Biochemical Pharmacology, 1964, Vol. 13, pp. 365–370. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF NATURAL AND UNNATURAL PYRIMIDINES AND PYRIMIDINE NUCLEOSIDES ON THE PHOSPHOROLYSIS OF 5-IODODEOXYURIDINE BY MOUSE LIVER EXTRACT*

JOSEPH P. KRISS and SHEILA B. BOND

Departments of Medicine and Radiology, Stanford University School of Medicine, Stanford, Calif., U.S.A.

(*Received* 22 *August* 1963; *accepted* 1 *October* 1963)

Abstract—The degradation of 5-iododeoxyuridine (IUdR) to 5-iodouracil (IU) by a supernatant fraction of mouse liver extract in the presence of added threefold-excess amounts of a variety of pyrimidines and pyrimidine nucleosides has been investigated. Phosphorolysis of IUdR was significantly reduced by the addition as substrates of the nucleosides, 5-bromodeoxyuridine, thymidine, deoxyuridine, and 5-trifluoromethyldeoxyuridine, and by the 5-halogenated pyrimidines, bromouracil and iodouracil. Slight effect was observed with addition of arabinosylthymine. Minimal or absent effects were observed with 21 other related compounds.

THE rapid degradation of 5-bromodeoxyuridine (BUdR) and 5-iododeoxyuridine (IUdR) *in vivo*[1–5] greatly limits the clinical usefulness of these drugs as antineoplastic agents,[6] radiosensitizers,[7] systemic antiviral compounds,[8] or detectors of malignant tissue.[3] On the other hand, when IUdR is applied topically as treatment against superficial virus infections,[9–11] or when BUdR or IUdR is used in *in vitro* systems to increase cellular sensitivity to ultraviolet light or X rays,[12] phosphorolytic cleavage is greatly reduced or absent, and striking drug effects are obtained.

Significantly enhanced clinical effectiveness of systemically administered BUdR and IUdR appears to require a safe, reliable method of reducing the rate or extent of their phosphorolysis. The same practical considerations also apply to the potential use of the related compounds 5-bromodeoxycytidine (BCdR) and 5-iododeoxycytidine (ICdR), since they are rapidly deaminated in man to BUdR and (IUdR), respectively,[3, 13, 14] and subsequently rapidly cleaved to their respective pyrimidine bases.

The present studies were designed to determine to what extent the phosphorolysis of IUdR may be inhibited by the addition of another competing substrate. Mouse liver was selected as the source of pyrimidine nucleoside phosphorylase. An extract of a homogenate of the organ was prepared that cleaved IUdR to 5-iodouracil (IU) without release of iodide due to sequential degradation of IU,[15] and the effect of 29 different compounds on the rate and extent of the cleavage of a standard quantity of IUdR-^{125}I was determined.

* This investigation was supported by Grants CA 06135-02 CY and C5838 from the National Cancer Institute, Bethesda, Md.

MATERIALS AND METHODS

IUdR-^{125}I, with a specific activity of 15 mc/mmole was prepared by a modification[16] of the method of synthesis of ICdR-^{3}H of Chang and Welch.[17] Purity of the compound was established by chromatographic analysis, with the solvent systems butanol–H_2O–

TABLE 1. EFFECT OF A VARIETY OF COMPOUNDS IN INHIBITING THE DEGRADATION BY A LIVER EXTRACT OF IUdR-^{125}I TO IU-^{125}I

Results are expressed as the increment in percentage IUdR-^{125}I radioactivity of 45-min and 90-min incubates containing 0·3 μmole of compound, plus 0·1 μmole of IUdR-^{125}I (in a volume of 80 μl) over those of control incubates containing IUdR-^{125}I alone. Compounds are listed in order of decreasing effect at the 90-min interval. The expected percentage variation is ±5%.

