Kinetic and Photochemical Studies of 3-N-Methyl-5-iodo-2'-deoxyuridine

SENSITIZATION OF ULTRAVIOLET INACTIVATION OF THYMIDINE KINASE BY 3-N-METHYL-5-IODO-2'-DEOXYURIDINE AND OTHER HALOGENATED ANALOGS OF THYMIDINE*

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SUMMARY

The effects of 5-iodo-2'-deoxyuridine, 5-bromo-2'-deoxyuridine, 5-chloro-2'-deoxyuridine, 5-fluoro-2'-deoxyuridine, and 3-N-methyl-5-iodo-2'-deoxyuridine, a new analog of thymidine, on the ultraviolet (UV) inactivation of thymidine kinase have been investigated. Of these compounds only 5-iodo-2'-deoxyuridine and 3-N-methyl-5-iodo-2'-deoxyuridine enhance the rate of inactivation of the enzyme by UV light. The other halogenated analogs neither protect nor sensitize thymidine kinase to UV inactivation. Whereas the inactivation of thymidine kinase by UV light in the presence of 5-iodo-2'-deoxyuridine can be prevented by the substrate, thymidine (CYSK, R., and PRUSOFF, W. H. (1969) Fed. Proc., 28, 473; CYSK, R. (1970) Ph.D. thesis, Yale University), that produced by 3-N-methyl-5-iodo-2'-deoxyuridine is not prevented by thymidine alone but requires the presence of both substrates, thymidine and MgATP. Kinetic studies with thymidine kinase show 5-iodo-2'-deoxyuridine to be a competitive inhibitor with respect to thymidine; however, 3-N-methyl-5-iodo-2'-deoxyuridine shows uncompetitive inhibition with thymidine and competitive inhibition with MgATP. The allosteric regulators, dCDP, an activator, and dTTP, an inhibitor, protect the enzyme against UV irradiation and decrease the rate of inactivation caused by 3-N-methyl-5-iodo-2'-deoxyuridine. The primary photostable compound formed during photolysis of 3-N-methyl-5-iodo-2'-deoxyuridine is 3-N-methyl deoxyuridine, which is analogous to the formation of deoxyuridine during photolysis of 5-iodo-2'-deoxyuridine (CYSK, R. (1970) Ph.D. thesis, Yale University).

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The present communication presents a comparison of the sensitization effects of 5-fluoro-, 5-chloro-, 5-bromo-, and 5-iodo-2'-deoxyuridine with cellular DNA, unequivocal evidence has not been presented that the DNA is the principal or sole target molecule. The various other biochemical sites of inhibition exerted by 5-iodo-2'-deoxyuridine and its phosphorylated derivatives have been reviewed (20).

Recent investigations by Cysyk (21) have demonstrated that 5-iodo-2'-deoxyuridine, when present at the active site of thymidine kinase, markedly augments the rate of inactivation of the enzyme by UV light (253.7 nm), and that thymidine not only decreases the rate of UV inactivation but also prevents the sensitizing effect of 5-iodo-2'-deoxyuridine.

The present communication presents a comparison of the sensitization effects of 5-fluoro-, 5-chloro-, 5-bromo-, and 5-iodo-2'-deoxyuridine with that of the newly synthesized analog, 3-N-methyl-5-iodo-2'-deoxyuridine, on the UV inactivation of thymidine kinase. A kinetic analysis of the mode of interaction of 3-N-methyl-5-iodo-2'-deoxyuridine with thymidine kinase is presented also.

**EXPERIMENTAL PROCEDURE**

**Materials**—[2-14C]Thymidine (43.5 μCi per μmole) was obtained from New England Nuclear and bovine albumin (Fraction V) from Armour Pharmaceutical Co. Thymidine and 5-iodo-2'-deoxyuridine were purchased from Nutritional Biochemicals Corporation. 5 Bromo-2'-deoxyuridine, dTTP, and dCDP were obtained from P-L Biochemicals, Inc. 5-Chloro-2'-deoxyuridine and 5-fluoro-2'-deoxyuridine were procured from CalBiochem and Hoffman La Roche, respectively. Thymidine kinase from E. coli, purified by ammonium sulfate fractionation (Step III) as described by Voytek, Chang, and Prusoff (22), was used for the radiation studies. An extensively purified preparation of this enzyme (Step VI) was used for the kinetic studies (22).

