Table 1. Kainic acid analogues as inhibitors of high affinity uptake of D-[^{3}H]aspartate into adult rat crude cerebellar synaptosomes

Inhibitor	IC_{50} (μM)
α-Dihydrokainate	110 ± 16.5
α-Kainic acid	350 ± 52.5
β-Dihydrokainate	825 ± 124
2-Carboxy-4-ethyl-3-pyrrolidine methanol	930 ± 140
2-Carboxy-4-propanol-3-pyrrolidine acetic acid	970 ± 144
α-Allokainic acid	1050 ± 158
β-Kainic acid	1325 ± 200

Adult rat cerebellar synaptosomes were incubated with each inhibitor over a concentration range of 100 μM to 1.5 mM for 3 min at 37°, in the presence of 10 nM D[^{3}H]aspartate. The IC_{50} values were determined from the plots shown in Fig. 2. Results are means ± S.E.M. from three independent inhibition curves. Inactive compounds tested: α-homokainate, β-homokainate, α-alloketokainate, β-alloketokainate, α-ketokainate, β-ketokainate, α-kainylglycine, β-kainylglycine, α-kainylaminomethylphosphonate, β-kainylaminomethylphosphonate and α-carboxykainate.

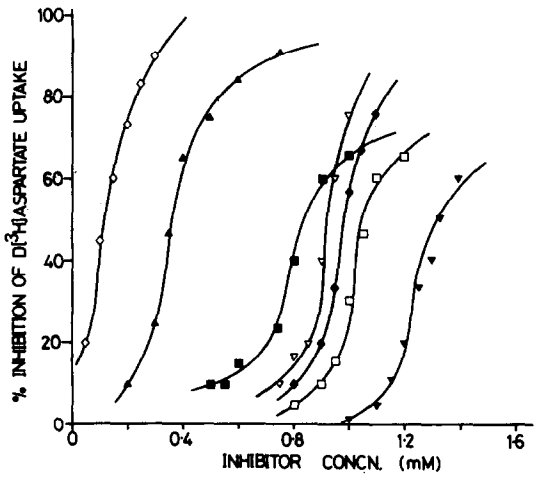

Fig. 2. Dose v/s % inhibition plots for D-[^{3}H]aspartate uptake in adult rat crude cerebellar synaptosomes incubated with α-dihydrokainate (◇), α-kainic acid (▲), β-dihydrokainate (■), 2-carboxy-4-ethyl-3-pyrrolidine methanol (▽), 2-carboxy-4-propanol-3-pyrrolidine acetic acid (◆), α-allokainic acid (□) and β-kainic acid (▼), for 3 min at 37° in the presence of 10 nM D-[^{3}H]aspartate. Each point is the mean of three independent experiments assayed in quadruplicate with S.E. of approximately 10–15%.

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REFERENCES

1. F. Fonnum, *J. Neurochem.* **42**, 1 (1984).
2. G. A. R. Johnston, in *Glutamate: Transmitter in the Central Nervous System* (Eds. P. J. Roberts, J. Storm-Mathisen and G. A. R. Johnston), p. 77. John Wiley, Chichester (1981).
3. G. A. R. Johnston, D. Lodge, J. C. Barnstein and D. R. Curtis, *J. Neurochem.* **34**, 241 (1980).
4. G. J. McBean and P. J. Roberts, *J. Neurochem.* **44**, 247 (1985).
5. V. J. Balcar and G. A. R. Johnston, *J. Neurobiol.* **3**, 295 (1972).
6. L. P. Davies and G. A. R. Johnston, *J. Neurochem.* **26**, 1007 (1976).
7. G. A. R. Johnston, S. M. E. Kennedy and B. Twitchin, *J. Neurochem.* **32**, 121 (1979).
8. H. Anand, P. J. Roberts and J. F. Collins, *Br. J. Pharmac.* (Proc. Suppl., in press).
9. H. Anand, P. J. Roberts, G. Badmen and J. F. Collins, *Biochem. Pharmac.* (in press).
10. O. H. Lowry, N. S. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).