1	2	3	4	5
No.	Substance	Obtained from*	Increment in IUdR-^{125}I activity over control, % remaining after, 45 min	90 min
1	5-Bromodeoxyuridine	1	32	45
2	5-Iododeoxyuridine	1	26	32
3	5-Bromouracil	4	23	32
4	Thymidine	1	22	26
5	5-Trifluoromethyl-2′-deoxyuridine	5	15	25
6	5-Iodouracil	1	23	24
	2′-Deoxyuridine + 5-fluorodeoxyuridine†		15	22
7	2′-Deoxyuridine	2	14	20
8	Arabinosylthymine	7	5	12
9	(+)-5-Bromo-6-methoxy-5,6-dihydro-thymidine	3	−6	9
10	6-Azauracil	1	5	9
11	Uridine	1	4	8
12	2-Deoxy-D-ribose-1-phosphate	1	3	8
13	5-Aminouridine	1	6	6
14	(+)-5-Bromo-5-fluoro-6-methoxy,5,6-dihydro-2′-deoxyuridine	3	3	4
15	5-Fluorouridine	3	7	4
16	6-Azadeoxyuridine	6	6	4
17	5-Aminouracil	1	−2	3
18	2-Deoxy-D-ribose	1	−5	2
19	6-Hydroxymethyluracil	1	−1	2
20	Deoxycytidine	1	−4	1
21	5-Fluorodeoxyuridine	3	−10	1
22	6-Azathymine	1	2	1
23	Thymine	1	1	1
24	6-Azathymidine	8	0	0
25	5-Methyldeoxycytidine	1	−3	−3
26	6-Azauridine	1	−9	−3
27	5-Fluorouracilarabinoside	5	6	−3
28	5-Fluorouracil	5	−3	−5
29	Uracil	2	−7	−6

* 1. California Corp. for Biochemical Research, Los Angeles, Calif.
2. Schwarz BioResearch, Orangeburg, N.Y.
3. Gift of Dr. R. Duschinsky, Hoffman-LaRoche, Inc., Nutley, N.J.
4. Sigma Chemical Co., St. Louis, Mo.
5. Courtesy of Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.
6. Gift of Dr. J. Škoda, Academy of Science, Praha, Czechoslovakia.
7. Gift of Dr. Leon Goodman, Stanford Research Institute, Palo Alto, Calif.
8. Gift of Dr. William Prusoff, Yale University, New Haven, Conn.

† 0·3 μmole UdR + 0·0004 μmole FUdR (5×10^{-6} M).

NH_3 (86 : 14 : 5%) and 2-propanol–water–HCl.[18] Also used in this study were 29 stable compounds, which are listed in Table 1, together with their respective sources of supply (columns 1–3).

METHODS

Male mice of the BLA strain, 90–120 days old, maintained on Purina chow, were used as a source of fresh liver for the preparation of a homogenate. The animals were killed by decapitation; immediately afterward the liver was excised and a 10% (w/v) homogenate prepared in ice-cold 0·25 M sucrose solution in a Schneider and Hogeboom tissue homogenizer. The homogenate was centrifuged at 700 *g* for 10 min to remove cell debris and nuclei. Fifty microliters of the supernatant fraction derived from 5 mg of wet liver tissue contained 0·6 mg of protein, as determined by the technique of Lowry *et al.*[19]