**Spectral Analyses**—UV spectrophotometric analyses were conducted on a Cary model 15 spectrophotometer. Nuclear magnetic resonance spectra were obtained from a Bruker 60 MHZ proton spectrometer.

**Preparation and Analysis of N-Methyl-5-iodo-2'-deoxyuridine—**To a clear solution of IdUrd1 (708 mg, 2 mmoles), in a mixture of tetrahydrofuran (30 ml), methanol (10 ml), and water (5 ml), was added ethereal diazomethane (50 ml containing approximately 300 mg of diazomethane). The mixture was allowed to stand overnight at room temperature. After removal of traces of insoluble impurities by filtration, the solution was concentrated in a vacuum at 40°C. The residue, when dissolved in methanol and the solution again concentrated in a vacuum, yielded the crystalline N-methyl derivative (670 mg, 90%).

The nuclear magnetic resonance spectrum of the N-methyl derivative shows the presence of a prominent N-methyl singlet at 3.17 ppm and the absence of the N—H singlet at 11.5 ppm, whereas the reverse is true with the spectrum of the parent compound (IdUrd).

UV spectra of N-MeIdUrd (Fig. 1) at both pH 2 and 13 gave a maximum absorption at 285 nm and a minimum at 247 nm, whereas with IdUrd a hypochromic shift is observed when the change is made from pH 2 (288 nm) to pH 13 (278 nm). The molar extinction coefficient of N-MeIdUrd was calculated to be 7.00 × 104 at pH 2.9 and 286 nm. The relative migrations of N-MeIdUrd as compared with several other pyrimidines by the use of descending chromatography on Whatman No. 1 paper in the N-butanol-water-formic acid (77:13:10) solvent system are: 2'-deoxyuridine 5'-monophosphate, 1.0; 2'-deoxyuridine, 4.9; 2'-deoxythymidine, 6.5; (IdUrd), 6.6; (N-MeIdUrd), 8.9.

**Kinetic Assays**—Thymidine kinase activity was measured according to the method described by Furlong (23). Volumes of reaction mixture were between 0.2 and 0.4 ml containing 4.9; 2'-deoxythymidine, 6.5; (IdUrd), 6.6; (N-MeIdUrd), 8.9.

**Ratiation of Thymidine Kinase—**Mercaptethanol (9 mm) present in the ammonium sulfate fractions of thymidine kinase (29) was removed by passage through a column of Sephadex G-25 (1 × 50 cm) and elution with a buffer containing 0.05 M Tris-HCl at pH 7.8 and 0.5 mM EDTA.

**UV irradiation experiments** were carried out at 0°C in 5-ml glass pH meter cups of 19 mm diameter, unless mentioned otherwise. The radiation source was a General Electric 15-watt germicidal lamp (G15T8) having 86% of its total radiant intensity at 253.7 nm and less than 1% at other wave lengths below 300 nm, giving 28 ergs per mm2 per sec at 25 cm. The distance between the light source and the top of the 0.5- to 1.0-ml solution

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**Fig. 1.** UV spectra of IdUrd and N-MeIdUrd at pH 2 and pH 13. - - -, IdUrd; ——, N-MeIdUrd.
Fig. 2. Effects of various halogenated deoxyribonucleosides on the inactivation rate of thymidine kinase activity by UV light. Irradiation mixtures contained 0.05 M Tris-HCl at pH 7.8, 0.5 mM of deoxyribonucleoside when present, and 0.2 mg per ml of the enzyme preparation. Fifty microliters were removed at different time points and assayed for thymidine kinase activity.

- no nucleoside present
- 5-fluoro-2'-deoxyuridine
- 5-chloro-2'-deoxyuridine
- 5-bromo-2'-deoxyuridine
- IdUrd
- N-MeIdUrd

Fig. 4. The effects of N-MeIdUrd with varying concentrations of thymidine and a constant concentration of MgATP (4.0 mM).

- no N-MeIdUrd
- 0.8 mM N-MeIdUrd
- 1.6 mM N-MeIdUrd

Fig. 5. The effects of N-MeIdUrd with varying concentrations of MgATP and a constant concentration of thymidine (2 mM).

