MOLECULAR PHARMACOLOGY, 6, 95-98 (1970)

SHORT COMMUNICATION

Inactivation by Mouse Serum of a Tightly Bound Inhibitor of Dihydrofolate Reductase

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(Received October 16, 1969)

SUMMARY

A potent inhibitor of dihydrofolate reductase, a substituted 4,6-diaminotriazine (NSC 113,423; a 1:1 compound of 4-[*p*-(4,6-diamino-2,2-dimethyl-*s*-triazine-1(2*H*)-yl)hydrocinnamido]-*o*-toluenesulfonyl fluoride and ethanesulfonic acid), was found to be rapidly inactivated by mouse serum. Preliminary studies on the mechanism of this inactivation indicate that a mouse serum protein hydrolyzes the sulfonyl fluoride group. However, this triazine inhibitor was not inactivated by the sera of other species, including rat, dog, and man. It is suggested that NSC 113,423 and related compounds may still be valuable chemotherapeutic agents against tumors occurring in man.

Certain substituted 4,6-diaminotriazines, recently synthesized by Baker and Lourens (1), have been shown to be potent inhibitors of the enzyme dihydrofolate reductase obtained from murine L1210 lymphoma cells (2) and from human leukemia cells.[1] These compounds were also shown to be extremely potent inhibitors of growth of the L1210 lymphoma propagated in tissue culture.[2]

This research was supported in part by United States Public Health Service Research Grant CA-08010 and by Contract PH 43-67-1193 from the Cancer Chemotherapy Service Center. The work reported in this paper was undertaken during the tenure of an Eleanor Roosevelt International Cancer Fellowship of the American Cancer Society awarded by the International Union Against Cancer, of which Dr. Fölsch is a recipient. Dr. Bertino is a Career Development Awardee of the National Cancer Institute.

[1] A. Cashmore, D. G. Johns, and J. R. Bertino, unpublished observations.

[2] F. White, personal communication.

Despite these encouraging results, only one of these inhibitors has shown modest chemotherapeutic activity when tested against the L1210 lymphoma *in vivo* (3). Furthermore, these compounds are toxic to the mouse only in high doses. This communication reports the results of studies using NSC 113,423 (a 1:1 compound of 4-[*p*-(4,6-diamino-2,2-dimethyl - *s* - triazine - 1(2*H*) - yl)hydrocinnamido]-*o*-toluenesulfonyl fluoride and ethanesulfonic acid) (Fig. 1) as a model compound for this series. This compound is rapidly inactivated by mouse serum, but not by rat, dog, fetal calf, and human serum, thus offering a possible explanation for the lack of antitumor effect in mice bearing the L1210 lymphoma.

Deoxyuridine-^{3}H (UdR-^{3}H), 3.1 Ci/mmole, was obtained from Schwarz BioResearch, Inc., New York. NADPH, horse serum, and fetal calf serum were obtained from commercial sources.

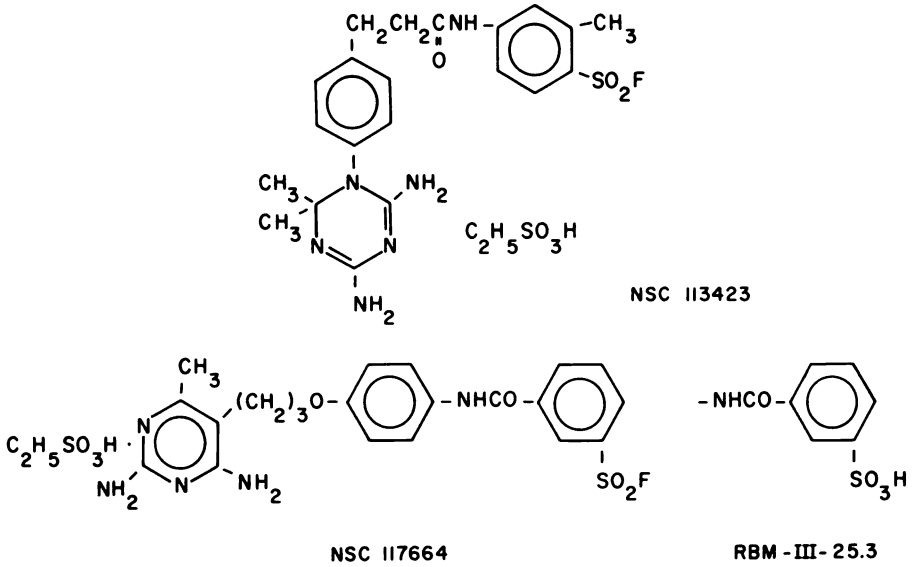

FIG. 1. *Structures of NSC 113,423 (a 1:1 compound of 4-[p-4,6-diamino-2,2-dimethyl-s-triazine-1 (2H)-yl)hydrocinnamido]-o-toluenesulfonyl fluoride and ethanesulfonic acid), NSC 117,664 (a 1:1 compound of m-[[p-[3-(2,4-diamino-6-methyl-5-pyrimidinyl)propoxy]phenyl]carbamoyl]benzenesulfonyl fluoride and ethanesulfonic acid), and RBM-III-25.3*

The structure of RBM-III-25.3 shown in this figure represents only the partial formula, and is identical with that of NSC 117,664 except for the substitution of the SO_3H group for the SO_2F moiety.

