ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1981, p. 420-423
0066-4804/81/090420-04$02.00/0
Vol. 20, No. 3

Differential Activity of Potential Antiviral Nucleoside Analogs on Herpes Simplex Virus-Induced and Human Cellular Thymidine Kinases

Y.-C. CHENG,[1]* G. DUTSCHMAN,[1] J. J. FOX,[2] K. A. WATANABE,[2] AND H. MACHIDA[3]

Departments of Pharmacology and Medicine, Drug Development Program, Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514[1]; Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, New York 10021[2]; and Research Laboratory, Yamasa Shoyu Co. Ltd., Choshi 288, Japan[3]

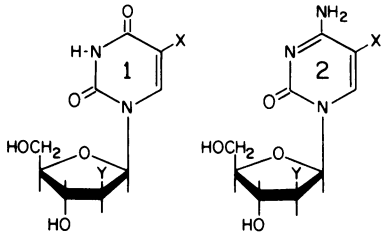
Received 20 March 1981/Accepted 26 June 1981

Potential antiviral nucleoside analogs 1-β-D-arabinofuranosylthymine, the 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-nucleosides of -5-methyluracil, -5-iodouracil, -5-methylcytosine, -5-iodocytosine, and -*E*-5-(2-bromovinyl)uracil, *E*-5-(2-bromovinyl)-2′-deoxyuridine, *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil, and 9-(2-hydroxyethyoxymethyl)guanine were studied to compare their phosphorylation rates relative to thymidine by purified thymidine kinases from human and herpes simplex virus sources. Most of these analogs are capable of being phosphorylated by both human and viral enzymes. On the assumption that inhibition constants (K_i) reflect binding affinity, K_i values were determined for these analogs with the same thymidine kinases. In general, these analogs have a greater affinity for the viral enzymes. The amount of the analogs phosphorylated to the monophosphate form, which is presumably necessary to produce cytotoxic effects, was determined by the combined effects of phosphorylation rates and binding affinities. All of these analogs act as preferential substrates for the viral thymidine kinases at low concentrations, which may be one of the main reasons for their selective antiviral action.

Ideally, therapeutically useful antiviral compounds should have good antiviral activity, while exhibiting low host cell toxicity. Some nucleoside analogs in this category are 1-β-D-arabinofuranosylthymine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine, *E*-5-(2-bromovinyl)-2′-deoxyuridine, *E*-5-(2-bromovinyl)-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil (Fig. 1), and 9-(2-hydroxyethyoxymethyl)guanine (1, 8, 10, 13-15). Many of these antiviral pyrimidine nucleoside analogs are clinically promising because of their low human cell toxicity. Although the exact mechanism of their antiviral activity is not known, the selective antiviral activity may be associated with their ability to be recognized as good substrates for herpes simplex virus (HSV)-induced thymidine kinase (dThd kinase), but not by the host enzymes. This "selective alternative substrate" mechanism (3) was found to be applicable to several other selective antiviral nucleoside analogs. This study examines the relative phosphorylation rates of these antiviral compounds by various kinds of dThd kinases and their ability to inhibit the formation of the normal product thymidine 5′-monophosphate as a reflection of their binding ability.

The inhibition constants of these nucleoside analogs for different types of dThd kinases are shown in Table 1. Based on the assumption that the K_i values are to a certain extent the reflection of the binding affinity of these analogs to the enzymes, the results in Table 1 could be summarized as follows: these analogs in general have a better affinity to HSV type 1 or 2 (HSV-1 or HSV-2) dThd kinase than to host cellular enzymes. The exceptions are *E*-5-(2-bromovinyl)-2′-deoxyuridine and *E*-5-(2-bromovinyl)-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil, which have good binding affinity to human mitochondrial dThd kinase. The sustitution of the -5-CH_3 group of dThd by an unsaturated, conjugated short-carbon-chain group, such as vinyl or propenyl, can also potentiate the binding to mitochondrial enzyme (4, 5, 12). The substitution of the -5-CH_3 group of dThd by an iodo group will affect the binding affinity to both viral enzymes to a lesser degree than to human