Into each of 30 small (12 × 100 mm) test tubes was placed 0·1 μmole IUdR-^{125}I. To each of 29 of these tubes was added 0·3 μmole of each of the test substances listed above (Table 1), respectively, in aqueous solution; the remaining tube, containing only IUdR-^{125}I, served as control. The mixtures were then taken to dryness by placing the tubes overnight in a vacuum desiccator. The experiment was started by the addition to each tube of 30 μl or 0·2 M phosphate buffer, pH 7·4, and 50 μl of fresh liver extract. The contents of the tube were well mixed, plugged with glass wool, and incubated at 37° for 2 hr in a metabolic shaker-incubator. At zero time and at intervals of 45 and 90 min, 20 μl of each mixture was removed and spotted on to Whatman 3 MM chromatographic paper strips. The strips were then subjected to descending chromatography using the solvent system of 2-propanol : water : HCl[18] for 18 hr. The paper chromatograms were then scanned for radioactivity in an automatic windowless gas-flow counter and recorder.* The areas under the recorded peaks of radioactivity on the chromatographed paper strips were measured with a compensating polar planimeter.† The percentage of the total radioactivity due to an identified component was calculated from the ratio of the area under its respective peak to that of all radioactive components. Birnie *et al.*[20] have described a similar design for an isotopic method to determine nucleoside phosphorylase, which gave values that agreed very well with those obtained by optical methods. In the present study peaks were usually found only at sites corresponding to IU and IUdR at R_f values of 0·78 and 0·88, respectively. The very small iodide peaks were rarely seen at the solvent front and averaged less than 1 per cent of the total activity in 93 determinations that were made. The zero-time samples were used to establish that in each case a single radioactive peak corresponding to IUdR was present initially.

RESULTS

When 0·1 μmole of IUdR-^{125}I was the sole substrate, 61 per cent was degraded to IU after 90 min. The addition of 0·3 μmole of nonradioactive IUdR resulted in a decreased degradation of IUdR-^{125}I to 29 per cent, while at the same time the total amount of IUdR catabolized to IU was increased from 0·061–0·117 ∂mole. The presence of any other compound capable of competing with IUdR-^{125}I for nucleoside phosphorylase (or capable of destroying enzymatic activity), or reversing

* Atomic Accessories, Inc., Valley Stream, N.Y.
† Keuffel and Esser Co., Germany.

the direction of the reaction (product inhibition), would likewise result in a decreased amount of IUdR-^{125}I cleaved, the degree of decrease depending on the amount of substrate added and the enzyme–substrate affinity.

Inasmuch as equimolar amounts of substrate (0·3 μmole) were added to each tube, observed differences in the amount of IUdR-^{125}I cleaved in the samples could be ascribed to the latter factor alone and permitted estimation of the relative substrate–enzyme affinities for the 29 compounds tested. The responses were expressed as the increase in percentage of radioactivity as IUdR-^{125}I remaining after incubation with added substrate over that percentage remaining in the control tube. Responses equal to or higher than that of IUdR indicated the presence of compounds with enzyme–substrate affinities equal to or higher than that of IUdR itself, while conversely, the lower the comparative response value, the less active was the compound as a competitor against IUdR. Values within a few per cent of zero indicate compounds that were judged to have no detectable activity under the experimental conditions described.

The results after 45 and 90 min of incubation are presented in Table 1, columns 4 and 5, respectively. The compounds are charted in order of declining activity after 90 min of incubation. At the later interval it will be noted that the addition of BUdR resulted in a response (45 per cent) in excess of that of IUdR (32 per cent), while responses equal to or slightly lower than that of IUdR were obtained with 5-bromouracil (BU), thymidine (TdR), 5-trifluoromethyl-deoxyuridine (CF_3-UdR), iodouracil (IU), and deoxyuridine (UdR). After the shorter interval of incubation of 45 min, the observed increments in IUdR-^{125}I activity were usually quantitatively smaller, but the same compounds gave evidence of activity. With the exception of some incubates, especially those containing compounds with very low responses, degradation of IUdR progressed between the intervals of 45 and 90 min, which indicates that the enzymatic reaction had not yet reached a plateau at 45 min.

That the observed effect of UdR cannot be ascribed to its conversion to thymidine was shown in another similar experiment in which 0·3 μmole UdR-^{3}H* was substituted for IUdR-^{125}I as substrate. The chromatographic system used with UdR-^{3}H was butanol–H_2O–NH_3 (86 : 14 : 5%) for 50 hr. Neither thymidine-^{3}H nor thymine-^{3}H was formed when UdR-^{3}H was the substrate. The effect of UdR in inhibiting IUdR phosphorolysis was not diminished in the presence of 5×10^{-6} M 5-fluorouracil-2′-deoxyribonucleoside (FUdR); FUdR alone (0·3 μmole) was inactive at both time intervals.

