)	
		PRK cells	E_6SM cells
BVdUrd		0.2	0.2
BVdUrd	+ dThd 413 nmol/ml	60	300
	+ dThd 41.3 nmol/ml	6	0.2
	+ dThd 4.1 nmol/ml	0.2	0.2
BVdUrd	+ dUrd 438 nmol/ml	0.6	2.1
	43.8 nmol/ml	0.2	0.2
	4.4 nmol/ml	0.2	0.1
EtdUrd		2.7	0.8
EtdUrd	+ dThd 413 nmol/ml	>1561	390
	+ dThd 41.3 nmol/ml	78	390
	+ dThd 4.1 nmol/ml	27	78
EtdUrd	+ dUrd 438 nmol/ml	78	780
	+ dUrd 43.8 nmol/ml	7.8	27
	+ dUrd 4.4 nmol/ml	7.8	7.8

* Required to reduce virus-induced cytopathogenicity by 50%; dThd and dUrd did not interfere with viral cytopathogenicity at the highest concentration tested.

Table 2. Phosphorolysis of several 5-substituted dUrd analogues by dThd Pase

Compounds	5-Substituent	Initial velocities of phosphorolysis*
dUrd	—H	9
dThd	—CH_3	10.3
EtdUrd	—CH_2—CH_3	6.5
ClEtdUrd	—CH_2—CH_2Cl	8.7
VdUrd	—CH=CH_2	19
ClVdUrd	—CH=CHCl	25
BrVdUrd	—CH=CHBr	22
IVdUrd	—CH=CHI	13.3

* Calculated as described in Materials and Methods, and expressed in μmol of deoxynucleosides degraded per min and per mg of protein.

RESULTS

The results presented in Table 1 clearly show that dThd counteracted the antiviral activity of BVdUrd and EtdUrd against HSV-1 (KOS) in both primary rabbit kidney cells and human embryonic skin fibroblasts. Similar results were obtained in other cell lines (data not shown). The reversing effect of dThd on the antiviral action of EtdUrd was even apparent at a concentration as low as 4 nmol/ml. In contrast, dUrd did not affect the antiviral activity of BVdUrd or EtdUrd when used at 4 or 44 nmol/ml. Only at 438 nmol/ml, dUrd reduced the antiviral activity of EtdUrd and, to a lesser extent, that of BVdUrd. EtdUrd was by itself active in inhibiting virus replication at a concentration of 3.9 nmol/ml and higher; it did not counteract the antiviral activity of BVdUrd (data not shown).

We then determined whether, in rats, BVdUrd could be regenerated from BVUra by a pentosyl transfer reaction with either dUrd or EtdUrd as the deoxyribosyl donor. Both dUrd and EtdUrd are substrates for dThd phosphorylase *in vitro* (ref. 2 and Table 2), and may also be subject to degradation by these PNPases *in vivo*, since their respective bases, Ura and EtUra, appear rapidly in the plasma after administration of dUrd or EtdUrd to rats (Figs. 1a and b). When dUrd or EtdUrd (200 μmol/kg)