cytosol dThd kinase. The differential effect of *E*-5-bromovinyl group substitution on HSV-1 and HSV-2 dThd kinases may be related to the fact that HSV-1 dThd kinase can tolerate a more bulky group substitution at the -5 position of dThd than HSV-2 dThd kinase (2, 4). Substitution of the hydrogen by an -F or -OH group in the 2'-"up" (arabino) configuration will have different effects on the binding affinity of these analogs to various dThd kinases. The decreased binding affinity of the dThd analogs to various dThd kinases caused by either -F or -OH substitution at the 2'-up position is in the order: cytosol > mitochondrial > HSV-2 > HSV-1. The -F substitution has a less pronounced effect than the -OH group substitution. The higher binding affinity of dThd analogs than deoxycytidine analogs to virus-induced enzymes may result from the fact that dThd is a much better substrate than deoxycytidine for both virus-induced dThd kinases (2).



NUCLEOSIDE	BASE	X	Y
dThd	1	CH_3	H
FMAU	1	CH_3	F
AraT	1	CH_3	OH
FIAU	1	I	F
BVDU	1	CH=CHBr	H
FBVAU	1	CH=CHBr	F
BVAU	1	CH=CHBr	OH
FMAC	2	CH_3	F
FIAC	2	I	F

FIG. 1. *Structural formulas for antiviral compounds. See Table 1, footnote a, for abbreviations.*

The phosphorylation rates of these analogs at 0.4 mM by different types of dThd kinases were compared with that of dThd (Table 2). Due to the sensitivity of the assay, the K_m values could not be determined. The results suggest that most of these analogs are capable of being phosphorylated by either viral or host dThd kinases. All of the analogs behaved as competitive inhibitors with respect to dThd phosphorylation. It should be noted that the concentration used was much higher than estimated K_i values of the analogs for virus-induced enzymes (Table 1). Thus, the relative rate is the reflection of the ratio of V_{max} of analogs to dThd by these virus-induced enzymes and also of the catalytic process of phosphorylation. It is apparent that the modifications of dThd affected not only binding affinity but also the V_{max} values. The effects on these

TABLE 1. *Inhibition constants of antiviral nucleoside analogs for various kinds of dThd kinases*

Compound[a]	Mean K_i (μM) ± SD for enzyme source[b]:			
	Human		HSV-1 (strain KOS)	HSV-2 (strain 333)
	Cytosol	Mitochondrial		
araT	>100	>100	2.99 ± 0.19	43.37 ± 2.97
FMAU	>100	>100	0.59 ± 0.04	2.50 ± 0.12
FIAU	26.36 ± 12.71	>100	0.68 ± 0.10	1.54 ± 0.51
FMAC		>100	15.88 ± 2.12	29.24 ± 2.16
FIAC		>100	1.09 ± 0.10	6.59 ± 0.26
BVDU[c]	>100	0.83 ± 0.12	0.24 ± 0.09	4.24 ± 1.30
BVAU	>100	>100	0.94 ± 0.06	54.67 ± 6.58
FBVAU	>100	7.44 ± 1.09	0.67 ± 0.07	10.26 ± 3.77

[a] araT, 1-β-D-Arabinofuranosylthymine; FMAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; FMAC, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine; FIAC, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; BVDU, *E*-5-(2-bromovinyl)-2'-deoxyuridine; BVAU, *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; FBVAU, *E*-5-(2-bromovinyl)-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil.

[b] The K_i values were determined by the procedure of Cheng and Prusoff (7). The means were calculated from a minimum of four determinations. The amount of enzyme used was 0.02, 0.05, 0.1, and 0.1 U for human cytosol, human mitochondrial, HSV-1 (strain KOS), and HSV-2 (strain 333) dThd kinases, respectively. The volume of the reaction mixture was 0.1 ml. The dThd kinase assay used has been previously published by this laboratory (6, 11). [^{14}C]dThd (>50 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. SD, Standard deviation.