At the 90-min interval a slight effect was observed with arabinosylthymine, and doubtful effects were noted with 5-bromomethoxydihydrothymidine, 6-azauracil, uridine, and deoxyribose-1-phosphate (column 5, Table 1). Such effects, however, were not clearly discernible with these compounds at the 45-min interval (column 4). The remaining compounds had responses judged to be negligible; under these test conditions, they were considered to be inactive as competitors with IUdR for enzymatic phosphorolysis.

DISCUSSION

It is appreciated that in crude extracts pyrimidine nucleoside phosphorylase may be complicated by the presence of tissue inhibitors[21] or other enzymes,[15, 22] or that there may be considerable variation in pH within cells or parts of cells. However, we chose

* New England Nuclear Corp., Boston, Mass.

to work with a crude extract at the approximate pH of extracellular fluid (pH 7·4) because we were interested in studying IUdR phosphorolysis under conditions that we believe most closely approximate those found *in vivo*. Experiments conducted under such conditions are likely not to yield results identical with those obtained with purified enzyme preparations at different levels of pH. Thus, Friedkin and Roberts found that the optimum activity of TdR phosphorylase is at pH 5·7–6·0, with activity still about 75 per cent of maximum at a pH of 7·4.[21] Similarly, Birnie *et al.*[20] noted that FUdR was degraded by nucleoside phosphorylase prepared from Ehrlich ascites cells at a pH optimum of 6·4, whereas about 64 per cent of the maximal activity was noted at pH 7·4. The same investigators used a high-speed centrifugation method to prepare pyrimidine phosphorylase from mouse liver, which degraded 0·17 μmole FUdR/mg protein/20 min. The mouse liver phosphorylase used in the present studies degraded 0·2 μmole IUdR/mg protein/90 min. As would be expected, because of their similarity to IUdR in structure and biochemical behavior, BUdR and TdR were active in reducing the rate of phosphorolysis of IUdR, but BUdR was considerably more potent. In addition, the recently synthesized thymidine analog, trifluoromethyldeoxyuridine,[23] was as active a competitor as was TdR. Deoxyuridine, in the presence or absence of FUdR, had activity slightly less than that of TdR, whereas arabinosylthymine had slight activity under the same experimental conditions. Of those nucleosides with activity, only BUdR inhibited the degradation of IUdR-^{125}I to a greater extent than an equivalent amount of stable IUdR. The results suggest that only BUdR has a greater affinity for phosphorylase than has IUdR, whereas the other compounds have lower affinities.

The products of the phosphorolysis of BUdR and IUdR (BU and IU, respectively) also reduced IUdR degradation, an example of the general reaction of product inhibition observed with nucleoside phosphorylases.[22] The observation that thymine, the product of TdR phosphorolysis, was inactive, whereas its analogs, BU and IU, were active is an interesting one which we are unable to explain adequately.

In the present study, FUdR, 5-fluorouracilribonucleoside (FUR), and uridine were either weak or impotent in competing with IUdR for phosphorylase. However, Birnie *et al.* found that these compounds were degraded by purified Ehrlich cell phosphorylase even more readily than thymidine, at either pH 6·4 or pH 7·4.[20] At concentrations greater than 1 μmole/ml, FU, azathymine, UdR, uridine, and FUR were active in inhibiting the degradation of FUdR. In another study from the same laboratory, trifluoromethyldeoxyuridine also was found to act as a competitive inhibitor of the nucleoside phosphorylase splitting FUdR to FU.[21] With this same group of compounds, using concentrations in excess of 3 μmole/ml, we found only CF_3-UdR and UdR definitely active in inhibiting the degradation of IUdR.