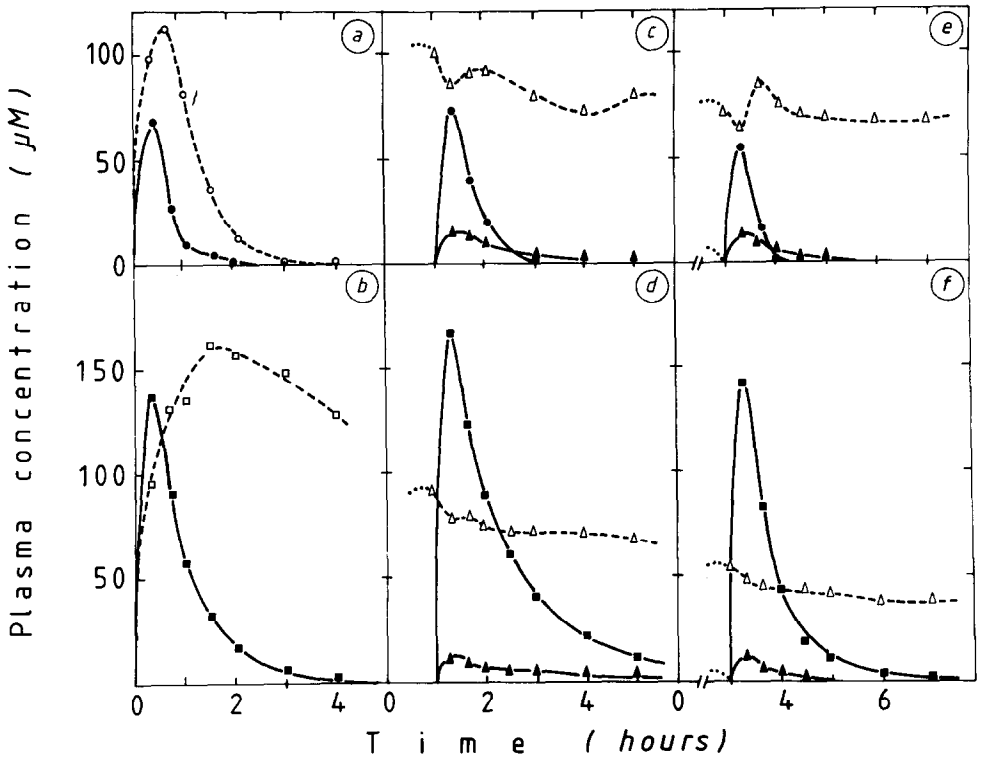

Fig. 1. Generation and regeneration of BVdUrd from BVUra in rats by deoxyribosyl transfer from dUrd or EtdUrd: (a) and (b), plasma concentrations of dUrd (●), Ura (○), EtdUrd (■) and EtUra (□) after i.p. administration of dUrd (a), or EtdUrd (b); (c) and (d), plasma concentrations of BVdUrd (▲) generated from BVUra (△) after administration of dUrd (c) and EtdUrd (d) one hour after a previous administration of BVUra; (e) and (f), plasma concentrations of BVdUrd regenerated from BVUra after administration of dUrd (e) and EtdUrd (f) 3 hr after a previous administration of BVdUrd. Each compound was administered at 200 μmol/kg.

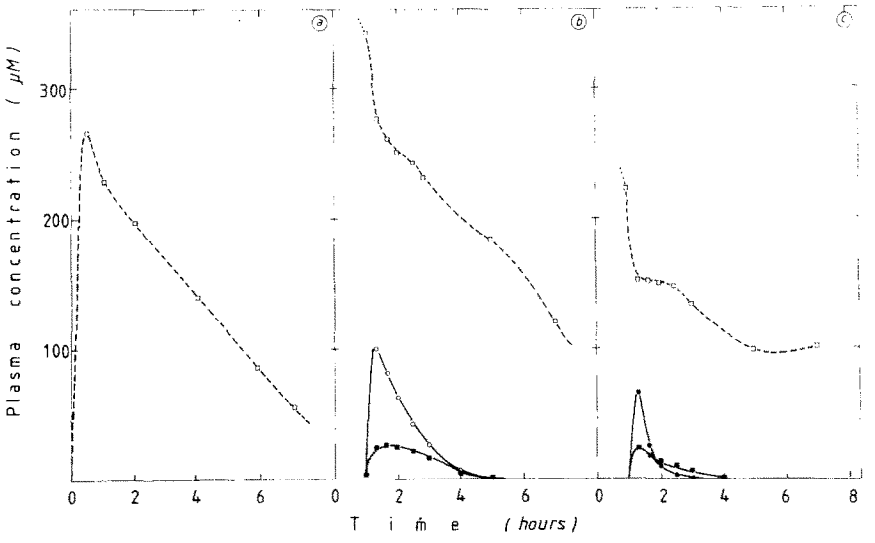

Fig. 2. Generation of EtdUrd from EtUra in rats: (a), (b) and (c), plasma concentrations of EtUra (□) after i.p. administration of EtUra; (b) and (c), plasma concentrations of EtdUrd (■) generated from EtUra after administration of (b) dThd (○) or (c) dUrd (●) 1 hr after a previous administration of EtUra. Each compound was administered i.p. at 200 μmol/kg.

were administered i.p. to rats one hour after the administration of BVUra (200 μmol/kg) (Figs. 1c and d), BVdUrd appeared in the blood, attaining plasma concentration of 15 μM within one hour after the administration of dUrd or EtdUrd. A BVdUrd concentration of 1 μM was still detectable 4–5 hr following the administration of dUrd or EtdUrd. BVdUrd could also be regenerated when dUrd or EtdUrd were administered 3 hr after the injection of 200 μmol/kg of BVdUrd, that is a time when BVdUrd had completely disappeared from the bloodstream (Figs 1e and f).