- 0.8 mM N-MeIdUrd
- 1.6 mM N-MeIdUrd

RESULTS

Fig. 2 compares the effects of several halogenated nucleosides on the UV inactivation of thymidine kinase, and all appear to follow first order reaction kinetics. IdUrd and N-MeIdUrd enhance the UV inactivation of the enzyme, whereas 5-bromo-, 5-chloro-, and 5-fluoro-2'-deoxyuridine have little or no effect on either protecting or increasing the rate of inactivation. At equal concentrations of the nucleoside analogs, the sensitization of the enzyme to UV light is greater for IdUrd as compared with N-MeIdUrd. However, unlike IdUrd, N-MeIdUrd is not phosphorylated by thymidine kinase but acts as an inhibitor of the enzyme. Figs. 3 and 4 show that two different types of inhibition are produced by IdUrd and N-MeIdUrd when deoxythymidine is the variable substrate. Both compounds bind to thymidine kinase, but their sites of binding are obviously different. As expected (Fig. 3), IdUrd is a competitive inhibitor of thymidine kinase, whereas N-MeIdUrd shows uncompetitive inhibition.
Fig. 6. Effects of increasing concentrations of N-MeIdUrd on the rate of inactivation of thymidine kinase. Irradiations were carried out 25 cm from the lamp source in a type 37 (Infrasil) cylindrical cell (1 × 22 mm) purchased from Precision Cells, Inc., New York. Otherwise the conditions were the same as described under "Experimental Procedure." Rate of inactivation equals first order rate constant for inactivation of the enzyme (min⁻¹).

Fig. 7. Effects of IdUrd and N-MeIdUrd alone and in combination on the rate of inactivation of thymidine kinase. *—* control; ○—○, 0.075 mM N-MeIdUrd; △—△, 0.075 mM IdUrd; ×—×, 0.075 mM N-MeIdUrd plus 0.075 mM IdUrd. Numbers in parentheses are the first order rate constants for inactivation of the enzyme (min⁻¹).

Table I

| Additions                  | Protection % |
|----------------------------|--------------|
| None                       | 0            |
| N-MeIdUrd                  | -292         |
| N-MeIdUrd + thymidine      | -208         |
| N-MeIdUrd + Mg-ATP         | -203         |
| N-MeIdUrd + thymidine + MgATP | -50        |
| Thymidine + Mg-ATP         | +33          |

* Concentration of compounds in the irradiation mixture were 0.08 mM N-MeIdUrd, 0.5 mM thymidine, and 0.5 mM MgATP. Conditions for irradiation are described under "Experimental Procedure."

The percentage of protection equals

\[
\text{Protection} = \left(\frac{1 - \text{rate with addition(s)}}{\text{rate no addition(s)}}\right) \times 100.
\]

with thymidine (Fig. 4) but competitive inhibition with MgATP (Fig. 5). The Kᵢ values were calculated from these inhibition patterns to be 20 μM for IdUrd and 1.7 mM for N-MeIdUrd, an indication that IdUrd binds more strongly to the enzyme.

Cysyk (21) has demonstrated that when thymidine kinase is irradiated in the presence of IdUrd the increased rate of inactivation is specific and directed to the active site of thymidine kinase. The effect of increasing concentrations of N-MeIdUrd on the rate of UV inactivation of thymidine kinase is shown in Fig. 6. The concentration of N-MeIdUrd at the half-maximal rate of inactivation was calculated to be 1.4 mM, which is the same order of magnitude as the Kᵢ calculated from the kinetic inhibition studies (Figs. 4 and 5). Therefore, the sensitization caused by N-MeIdUrd, like IdUrd (21), appears to be specific and directly related to the binding of the compound to thymidine kinase. Furthermore, Fig. 7 shows that the increased rates of inactivation of the enzyme caused by N-MeIdUrd and IdUrd are independent of each other and are additive.

Since Cysyk (21) has shown that thymidine can prevent the enhancement of UV inactivation by IdUrd the effect of the substrates thymidine and MgATP on the sensitization of thymidine kinase by N-MeIdUrd was investigated. Neither thymidine nor MgATP alone decreased the sensitization by N-MeIdUrd, but a combination of both substrates (thymidine + MgATP) prevented almost completely the inactivation caused by N-MeIdUrd (Table I). It should be noted further that a combination of MgATP and thymidine per se does not protect significantly against inactivation by UV light. Iwatsuki and Okazaki (25) have shown that the nucleotides, dTTP and dCDP, cause dimerization of thymidine kinase from Escherichia coli. The former is an allosteric inhibitor, and the latter is an allosteric
to photoinactivation. IdUrd at room temperature is much more sensitive to photolysis than either 5-bromo- or 5-chloro-2' deoxyuridine (4). It was found that 5-fluoro-2' - deoxyuridine is more sensitive to UV light than IdUrd (4); however, the photoproducts are distinctly different, being 5-hydroxyhydro-5-fluoro-2'-deoxyuridine (15-17) and the deoxyuridine free radical (5, 6, 21), respectively. The subsequent dehalogenation of the hydrated fluorouracil derivative does not involve a free radical mechanism but rather an elimination reaction. This accounts for the apparent paradox of 5-fluoro-2'- deoxyuridine having a greater photolability than IdUrd yet not sensitizing thymidine kinase.