The fact that the phosphorylation of IUdR *in vitro* may be reduced by the addition of one of several pyrimidine nucleosides or 5-halogenated pyrimidines warrants further investigation of their effects on IUdR degradation *in vivo*. Of particular interest with regard to potential clinical applicability are the compounds 5-trifluoromethyldeoxyuridine, deoxyuridine (with FUdR), arabinosylthymine, 5-bromouracil, and 5-iodouracil.

REFERENCES

1. A. D. Welch and W. H. Prusoff, *Cancer Chemother. Rep.* **6**, 29 (1960).
2. W. H. Prusoff, J. J. Jaffe and H. Gunther, *Biochem. Pharmacol.* **3**, 110 (1960).

3. J. P. KRISS, Y. MARUYAMA, L. A. TUNG, S. B. BOND and L. RÉVÉSZ, *Cancer Res.* **23**, 260 (1963).
4. J. P. KRISS and L. RÉVÉSZ, *Cancer Res.* **22**, 254 (1962).
5. M. L. EIDINOFF, L. CHEONG, E. GAMBETTA GURPIDE, R. S. BENUA and R. R. ELLISON, *Nature (Lond.)* **183**, 1686 (1959).
6. P. CALABRESI, S. S. CARDOSO, S. C. FINCH, M. M. KLIGERMAN, C. F. VON ESSEN, M. Y. CHU and A. D. WELCH, *Cancer Res.* **21**, 550 (1961).
7. G. S. RICHARDSON, T. C. HALL, T. H. GREEN and H. ULFELDER, *Ann. N.Y. Acad. Sci.* **97**, 841 (1962).
8. P. CALABRESI, R. W. MCCOLLUM and A. D. WELCH, *Nature (Lond.)* **197**, 767 (1963).
9. H. E. KAUFMAN, *Proc. Soc. exp. Biol. (N.Y.)* **109**, 251 (1962).
10. H. E. KAUFMAN, A. B. NESBURN and E. D. MALONEY, *Virology* **18**, 567 (1962).
11. E. S. PERKINS, R. M. WOOD, M. L. SEARS, W. H. PRUSOFF and A. D. WELCH, *Nature (Lond.)* **194**, 985 (1962).
12. B. DJORDJEVIC and W. SZYBALSKI, *J. exp. Med.* **112**, 509 (1960).
13. P. CALABRESI, W. H. PRUSOFF and A. D. WELCH, *Proc. Amer. Ass. Cancer Res.* **3**, 309 (1962).
14. P. CALABRESI, W. A. CREASEY, W. H. PRUSOFF and A. D. WELCH, *Cancer Res.* **23**, 583 (1963).
15. W. A. CREASEY, *J. biol. Chem.* **238**, 1772 (1963).
16. J. P. KRISS, L. TUNG and S. BOND, *Cancer Res.* **22**, 1257 (1962).
17. P. K. CHANG and A. D. WELCH, *Biochem. Pharmacol.* **8**, 327 (1961).
18. J. D. SMITH and L. R. WYATT, *Biochem. J.* **49**, 144 (1951).
19. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
20. G. D. BIRNIE, H. KROEGER and C. HEIDELBERGER, *Biochemistry* **2**, 566 (1963).
21. M. FRIEDKIN and D. ROBERTS, *J. biol. Chem.* **207**, 245 (1945).
22. M. FRIEDKIN and H. KALCKAR, in *The Enzymes*, P. D. BOYER, H. LARDY and K. MYRBACK, Eds. vol. 5, chap. 15. Academic Press, New York (1961).
23. C. HEIDELBERGER, D. PARSONS and D. C. REMY, *J. Amer. chem. Soc.* **84**, 3597 (1962).
24. C. HEIDELBERGER, H. GOTTSCHLING and G. D. BIRNIE, *Fed. Proc.* **22**, 532 (1963).