TABLE 1

Reversal by mouse serum of the inhibition of UdR-^{3}H incorporation into DNA caused by NSC 113,423

The L1210 cells were incubated as described in the text. The concentration of NSC 113,423 employed was 1.6×10^{-6} M.

Reaction mixture	UdR-^{3}H incorporation	Inhibition
	dpm/10^6 cells/min	%
Control (10% horse serum)	1450	
+ NSC 113,423	334	77
Control (15% horse serum and 5% mouse serum)	705	
+ NSC 113,423	565	20
Control (10% horse serum and 10% mouse serum)	615	
+ NSC 113,423	490	20
Control (20% mouse serum)	376	
+ NSC 113,423	395	−4

Inhibition of DNA synthesis in L1210 cells *in vitro* by NSC 113,423 was measured by comparing the rate of UdR-^{3}H incorporation in the absence and presence of this inhibitor (4). L1210 lymphoma cells were harvested in an ascitic form from BDF mice 3 days after the intraperitoneal inoculation of 1×10^6 cells per mouse. The cells (10^6/flask) were incubated at 37° in Eagle's basal medium with Earle's balanced salt solution containing horse serum and/or mouse serum, as indicated in Table 1, for 30 min; UdR-^{3}H (9×10^{-4} μmole) was then added to the flasks and incubation was continued for an additional 60 min. The incorporation of radioactivity into the cells was determined as described previously (4).

Dihydrofolate reductase activity was measured as described previously (5). The enzyme employed was partially purified from a methotrexate-resistant subline of the L1210 lymphoma, at the Sephadex stage. This subline differs from the wild type tumor used for UdR-^{3}H incubation studies as described above, in that the level of dihydrofolate reductase is increased but is identical in its properties with the wild type enzyme (5). Inactivation of NSC 113,423 was measured by a comparison of the inhibition of dihydrofolate reductase produced by the inhibitor before and after incubation with mouse serum.

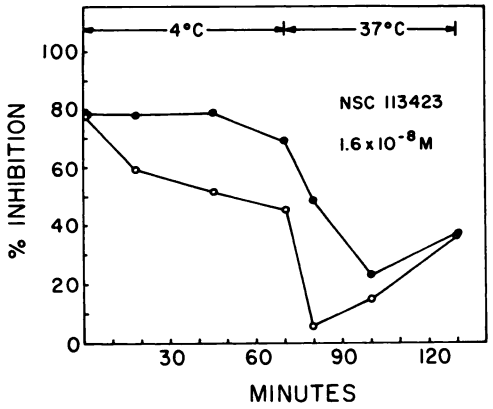

FIG. 2. *Effect of time, temperature, and concentration on inactivation of NSC 113,423 by mouse serum*

NSC 113,423 (1.6×10^{-7} M) was incubated in 1 ml of 0.9% NaCl containing 0.25 ml of mouse serum (15 mg of protein; ●——●) or 0.05 ml of mouse serum (3 mg of protein; ○——○) at the temperatures shown. At the indicated times, 0.1-ml portions were removed and added to a complete assay mixture (0.8 ml) for dihydrofolate reductase, less dihydrofolate. After mixing, dihydrofolate (0.01 μmole; 0.1 ml) was added to initiate the reaction. The control reaction rate gave an absorbance change of 0.150 at 340 mμ in 5 min at 37°.

There was a 77% decrease in the rate of incorporation of UdR into DNA in the presence of 1.6×10^{-6} M NSC 113,423; an 88% decrease was produced by methotrexate at an equimolar dose. The addition of mouse serum to L1210 cells incubated in Eagle's-Earle's media with horse serum reversed the inhibition of DNA synthesis produced by NSC 113,423 (Table 1). The inhibitory effect of mouse serum itself on UdR-^{3}H incorporation into DNA has been previously noted.[3]

Inhibition of L1210 dihydrofolate reductase by NSC 113,423 was extremely potent and was "stoichiometric," similar to the inhibition produced by methotrexate (6). This inhibition was reduced markedly if the inhibitor was incubated with mouse serum prior to being added to the enzyme assay. As shown in Fig. 2, this inactivation was found to be dependent on temperature, time, and serum concentration.