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Enzymatic cleavage of 5-substituted-2′-deoxyuridines by pyrimidine nucleoside phosphorylases

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Some 5-substituted derivatives of 2′-deoxyuridine (5-ethyl-, 5-propyl-, 5-isopropyl-, (E)-5-(2-bromovinyl)-2′-deoxyuridine) have antiviral activity [1–6]. The corresponding pyrimidine bases have only very weak antiviral activity compared with nucleosides [7]. It has also been reported that 5-hexyl-2′-deoxyuridine is active towards Ehrlich and

NK/Ly ascites tumors and Novikoff hepatoma whereas 5-hexyluracil has no antitumor activity [8]. Therefore cleavage of the N-glycosidic bond of these nucleosides catalyzed by pyrimidine nucleoside phosphorylases leads to a loss of biological activity. There are two distinct pyrimidine nucleoside phosphorylases in mammalian cells [9–11]. One is thymidine phosphorylase (TdRPase EC 2.4.2.4.).* This enzyme is specific for the 2′-deoxyribosyl moiety of the nucleosides [9, 12]. The other phosphorylase is uridine phosphorylase (URPase, EC 2.4.2.3) which has broader substrate specificity.

The subject of our investigations was the study of phosphorolysis of some 5-substituted-2′-deoxyuridines by mammalian TdRPase and URPase isolated from the liver of mice and from rat intestinal mucosa.

Materials and methods

Materials. [2-^{14}C] Thymidine (53.2 mCi/mmole) and [6-^{3}H]uridine (21.6 Ci/mmole) were obtained from Amersham International Ltd. (Amersham, U.K.). [2-^{14}C] 5-ethyl-2′-deoxyuridine (4.8 mCi/mmole) [2-^{14}C] 5-butyl-2′-deoxyuridine (2.6 mCi/mmole), [2-^{14}C]5-hexyl-2′-deoxyuridine (3.2 mCi/mmole), [2-^{14}C]5-isopropyl-2′-deoxyuridine (23.4 mCi/mmole) and [2-^{14}C] (E)-5-(2-bromovinyl)-2′-deoxyuridine (0.6 mCi/mmole) were synthesized by methods previously published [13, 14].

DEAE-cellulose (DE-32) was purchased from Whatman Biochemicals (Maidstone, Kent, U.K.). Silica gel plates (DC-Alufolien Kieselgel 60 F_{254}) and all other chemicals were obtained from Merck (Darmstadt, F.R.G.).

Enzyme preparation and partial purification. URPase was prepared and partially purified from rat intestinal mucosa as previously described [15]. There was no detectable phosphorolysis of TdR by URPase in the presence of 2,2′-anhydro-5-ethyluridine, showing that our URPase does not contain TdRPase. (2-2′-Anhydro-5-ethyluridine is a potent and specific inhibitor of URPase as previously described [15].) TdRPase was isolated from mouse liver, using DEAE-cellulose chromatography to remove URPase contamination from the preparation as described elsewhere [15].

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [16].

Enzyme assay. The phosphorolysis of pyrimidine nucleosides by the extract of mouse liver and rat intestinal mucosa was assayed in a 125 μl incubation mixture which contained 0.04 M potassium phosphate buffer (pH 7.4), 2 mM 2-mercaptoethanol, 0.001–1 mg protein and substrate at the desired concentration. Incubations were carried out at 37°.

Thin-layer chromatography. The enzyme reaction was stopped with ice-cold methanol, and reaction mixtures were applied on the silica gel plates. (The appropriate base and nucleoside were also applied to facilitate visualization on TLC plates.) Silica gel plates were developed with ethylacetate–methanol–chloroform (8:1:1, v/v/v) or ethylacetate–methanol (9.5:0.5,v/v) (Table 1). Spots containing substrate or product were identified by u.v. quenching, cut out, eluted with 1 ml of methanol and then counted in a scintillation cocktail containing toluene, using a Packard model 2650 scintillation spectrometer. The recovery of radioactivity from the plates was 95%.