The generation of BVdUrd from BVUra is readily achieved because BVUra has a long half-life in the bloodstream in comparison with other uracil (Ura) analogues, such as thymine (Thy), 5-trifluoromethyl-Ura (CF_3Ura), 5-fluoro-Ura (FUra) or 5-iodo-Ura (IUra) [10]. However, as shown in Fig. 1b, EtUra, formed *in vivo* by the phosphorolysis of EtdUrd, also has a long half-life in rat plasma, probably because this base is not an efficient substrate for H_2Thy dehydrogenase [18]. When administered i.p. at 200 μmol/kg, EtUra attains its maximum plasma concentration 30–60 min after its injection, then disappears slowly from the plasma so that plasma concentrations above 50 μM are maintained for at least 7 hr (Fig. 2a), while other bases are cleared completely within 3–4 hours.

Consequently, we attempted to generate EtdUrd from EtUra by a deoxyribosyl transfer reaction *in vivo*. When dThd (200 μmol/kg) was injected i.p. to rats 1 hr after a previous administration of EtUra, EtdUrd appeared in the plasma with a peak concentration of 20–30 μM and maintained a concentration of 1 μM for at least 5 hr (Fig. 2b). EtdUrd generation from EtUra also occurred when dUrd was administered 1 hr after EtUra (Fig. 2c).

In attempts to regenerate EtdUrd from EtUra, dThd or dUrd (200 μmol/kg) were administered 3 hr after a previous injection of EtdUrd, when EtdUrd had completely disappeared from the plasma. At that time, only EtUra was present in the plasma; and injection of dThd (Fig. 3a) or dUrd (Fig. 3b) led to a prompt reappearance of EtdUrd in the circulation.

Akin to the other 5-substituted dUrd analogues, ClEtdUrd is also a substrate for human dThd phosphorylase; its phosphorolysis occurs with an initial velocity similar to that of dUrd, but 3 times lower than that of its unsaturated analogue ClVdUrd (Table 2). *In vivo*, ClEtdUrd is rapidly cleared from the bloodstream with an half-life time similar to that of the other dUrd analogues (Table 3). The free base resulting from the phosphorolysis of ClEtdUrd, i.e. ClEtUra, has, like BVUra and EtUra, a long half-life in the rat, since a concentration of 25 μM ClEtUra is found in the plasma 7 hr after the administration of ClEtdUrd (Fig. 4a). When dThd (Fig. 4b) or dUrd (Fig. 4c) are administered 3 hr after ClEtdUrd, at a time when ClEtdUrd is almost completely cleared from the plasma and when ClEtUra is present at a plasma concentration of 50 μM, ClEtdUrd reappears

Table 3. Half-lifes of 5-substituted dUrd analogues in the plasma of rats following intraperitoneal administration

Compound	Mean $T_{1/2}$ (min)
dUrd	15.6 ± 5.2 (6)
dThd	25 ± 1.4 (3)
EtdUrd	27 ± 9.5 (3)
ClEtdUrd	24.3 ± 7.4 (3)
BVdUrd	25.6 ± 5.5 (3)

200 μmol/kg of each compound was administered i.p. to rats. The half-life time ($T_{1/2}$) was determined in the linear part of the semilogarithmic representation of plasma drug concentrations as a function of time. The number of experiments for each nucleoside is indicated in parentheses.

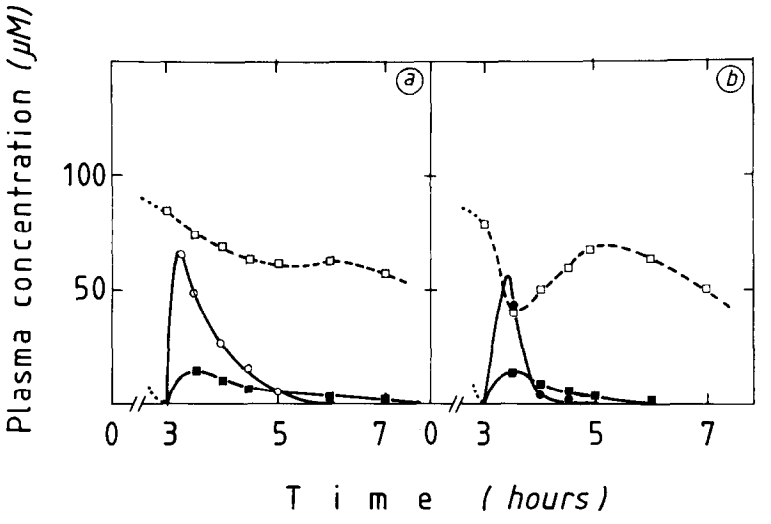

Fig. 3. Regeneration of EtdUrd in rats. Plasma concentrations of EtdUrd (■) regenerated from EtUra (□) upon administration of (a) dThd (○) or (b) dUrd (●) 3 hr after a previous administration of EtdUrd. Each compound was administered i.p. at 200 μmol/kg.

in the plasma; the regeneration of ClEtdUrd is accompanied by a decrease in the plasma concentration of ClEtUra (Figs. 4b and 4c). Such a decrease in plasma concentration has also been noted for BVUra (Fig. 1) and EtUra (Figs. 2 and 3) during the pentosyl transfer reaction.