[c] Results of Cheng et al. (Y.-C. Cheng, G. Dutschman, E. DeClercq, A. S. Jones, S. G. Rahim, G. Verhelst, and R. T. Walker, Mol. Pharmacol., in press) were included for comparison.

TABLE 2. *Relative phosphorylation rates of antiviral nucleoside analogs by various kinds of dThd kinases*

Compound[a] (0.4 mM)	Velocity (%) relative to dThd for enzyme source[b]:			
	Human		HSV-1 (strain KOS)	HSV-2 (strain 333)
	Cytosol	Mitochondrial		
araT	33.3 (28-39)	82.2 (60-97)	61.8 (53-80)	43.4 (31-53)
FMAU	81.7 (63-98)	219.0 (200-254)	42.0 (39-45)	146.6 (118-180)
FIAU	88.3 (79-100)	199.3 (164-229)	104.0 (84-134)	194.2 (171-229)
FMAC	4.2 (0-13)	177.8 (165-186)	55.0 (52-58)	43.8 (33-57)
FIAC	4.2 (0-13)	201.0 (178-229)	31.2 (22-44)	79.8 (71-84)
BVDU[c]	4.9 (0-14)	33.0 (30-36)	90.0 (81-99)	127.0 (116-148)
BVAU	0.0	176.5 (166-187)	71.0 (68-74)	27.0 (17-38)
FBVAU	4.3 (3-7)	71.5 (71-72)	234.0 (229-237)	162.5 (147-171)
ACG	0.0	0.0	27.5 (25-30)	25.0 (18-32)

[a] ACG, 9-(2-Hydroxyethyoxyethyl)guanine. For other abbreviations, see Table 1, footnote *a*.

[b] The amount of enzyme used and the reaction volume are the same as indicated in Table 1, footnote *b*. Values are expressed as means (range). The means are the averages of 4 to 10 datum points from several experiments; the range reflects the extremes in these experiments. The adenosine 5′-triphosphate transfer assay used to determine the relative phosphorylation rate is essentially the same as that used by Dobersen and Greer (9). [γ-^{32}P]adenosine 5′-triphosphate (3,000 Ci/mmol) was purchased from New England Nuclear Corp.

[c] Results of Cheng et al. (in press) were included for comparison.

two parameters do not correlate with each other. A substrate with poor binding affinity may give a better V_{max} value.

The rate of phosphorylation by dThd kinase at a defined concentration of analog is determined by two parameters, binding affinity and V_{max}. At low concentrations (less than or around the K_m), the binding affinity plays a more important role, but at higher concentrations (severalfold above the K_m), V_{max} is the determining factor for the phosphorylation. Thus, it is conceivable that these analogs at low concentrations could be preferentially phosphorylated in virus-infected cells. This may be one of the key determinants for their selective antiviral effects without toxifying uninfected cells. At high concentrations, these analogs could exert their cytotoxic effects through phosphorylation by the host enzymes.

The nucleoside analogs were obtained as follows, 1-β-D-arabinofurosylthymine was provided by Jerry Ruth of Calbiochem-Behring Corp., La Jolla, Calif.; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine, 1-(2-deoxy-2-fluoro-β-D-arabinopuranosyl)-5-iodocytosine, and *E*-5-(2-bromovinyl)-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil were prepared in the laboratory of Jack Fox; *E*-5-(2-bromovinyl)-2′-deoxyuridine was provided by R. T. Walker; *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil was provided by Haruhiko Machida; and 9-(2-hydroxyethyoxymethyl)guanine was provided by G. B. Elion of Burroughs Wellcome Co., Research Triangle Park, N.C.

This investigation was supported by American Cancer Society grant CH-29 (to Y.-C.C.) and Public Health Service grants CA 08748, CA 18601, and CA 18856 from the National Institutes of Health (to J.J.F. and K.A.W.). Y.-C.C. is an American Leukemia Society Scholar.

