Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1068-1075

5(E)-(3-AZIDOSTYRYL)-2'-DEOXYURIDINE 5'-PHOSPHATE IS A PHOTO-ACTIVATED INHIBITOR OF THYMIDYLATE SYNTHETASE

E. De Clercq[1], J. Balzarini[1], C.T.-C. Chang[2], C.F. Bigge[2], P. Kalaritis[2], and M.P. Mertes[2]

1. Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium
2. Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045, U.S.A.

Received October 23,1980

Summary

The title compound is a photoaffinity labeling reagent for thymidylate synthetase, a key enzyme for the de novo biosynthesis of DNA. This compound is also a light-dependent inhibitor of murine (L-1210) and human (Namalva, Raji) tumor cell growth, and vaccinia virus replication.

INTRODUCTION

One contemporary approach to the design of chemotherapeutic agents employs the concept of affinity labeling reagents to achieve a high degree of selective pathogen toxicity with minimal effects on the host organism (1). Photoaffinity labeling utilizes a light induced reaction to provide a highly reactive functional group which then interacts with the (bound) enzyme to give enzyme inactivation (2). Our observation that the title compound is a light-dependent inhibitor of viral replication and tumor cell growth opens an unexplored area in the development of novel chemotherapeutic agents, that of providing a selective basis for the treatment of dermatological diseases through activation of a specific enzyme-inhibitor on the light-exposed skin surface.

MATERIALS AND METHODS

5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate (I) was designed as a photoaffinity labeling reagent for thymidylate synthetase (3).

Thymidylate synthetase was purified from amethopterin-resistant Lactobacillus casei. The crystalline enzyme had a specific activity of 3.1 µmole of product formed per minute per mg protein. It was purified according to Galivan et al. (4). In the enzyme inactivation studies it was used at a concentration of 0.05 µM. Enzyme activity was measured as described by Brouillette et al. (5).

Antiviral activity was based upon the inhibition of virus-induced cytopathogenicity in primary rabbit kidney (PRK) cell cultures infected with vesicular stomatitis virus, herpes simplex virus type 1 (KOS) or vaccinia virus (6). As

0006-291X/80/231068-08$01.00/0

criterion of antitumor cell activity served the inhibition of growth of a murine leukemic (L-1210) cell line and three human lymphoblastoid (Namalva, Raji, TK⁻ (thymidine kinase deficient) Raji) cell lines (7).

The enzyme inactivation studies were done by photolysis in glass cuvettes in a Rayonet Model RPR 100 Photochemical Reactor using 7 lamps of 350 nm wavelength. In the antiviral and tumor cell studies, the cell cultures were irradiated with a Sylvania lamp of 366 nm at a rated intensity of 16 lux.

RESULTS AND DISCUSSION

Inhibition of thymidylate synthetase

Compound 1 was found to be a potent reversible inhibitor of *Lactobacillus casei* thymidylate synthetase. A double reciprocal plot of the velocity of product formation *vs* the concentration of substrate at two inhibitor concentrations showed the inhibition to be competitive with substrate. The calculated inhibitory constant (K_i) was 0.23 μM. Comparing the K_i/K_m ratio (0.04), 1 had substantially greater affinity for the enzyme than did the substrate 2'-deoxyuridine 5'-phosphate.

Arylazides such as compound 1 are decomposed by light to form nitrenes, chemically reactive groups that are useful for active site-labeling of enzymes (2). Photolysis (254, 310, or 350 nm) of an aqueous solution of 1 was accompanied by a light-dependent decrease in the ultraviolet spectra at 325 and 256 nm. The change in the latter absorption band was rapid and could be attributed to a photodecomposition of the azido group.

The enzyme was stable to light of 350 nm wavelength. When 2 μM of compound 1 (10 times the K_i) was added to the buffered enzyme solution and the mixture exposed to light, a rapid, time-dependent inactivation of the enzyme was observed (Fig. 1A). Under the conditions of the experiment the half-life for enzyme inactivation was 3 minutes.