Since BVUra and EtUra have a relatively long half-life *in vivo*, the generation of BVdUrd and EtdUrd can be achieved several times upon a single administration of the base followed by repeated administrations of dUrd, i.e. 1, 3 and 5 hr after injection of BVUra or EtUra (Figs. 5a and b). The repeated formation of ClEtdUrd could also be accomplished when a single injection of ClEtdUrd was followed, 3 hr later, by repeated administrations of dUrd although the maximum plasma levels of regenerated ClEtdUrd did not exceed 5–7 μM (data not shown).

DISCUSSION

It has previously been demonstrated that BVdUrd can be restored *in vivo* from its degradation product, BVUra, upon administration of dThd or some dThd analogues, i.e. FdUrd, IdUrd or CF_3dUrd [10]. To the extent that these compounds potentiate the *in vivo* efficacy of BVdUrd as an antiviral drug, their combined use with BVdUrd may be of clinical importance. A limiting factor, however, is that dThd

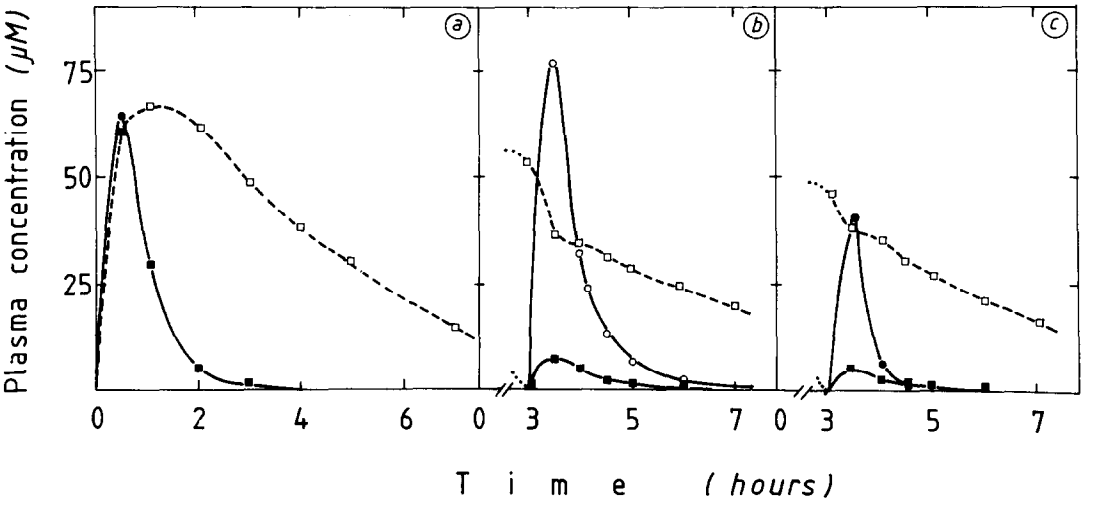

Fig. 4. Regeneration of ClEtdUrd in rats. (a), plasma concentrations of ClEtdUrd (■) and ClEtUra (□) after i.p. administration of ClEtdUrd; (b) and (c), plasma concentrations of ClEtdUrd regenerated from ClEtUra upon administration of (b) dThd (○) or (c) dUrd (●) 3 hr after a previous administration of ClEtdUrd. Each compound was administered i.p. at 200 μmol/kg.