Studies concerned with the nature and mechanism of this inactivation have been initiated. The inactivating serum factor is probably a macromolecule, since it was not dialyzable, was precipitated by ammonium sulfate, and was partially destroyed by heating to 55°. Some evidence has been obtained to indicate that the mechanism of this inactivation involves binding of the inhibitor to the protein, with subsequent slow hydrolysis of the terminal sulfonyl fluoride group to sulfonic acid. The following data support this possibility. Prolonged incubation (60–130 min) of NSC 113,423 with mouse serum resulted in partial regeneration of dihydrofolate reductase inhibitor activity (Fig. 2). The same effect occurred immediately when the inhibitor-serum complex generated by incubation for 30 min at 37° was heated at 100° for 2 min. Once partial regeneration occurred, the addition of fresh mouse serum (1.8 mg of protein per milliliter) failed to inactivate the inhibitor

[3] W. Hryniuk and J. R. Bertino, unpublished data.

TABLE 2

Inactivation of NSC 113,423 by sera from several mammalian species

Dihydrofolate reductase from the L1210 lymphoma was assayed as described in the text and in Fig. 2. The final inhibitor concentration was 1.6×10^{-8} M. In experiment I, the serum was incubated for 2 min at 37° with the inhibitor before the mixture (0.1 ml) was added to the dihydrofolate reductase assay system (0.9 ml); in experiment II, the incubation time of serum with inhibitor was 45 min.

Serum			Inhibition of dihydrofolate reductase
Source	Amount		
	ml	*mg protein*	%
Experiment I			
Control			83
Mouse	0.03	1.6	50
Rat	0.03	2.3	75
Dog	0.03	2.0	82
Fetal calf	0.05	1.8	72
Horse	0.05	3.8	75
Human	0.05	3.6	81
Experiment II			
Control			90
Mouse	0.03	1.6	30
Rat	0.03	2.3	73
Human	0.03	2.2	82

further. These data are consistent with the interpretation that NSC 113,423 is metabolized to the sulfonic acid derivative; this compound, a weaker inhibitor of dihydrofolate reductase than is the parent compound, cannot be further inactivated by mouse serum.

Additional evidence in support of this interpretation was obtained with NSC 117,664, a 2,4-diaminopyrimidine also containing a sulfonyl fluoride moiety (Fig. 1), and its sulfonic acid derivative (RBM-III-25.3). NSC 117,664 behaves as does NSC 113,423 with respect to dihydrofolate reductase inhibition and inactivation by mouse serum. However, the sulfonic acid derivative, a poorer inhibitor of dihydrofolate reductase than NSC 117,664, was not inactivated by mouse serum.

The suggestion that an enzyme-catalyzed hydrolysis of inhibitors of the sulfonyl fluoride type has previously been made by Baker and Meyer (7) and Baker and Hurlbut (8). These authors showed that a compound differing from NSC 117,664 only in the replacement of the methyl group by an amino group, and a compound differing from NSC 113,423 only in a methyl group, were inactivated by a relatively impure mouse liver preparation of dihydrofolate reductase; therefore, whether this hydrolysis was due to dihydrofolate reductase or to another liver protein could not be ascertained. These authors also observed enzyme-catalyzed inactivation of sulfonyl fluoride inhibitors of chymotrypsin, trypsin, and xanthine oxidase. The question as to whether the modest antitumor activity of a sulfonyl fluoride compound (compound 3, ref. 3) against the L1210 lymphoma is due to lack of hydrolysis of this compound by mouse serum or to significant activity of the sulfonic acid derivative has not yet been resolved.

The results shown in Table 2 are of potential importance to the further evaluation of these compounds as chemotherapeutic agents. Thus, although mouse serum decreased the ability of NSC 113,423 to inactivate L1210 dihydrofolate reductase, the sera of other species, including rat, dog, and man had little or no inactivating ability.[4] Therefore, NSC 113,423 and related compounds may still be valuable antineoplastic agents when tested against tumors occurring in man. The ability of a certain mammalian species but not others to inactivate a drug is not without precedent; the rabbit and guinea pig can rapidly inactivate the antifolates methotrexate and aminopterin, while the mouse, rat, and man cannot (9).

ACKNOWLEDGMENTS

NSC 113,423 and 117,664 were obtained from Dr. Florence White of the Cancer Chemotherapy Service Center, and from Dr. B. R. Baker, who also generously supplied the sulfonic acid derivative of NSC 117,664 as well as valuable advice. The expert technical assistance of Mrs. Inger-Anne Skjeltorp is also acknowledged.

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[4] Based on these observations, NSC 113,423 has recently been screened for anti-tumor activity against the Walker carcinoma in rats and found to be highly active (F. White, unpublished observations).