The mechanism of affinity labeling reactions dictates that the addition of substrate to the enzyme inhibitor solution prior to light exposure should afford protection if the photoactivated inhibitor is acting at the active site of the enzyme. Photolysis of a buffered solution of enzyme, 2 μM of inhibitor 1, and 20 μM of 2'-deoxyuridine 5-phosphate, substantially reduced the rate of inactivation from a half-life of 3 minutes in the absence of substrate to about 15 minutes in the presence of substrate; protection also was observed when a solution of 20 μM of substrate and 0.25 μM of 1 was photolyzed. This protective effect could not be attributed to a decrease of light intensity in the reaction mixture since the substrate did not absorb light of 340 nm wavelength.

To exclude any possibility that enzyme inactivation was the consequence of an indirect or non-specific interaction of the photolysis products of 1 with the enzyme, the inhibitor (1 μM) was photolyzed for 90 minutes prior to

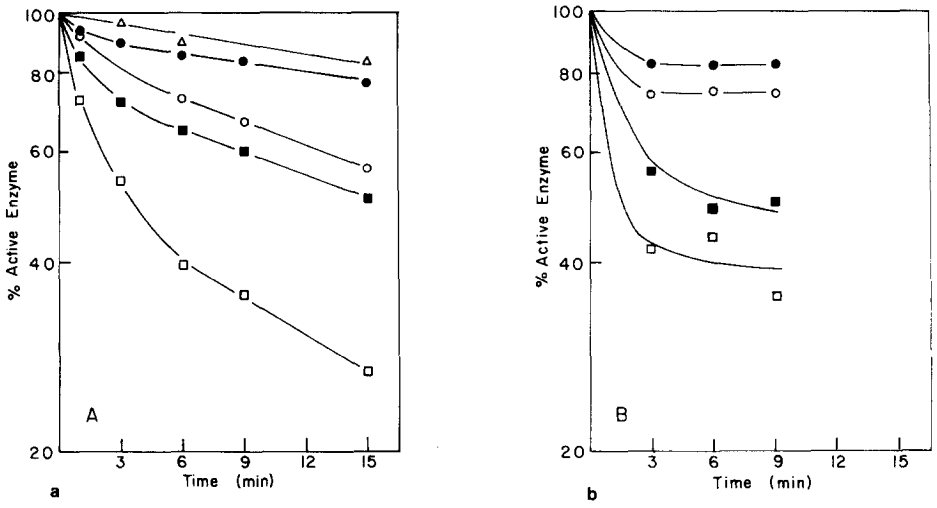
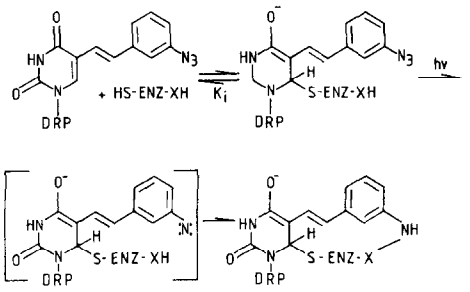


Figure 1. Semi-log plot of the percentage of remaining thymidylate synthetase activity vs time in the presence of varying amounts of 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate (1) with and without added substrate 2'-deoxyuridine 5'-phosphate (dUMP).
A. Lactobacillus casei enzyme : 0.25 μM 1 (○); 0.25 μM 1 plus 20 μM dUMP (●); 2 μM 1 (□); 2 μM 1 plus 20 μM dUMP (■); control, no inhibitor (Δ).
B. L-1210 enzyme : 1 μM 1 (○); 1 μM 1 plus 10 μM dUMP (●); 10 μM 1 (□); 10 μM 1 plus 10 μM dUMP (■).

addition of the enzyme. After the enzyme was added photolysis was continued; only minimal enzyme inactivation was observed (86 % activity recovered after 9 minutes of light exposure). Finally, as a control experiment 5(E)-(3-nitrostyryl)-2'-deoxyuridine 5'-phosphate did not inactivate thymidylate synthetase when 10 μM of this analog, buffer, and enzyme were exposed to 350 nm light for periods up to 12 minutes. Thus, under the experimental conditions applied for the azido compound, the corresponding nitro analog was not a photoaffinity label.