Table 1. Mobilities of nucleosides and their bases on TLC plates

Compound	R_f in solvent system* A	B
UR	0.40	—
U	0.12	—
TdR	0.53	—
T	0.38	—
EdUrd	0.64	—
EUra	0.50	—
BdUrd	0.74	—
BUra	0.62	—
HdUrd	—	0.77
HUra	—	0.64
iPdUrd	—	0.71
iPUra	—	0.53
BVdUrd	—	0.79
BVUra	—	0.69

* Solvent systems: (A) Ethylacetate–methanol–chloroform (8:1:1,v/v/v); (B) ethylacetate–methanol (9.5:0.5, v/v).

Results and discussion

The appK_m and appV_{max} values for 5-substituted-2′-deoxyuridines of TdRPase and URPase are presented in Table 2.

The appK_m value for EdUrd of TdRPase is about 4 times higher than that for TdR. Introduction of a longer alkyl chain in position C-5 further increases the appK_m value. The appK_m value is greatly increased by substitution of a branched side chain at C-5 of 2′-deoxyuridine.

The appK_m value of TdRPase for iPdUrd is by more than one order of magnitude higher than that for TdR. The appK_m of TdRPase for BVdUrd does not differ from the appK_m for TdR. In contrast to TdRPase the appK_m values of URPase for 5-alkyl-2′-deoxyuridines are practically not related to the number of carbon atoms of the C-5 substituent and are similar to the appK_m for UR. The only exception is iPdUrd with branched side chain and higher appK_m value. The appK_m values of URPase for 5-alkyl-2′-deoxyuridines are much lower than those of TdRPase, showing that these nucleosides may be better substrates for URPase.

While the appK_m value of TdRPase for BVdUrd was equal to that for TdR, the appK_m of URPase for BVdUrd was much higher than that for TdR.

The rate of phosphorolysis of 5-alkyl-2′-deoxyuridines was found to be related to the length of the C-5 side chain. The longer the side chain the slower the rate of phosphorolysis. This observation is true for both enzymes. BVdUrd underwent very quick phosphorolysis in the presence of both enzymes. This finding is in agreement with the observations of Desgranges *et al.* [17] and Liermann and Hermann [18], according to whom this analog is a good substrate for pyrimidine nucleoside phosphorylases.

The parameter V^{rel}_{max}/K_m as described by Nakayama represents the "efficiency of catalysis" of the reaction of a given substrate [12]. This means that analogs with higher V^{rel}_{max}/K_m values are more rapidly catabolized.

EdUrd, iPdUrd and, in particular, BVdUrd are efficient antiviral agents. These analogs are phosphorolysed by pyrimidine nucleoside phosphorylases. The data presented here show that TdRPase from mouse liver catabolises these nucleosides in the following order: BVdUrd>EdUrd>iPdUrd, while in the rate of phosphorolysis by URPase of rat intestinal mucosa follows the order: EdUrd>BVdUrd>iPdUrd.

* Abbreviations used: URPase, uridine phosphorylase; TdRPase, thymidine phosphorylase; UR, uridine; U, uracil; TdR, thymidine; T, thymine; EdUrd, 5-ethyl-2′-deoxyuridine; EUra, 5-ethyluracil; BdUrd, 5-butyl-2′-deoxyuridine; BUra, 5-butyluracil; HdUrd, 5-hexyl-2′-deoxyuridine; HUra, 5-hexyluracil; iPdUrd, 5-isopropyl-2′-deoxyuridine; iPUra, 5-isopropyluracil; BVdUrd, (E)-5-(-2-bromovinyl)-2′-deoxyuridine; BVUra, (E)-5-(2-bromovinyl)-uracil; TLC, thin-layer chromatography; u.v., ultra violet; appK_m, apparent K_m; appV_{max}, apparent V_{max}.