LITERATURE CITED

1. **Aswell, J. F., G. P. Allen, A. T. Jamieson, D. E. Campbell, and G. A. Gentry.** 1977. Antiviral activity of arabinosylthymine in herpesviral replication: mechanism of action in vivo and in vitro. Antimicrob. Agents Chemother. **12**:243-254.
2. **Cheng, Y.-C.** 1976. Deoxythymidine kinase induced in Hela TK$^-$ cells by herpes simplex virus type 1 and type 2. 2. Substrate specificity and kinetic behaviors. Biochim. Biophys. Acta **452**:370-381.
3. **Cheng, Y.-C.** 1977. A rational approach to the development of antiviral chemotherapy: alternative substrates of HSV-1 and HSV-2 thymidine kinase. Ann. N.Y. Acad. Sci. **284**:594-598.
4. **Cheng, Y.-C.** 1979. Strategy for the development of selective antiherpes virus agents based on the unique properties of viral induced enzymes—thymidine kinase, DNase, and DNA polymerase, p. 263-274. *In* J. Skoda and P. Langen (ed.), Antimetabolites in biochemistry, biology and medicine. Pergamon Press, Ltd., Oxford.
5. **Cheng, Y.-C., S. Grill, J. Ruth, and D. E. Bergstrom.** 1980. Anti-herpes simplex virus and anti-human cell growth activity of *E*-5-propenyl-2′-deoxyuridine and the concept of selective protection in antivirus chemotherapy. Antimicrob. Agents Chemother. **18**:957-961.
6. **Cheng, Y.-C., and M. Ostrander.** 1976. Herpes simplex type 1 and type 2 specific thymidine kinase. I. Induction, purification, and general properties. J. Biol. Chem. **251**: 2605-2610.
7. **Cheng, Y.-C., and W. H. Prusoff.** 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. **22**: 3099-3108.
8. **DeClercq, E., J. Descamps, P. DeSomes, P. J. Barr, A. S. Jones, and R. T. Walker.** 1979. *E*-5-(2-bromovinyl)-2′-deoxyuridine: a potent and selective antiherpes agent. Proc. Natl. Acad. Sci. U.S.A. **76**:2947-2951.
9. **Dobersen, M. J., and S. Greer.** 1975. An assay for pyrimidine deoxyribonucleoside kinase using γ-^{32}P-labeled ATP. Anal. Biochem. **67**:602-610.
10. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. DeMiranda, L. Beauchamp, and H. J. Shaeffer.** 1977. Selectivity

of action of an antiherpetic agent, 9-(2-hydroxyethyomethyl)guanine. Proc. Natl. Acad. Sci. U.S.A. **74:** 5716–5720.

11. **Lee, L. S., and Y.-C. Cheng.** 1976. Human thymidine kinase. I. Purification and general properties of the cytosol and mitochondria thymidine kinase derived from human acute myelocytic blast cells. J. Biol. Chem. **251:**2600–2604.
12. **Lee, L. S., and Y.-C. Cheng.** 1976. Human deoxythymidine kinase. II. Substrate specificity and kinetic behaviors of the cytoplasmic and mitochondria isozymes derived from blast cells of the acute myelotic leukemia. Biochemistry **15:**3686–3690.
13. **Lopez, C., K. A. Watanabe, and J. J. Fox.** 1980. 2'-Fluoro-5-iodo-aracytosine, a potent and selective antiherpesvirus agent. Antimicrob. Agents Chemother. **17:** 803–806.
14. **Sakata, S., S. Shibuya, H. Machida, H. Yoshino, K. Hirota, and S. Senda.** 1980. Synthesis and antiherpesviral activity of 5-C-substituted uracil nucleosides. Nucleic. Acids Res. Spec. Publ. **8:**S39–S42.
15. **Watanabe, K. A., A. Reichman, K. Hirota, C. Lopez, and J. J. Fox.** 1979. Nucleosides. 110. Synthesis and antiherpes virus activity of some 2'-fluoro-2'-deoxyarabinofuranosyl-pyrimidine nucleosides. J. Med. Chem. **22:**21–24.