Compound 1 was also found to be relatively potent inhibitor of the thymidylate synthetase isolated from L-1210 cells (K_i = 1.90 μM; K_m = 1.29 μM). The inhibition was competitive with respect to the natural substrate (2'-deoxyuridine 5'-phosphate). Exposure of the inhibitor-enzyme solution to light at 366 nm wavelength gave rapid inactivation of the enzyme. After three minutes at a concentration of 1 μM of 1 26 % inactivation was observed; 10 μM of inhibitor gave 58 % inactivation (Fig. 1B). At both concentrations the addition of substrate afforded protection. Thus, L-1210 thymidylate synthetase also showed inactivation by photo-activated 1 (Fig. 1B), which was comparable to that noted for the Lactobacillus casei thymidylate synthetase (Fig. 1A).



Scheme I. Proposed mechanism of inactivation of thymidylate synthetase by the photoaffinity label 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate (1) (DRP = 2'-deoxyuridine 5'-phosphate).

Scheme I illustrates the mechanism by which compound 1 may interact with thymidylate synthetase upon exposure to light.

Antiviral and antitumor cell activity

Compound 1 was not active against vesicular stomatitis virus or herpes simplex virus, and did not become active against these viruses after exposure to light (Table 1). However, compound 1 proved to be an effective inhi-

Table 1

Effect of irradiation (λ = 366 nm) on antiviral activity of 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate in PRK cell cultures

Time of irradiation[a] (min.)	ID_{50} (µg/ml)[b]		
	Vesicular stomatitis virus	Vaccinia virus	Herpes simplex virus type 1 (KOS)
0	> 200	20, 20, 20	> 200
5	> 200	10, 20, 20	> 200
10	> 200	4, 4, 7	> 200
20	> 200	1, 2, 2	> 200
30	> 200	1, 2, 2	> 200

[a] Irradiation of the exposed cultures was started immediately after virus adsorption and after the compound (at various doses) had been added to the cell cultures.

[b] Inhibitory dose-50 or dose of compound required to reduce virus-induced cytopathogenicity by 50 %. The minimal toxic dose, or dose causing a microscopically visible alteration of cell morphology was ≥ 200 µg/ml, irrespective of the time of irradiation. Virus input : 100 $CCID_{50}$ (cell culture infecting dose-50) per microtiter well. Viral cytopathogenicity was recorded as soon as it reached completion in the control (infected, untreated) cell cultures (usually at 2 - 3 days after virus inoculation). Irradiation itself did not cause an inhibition of viral cytopathogenicity.

bitor of vaccinia virus replication with an ID_{50} (or dose required to reduce virus-induced cytopathogenicity by 50 %) of 20 µg/ml. Irradiation of the cell cultures with light of 366 nm wavelength resulted in a tenfold increase in the potency of compound 1 : i.e. the ID_{50} decreased from 20 µg/ml to 1 - 2 µg/ml after 20 minutes of light exposure.

A similar light-dependence was observed for the inhibitory effects of compound 1 on the growth of murine L-1210 tumor cells (Table 2) and human lymphoblastoid (Namalva, Raji) cells (Table 3). For the L-1210 cells the ID_{50} of 1 decreased from 80 - 90 µg/ml to 10 - 20 µg/ml if the cell cultures were exposed to light following addition of the compound (Table 2). Likewise, irradiation caused a 2- to 4.5-fold decrease in the ID_{50} of 1 for Namalva, Raji and TK^- (thymidine-kinase deficient) Raji cells (Table 3).