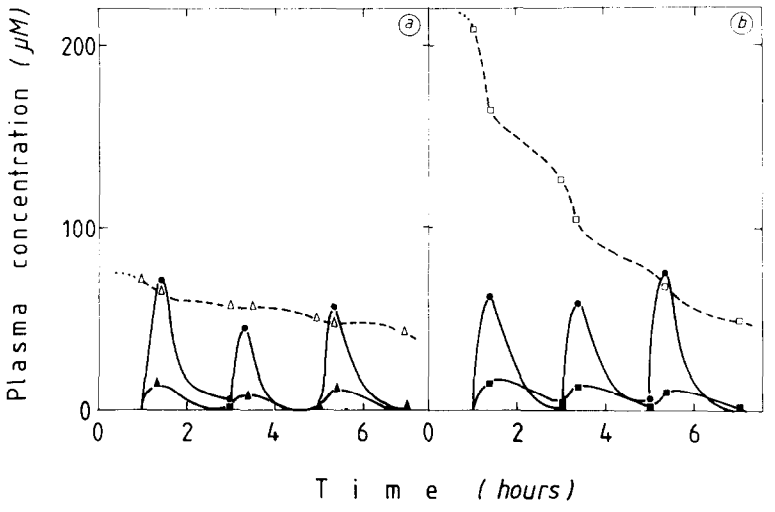

Fig. 5. Repeated generation of BVdUrd and EtdUrd in rats after a single administration of their base (BVUra and EtUra, respectively), followed by repeated administrations of dUrd: (a) and (b) plasma concentration of BVUra (△) and EtUra (□) after i.p. administration of BVUra and EtUra; (a) plasma concentrations of BVdUrd (▲) generated from BVUra after administration of dUrd (●) 1, 3 and 5 hr after the administration of BVUra; (b), plasma concentrations of EtdUrd (■) generated from EtUra after administration of dUrd (●) 1, 3 and 5 hr after the administration of EtUra. Each compound was administered i.p. at 200 μmol/kg.

antagonizes the antiviral activity of BVdUrd [12–15] and FdUrd, IdUrd or CF_3dUrd are known to be toxic, mutagenic, teratogenic and/or carcinogenic.

From the present findings it appears that dUrd, which does not counteract the antiviral activity of BVdUrd *in vitro*, can be used as deoxyribosyl donor to regenerate BVdUrd from BVUra *in vivo*. EtdUrd, which is relatively non-toxic and by itself an antiviral agent [12, 16, 17], can also be used as pentosyl donor to regenerate BVdUrd *in vivo*; and the regeneration of an antiherpetic agent by another one may have interesting therapeutic implications.

Like BVUra, EtUra has a long half-life in the plasma of the rats, probably because EtUra is not a substrate for H_2Thy-dehydrogenase [18], the first enzyme of the catabolic pathway of pyrimidines. Therefore, EtdUrd can also be generated *in vivo* from EtUra via a pentosyl transfer reaction with dThd or dUrd as the pentosyl donors. Following i.p. administration of EtUra and dUrd at 200 μmol/kg, blood levels of EtdUrd may reach 25 μM, which is far above the minimum antiviral concentration of EtdUrd [17]. ClEtdUrd, another potent antiherpetic agent [19, 20], can also be regenerated from its base ClEtUra, after dThd or dUrd administration; while the maximum concentration of ClEtdUrd achieved in the plasma did not exceed 5–7 μM; a concentration of 1 μM was maintained for at least 4 hr following administration of the deoxyribosyl donor (dThd or dUrd).

We have previously demonstrated that BVdUrd can be (re)generated several times after a single administration of BVdUrd or BVUra followed by repeated doses of dThd [6], apparently because high levels of BVUra are maintained in the plasma for a long time. Like BVUra, EtUra has a long half-life in plasma, and our present findings indicate that the generation of both BVdUrd and EtdUrd can be repeated at least 3 times, after a single administration of BVUra or EtUra followed by repeated injections of dUrd. By this process, drug concentrations well above 1 μM can be maintained for both BVdUrd and EtUrd for at least 6 hr; this is still far in excess of the minimum concentration required for BVdUrd to express antiviral activity [9]. For ClEtdUrd, repeated regeneration by repeated administrations of dUrd after a single administration of ClEtdUrd (ClEtUra not being available) has also been demonstrated (data not shown). This regeneration process yielded for up to 9 hr plasma concentrations of 1 μM ClEtdUrd, which is again in excess of the minimum antiviral concentration of ClEtdUrd [19, 20].

In conclusion, our studies demonstrate that: (i) BVdUrd can be regenerated or even generated *de novo* from BVUra by a deoxyribosyl transfer with as pentosyl donor, either dUrd which, unlike dThd, does not interfere with the antiviral activity of BVdUrd, or EtdUrd which has by itself marked antiviral activity; (ii) this process can be extended to other antiviral agents, like EtdUrd or ClEtdUrd, of which the base has a long half-life in the plasma; and finally (iii), this process can be repeated several times allowing to maintain during several hours, after a single administration of the nucleoside or its base, sufficient plasma levels of the active compound to achieve an antiviral effect.

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