Table 2. Apparent kinetic constants for phosphorolysis of 5-substituted-2′-deoxyuridines by TdRPase and URPase*

Compound	appK_m (mM)		appV_{max} (nmole/min/mg protein)		appV^{rel}_{max}/appK_m	
	TdRPase	URPase	TdRPase	URPase	TdRPase	URPase
UR	ND†	0.0219	ND	89.4	ND	365.5
TdR	0.236	0.0292	4.4	11.17	4.24	34.2
EdUrd	0.912	0.0226	4.31	6.7	1.07	26.5
BdUrd	2.1	NT‡	1.78	NT	0.19	NT
HdUrd	3.97	0.0224	1.92	0.079	0.11	0.32
iPdUrd	9.15	0.178	0.24	0.61	0.006	0.31
BVdUrd	0.226	1.06	3.61	76.8	3.63	6.5

*appV^{rel}_{max} = appV_{max}/appV^{TdR}_{max}. All nucleosides have β-D-configuration. AppK_m and appV_{max} values were determined from Lineweaver–Burk plots of the data by a computer program with least-squares fitting.

†ND, not detectable.

‡NT, not tested.

In summary, our data show that the appK_m values of URPase for 5-alkyl-2′-deoxyuridines are not related to the length of the side chain at C-5, while the appK_m values of TdRPase for these analogs increase with the elongation of the side chain. Nucleoside analogs containing a branched or a long linear alkyl chain are more resistant to phosphorolytic cleavage than nucleoside analogs with a short linear side chain at C-5.

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REFERENCES

1. E. De Clercq, *Meth. Find exp. clin. Pharmac.* **2**, 253 (1980).
2. E. De Clercq and P. F. Torrence, *J. Carbohydr. Nucleosides, Nucleotides,* **5**, 187 (1978).
3. Y.-C. Cheng, S. Grill and G. Dutschman, *Biochem. Pharmac.* **28**, 3529 (1979).
4. E. De Clercq, J. Descamps, P. De Somer, P. J. Barr, A. S. Jones and R. T. Walker, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2947 (1979).
5. H. S. Allaudeen, M. S. Chen, J. J. Lee, E. De Clercq and W. H. Prusoff, *J. biol. Chem.* **257**, 603 (1982).
6. E. De Clercq, in *Proceedings of the 4th International Round Table* (Eds. F. C. Alderweireldt and E. L. Esmans), p. 25. University of Antwerp (R.U.C.A.), Belgium (1982).
7. M. Muraoka, A. Takada and T. Ueda, *Chem. Pharm. Bull.* **18**, 261 (1970).
8. L. Ötvös, K. Lapis, A. Szabolcs, V. Kemény, A. Jeney and L. Kopper, XII. Congreso Internacional del Cáncer 5 al 11 de Octubre de 1978, Buenos Aires, Abstr. 2, 14.
9. T. A. Krenitsky, J. W. Mellors and R. K. Barclay, *J. biol. Chem.* **240**, 1281 (1965).
10. H. Ishitsuka, M. Miwa, K. Takemoto, K. Fukuoka, A. Itoga and H. B. Maruyama, *Gann* **71**, 112 (1980).
11. P. W. Woodman, A. M. Sarrif and C. Heidelberger, *Cancer. Res.* **40**, 507 (1980).
12. C. Nakayama, Y. Wataya, R. B. Meyer, Jr., D. V. Santi, M. Saneyoshi and T. Ueda, *J. med. Chem.* **23**, 962 (1980).
13. J. Sági, A. Szabolcs, A. Szemző and L. Ötvös, *Nucleic Acids Res.* **9**, 6985 (1981).
14. A. Szabolcs, G. Kruppa, J. Sági and L. Ötvös, *J. Lab. Comp. Radiopharm.* **14**, 713 (1978).
15. Zs. Veres, A. Szabolcs, I. Szinai, G. Dénes, M. Kajtár-Peredy and L. Ötvös, *Biochem. Pharmac.* **34**, 1737 (1985).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. C. Desgranges, G. Razaka, M. Rabaud, H. Bricaud, J. Balzarini and E. De Clercq, *Biochem. Pharmac.* **32**, 3583 (1983).
18. B. Liermann and G. Hermann, *Biomed. biochim. Acta* **42**, K 35 (1983).

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