Irradiation (λ = 366 nm) did not markedly affect the antitumor properties of compounds that do not contain the same photoaffinity label as 1. For example, the ID_{50} of 5-iodo-2'-deoxyuridine and 5(E)-(2-bromovinyl)-2'-deoxyuridine for L-1210 cell growth were 250 and 30 µg/ml, respectively. Upon 10 min. irradiation, these ID_{50} values became 220 and 29 µg/ml, respectively. Thus, the photo-activating effect cannot be attributed to a non-specific killing of the tumor cells, since it is only observed with compound 1, and not with other nucleoside analogues.

According to the enzyme inactivation studies the mechanism of the antiviral and antitumor effect of 1 could be related to an inhibition of thymidylate synthetase. To evaluate the possibility that the antitumor activity of 1 was due to an inhibition of thymidylate synthetase within the cell, we determined (i) the ability of dThd (deoxythymidine), relative to dUrd (deoxyuridine), to reverse the inhibitory effect of 1 on L-1210 cell growth, and (ii) the capacity of 1 to inhibit the incorporation of (6-^3H)dUrd, relative to $(\underline{\text{methyl}}\text{-}^3H)$dThd, into L-1210 cell DNA. Both parameters can be considered as valuable indexes of thymidylate synthetase inhibition in cell culture (7). As shown in Table 4, the antitumor activity of 1, whether irradiated or not, was not reversed by either dUrd or dThd. Neither did irradiated or non-irradiated 1 inhibit the incorporation of (6-^3H)dUrd into DNA to a greater extent than $(\underline{\text{methyl}}\text{-}^3H)$dThd incorporation. Thus, the cytotoxic action of photo-activated 1 cannot simply be equated to an inhibition of thymidylate synthetase. Within the cell it may act by inactivating proteins other than, or in addition to, thymidylate synthetase.

Photoaffinity labeling reagents such as that described herein should be further pursued as potential chemotherapeutic agents in the treatment of those cutaneous disorders that are accompanied by excessive DNA synthesis.

Table 2

Effect of irradiation (λ = 366 nm) on inhibitory activity of 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate towards L-1210 cell growth

Time of irradiation[a] (min)	ID_{50} (μg/ml)[b]		
	Exp. ≠ 1	Exp. ≠ 2	Exp. ≠ 3
0	86	73	92
1	...	54	...
3	...	25	...
5	18	...	...
10	13	28	21
20	16	...	...
30	14	...	...

[a]Irradiation of the exposed cultures was started immediately after the cells had been seeded (in the presence of various doses of the compound).
[b]Inhibitory dose-50 or dose of compound required to reduce the number of living L-1210 cells (after a 48 hour proliferation period) by 50 %, as compared to the control irradiated cell cultures. Cell input : 40,000 cells per cup; cell out-put (not irradiated and not treated) : approximately 400,000 cells per cup. Irradiation itself caused some inhibition of tumor cell growth which amounted up to 4 % after 1 min. irradiation, 8 % after 3 min. irradiation, 13 % after 10 min. irradiation and 67 % after 30 min. irradiation.

It should be pointed out that our photoaffinity approach is fundamentally different from at first glance similar photochemotherapeutic approaches that have been previously advocated for the treatment of psoriasis (8-methoxypsoralen and longwave ultraviolet (365 nm) irradiation (8,9)) and herpes simplex skin infections (proflavine or other "photodynamically" active dyes and irradiation with longwave blue light (450 nm) (10)). Indeed, the target

Table 3

Effect of irradiation (λ = 366 nm) on inhibitory activity of 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate towards growth of human lymphoblastoid cell lines

Time of irradiation[a] (min.)	ID_{50} (μg/ml)[b]		
	Namalva	Raji	TK^- Raji
0	34	60	58
10	17	19	13

[a,b]For further details see footnotes to Table 2.