The primary photochemical product is the hydrate when solutions of 5-fluorouracil, 5-fluoro-2'-deoxyuridine, N-methyl-5fluoro-2'-deoxyuridine or 5-fluorouridine-5'-phosphate are irradiated with UV (15). Spectral analyses of the irradiated solutions of IdUrd and N-MeIdUrd in 0.05 M Tris buffer at pH 7.8 (Fig. 9) indicate that dehalogenation occurs with the formation of a substance that has a maximum absorbance at lower wavelength. Studies of the photochemical transformation of IdUrd by Cysyk (21) showed unequivocally that dUrd is the initial stable photoproduct formed, and, in analogy to the photochemistry of 5-iodouracil (5, 6), dUrd is formed via formation of the deoxyuridine free radical with subsequent abstraction of a hydrogen from the Tris buffer. Upon continued irradiation, however, deoxyuridine forms a hydrate which can be reverted back to deoxyuridine by heating in acid (21). When the irradiated solution of N-MeIdUrd was acidified and heated (Fig 9B), an increase in absorbance with a peak of 264 nm was observed. These findings are in accord with the formation of a deoxyuridine hydrate analogous to that observed with IdUrd (Fig 9B (21)). Since the spectral studies of IdUrd and N-MeIdUrd indicate a similar behavior, one may assume that a similar photochemical reaction mechanism is involved.

Studies by Cysyk and Prusoff (26) and Cysyk (21) on the enhanced UV inactivation of thymidine kinase by IdUrd have shown that the sensitizing effect of the halogenated nucleoside is active site directed and related to its binding constant. The present study shows that although both N-MeIdUrd and IdUrd increase the rate of inactivation of thymidine kinase, N-MeIdUrd is not a substrate at the thymidine binding site, but rather inhibits the enzyme uncompetitively with respect to thymidine and competitively with MgATP (Figs. 4 and 5). Like IdUrd (21), the rate of inactivation caused by N-MeIdUrd is related to the binding potential of the compound to the enzyme (Fig. 6). The $K_i$ values calculated from the kinetic plots indicate that the binding of IdUrd to thymidine kinase is approximately 100-fold greater than N-MeIdUrd and because of this one might expect proportionately a greater UV inactivation with IdUrd as compared with the methyl derivative. However, this does not seem to be the case since at equal concentrations (Fig. 2) the inactivation caused by IdUrd is only twice that of N-MeIdUrd. Since N-MeIdUrd, (a) unlike IdUrd, is not phosphorylated by the enzyme, (b) has different inhibition patterns than IdUrd, and (c) appears to inactivate the enzyme by UV independently from IdUrd (Fig. 7), it is most probable that the site of binding of N-MeIdUrd to thymidine kinase is not only different than IdUrd but also more susceptible to UV inactivation in the presence of N-MeIdUrd as compared with the corresponding site of inactivation with IdUrd. It is conceivable that the photochemical lability of N-MeIdUrd is markedly greater than that of IdUrd when these two compounds are bound to the
enzyme at their respective bindings sites (due to differences, for example, in formation of charge transfer complexes) in spite of the fact that the photochemical lability of these compounds in aqueous solution is similar.

Product inhibition studies carried out by Voytek and Prusoff have revealed that the reaction mechanism of thymidine kinase from E. coli is Leu Ordered Bi Bi with thymidine binding to the enzyme before the second substrate, MgATP. UV inactivation experiments indicate that neither MgATP nor thymidine alone can overcome the enhanced inactivation caused by N-MeldUrd (Table I); however, a combination of both substrates (thymidine + MgATP) does exert marked protection. This is in agreement with our study of the reaction mechanism of this enzyme, which indicated that thymidine must be bound to the enzyme prior to the addition of MgATP of which N-MeldUrd is a competitive inhibitor.

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