Table 4

Inhibitory effects of photo (λ = 366 nm)-activated 5(E)-(3-azidostyryl)-2'-deoxyuridine 5'-phosphate on (A) L-1210 cell growth in the presence of dThd or dUrd; (B) incorporation of (methyl-^{3}H)dThd or (6-^{3}H)dUrd into L-1210 cell DNA

Time of irradiation[a] (min.)	ID_{50} (μg/ml)		
A. L-1210 cell growth in the presence of :	dThd (5 μg/ml)[b]	dUrd (125 μg/ml)[b]	Control
0	76	85	92
10	23	23	21
B. Incorporation into L-1210 cell DNA of :	(methyl-^{3}H)dThd[c]		(6-^{3}H)dUrd[c]
0	19		26
10	21		32

[a] See footnote to Table 2.
[b] Maximum concentrations of dThd and dUrd which were themselves not inhibitory to L-1210 cell growth.
[c] Incorporation measured according to previously described procedures (7).

for our photoinactivation procedure is not DNA itself, as is the case for 8-methoxypsoralen and proflavine, but an enzyme (thymidylate synthetase) that is involved in its biosynthesis. Additional targets could be considered, however. It is unclear whether the photochemotherapeutic approach described herein would be complicated by those risks (mutagenicity, carcinogenicity) that psoralen photochemotherapy and photodynamically active dyes are confronted with (11,12).

Another example of photoactivation is the increased sensitivity to short- and long-wave ultraviolet light that results from the incorporation of 5-bromo-2'-deoxyuridine into viral or cellular DNA (13-15). However, this approach is not used therapeutically, probably because of the risks for mutagenicity and carcinogenicity.

Acknowledgements. This research was supported by a research grant (CA 7522) from the National Cancer Institute of the National Institutes of Health, and by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Krediet No 3.0048.75) and the Geconcerteerde Onderzoeksacties (Conventie No 76/81-IV).

References

1. Baker, B.R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, pp. 122-129, Wiley, New York.
2. Bayley, H., and Knowles, J.R. (1970) Affinity Labeling, Methods in Enzymology Vol. 46. W.P. Jakoby and M.W. Wilchek, Eds. Academic Press, New York, pp. 69-114.

3. Bigge, C.F., Kalaritis, P., Deck, J.R., and Mertes, M.P. (1980) J. Amer. Chem. Soc. 102, 2033-2038.
4. Galivan, H.J., Maley, G.F., and Maley, F. (1975) Biochemistry 14, 3338-3344.
5. Brouillette, C.B., Chang, C.T.-C., and Mertes, M.P. (1979) J. Med. Chem. 22, 1541-1544.
6. De Clercq, E., Descamps, J., Huang, G.-H., and Torrence, P.F. (1978) Mol. Pharmacol. 14, 422-430.
7. De Clercq, E., Balzarini, J., Torrence, P.F., Mertes, M.P., Schmidt, C.L., Shugar, D., Barr, P.J., Jones, A.S., Verhelst, G., and Walker, R.T. (1980) Mol. Pharmacol. (submitted).
8. Parrish, J.A., Fitzpatrick, T.B., Tanenbaum, L., and Pathak, M.A. (1974) New Engl. J. Med. 291, 1207-1211.
9. Epstein, J.H. (1979) New Engl. J. Med. 300, 852-853.
10. Melnick, J.L., and Wallis, C. (1977) Ann. N.Y. Acad. Sci. 284, 171-181.
11. Stern, R.S., Thibodeau, L.A., Kleinerman, R.A., Parrish, J.A., Fitzpatrick, T.B., and 22 participating investigators (1979) New Engl. J. Med. 300, 809-813.
12. Verwoerd, D.W., and Rapp, F. (1978) J. Virol. 26, 200-202.
13. Stahl, F.W., Crasemann, J.M., Okun, L., Fox, E., and Laird, C. (1961) Virology 13, 98-104.
14. Wacker, A., Menigmann, H.D., and Szybalski, W. (1962) Nature 196, 685-686.
15. Puck, T.T., and Kao, F.-T. (1967) Proc. Nat. Acad. Sci. USA 58, 1227-1234.