PYRIMIDO(4,5-b)(1,4)THIAZINE DERIVATIVES. A NEW TYPE OF FOLIC ACID ANTAGONIST

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The antimetabolites of folic acid – aminopterin and methotrexate – are widely used for the medical treatment of malignant neoplasms. The mechanism of action of these compounds is associated with the inhibition of dihydrofolate reductase (DFR) with the help of which the conversion of folic acid into its coenzyme form, tetrahydrofolic acid, is achieved. The latter, as is known, is the carrier of one-carbon units and participates in the synthesis of such vitally-important compounds as amino acids and pyrimidine and purine bases. However, aminopterin and methotrexate are highly toxic. The therapeutic effect is generally attained on using doses which cause the development of a secondary action (depression of the activity of bone marrow, ulcerous stomatitis, diarrhea, etc.) [1].

With the object of searching for new folic-acid antagonists possessing a greater selectivity of action towards the tumor process than the known compounds, a series of pyrimido(4,5-b)(1,4)thiazine derivatives was prepared [2, 3]. The bicyclic system of pyrimido(4,5-b)(1,4)thiazine differs from pteridine, which is the framework of the structure of folic acid, by the presence of the 1,4-thiazine ring in place of the pyrazine ring. One might expect that the substitution of a nitrogen atom by a sulfur atom while retaining in the molecule the functional groups which are responsible in folic acid for the realization of the enzymic reactions would lead to the development of compounds having antimetabolic properties. In connection with this we have studied in the present work the effect of pyrimidothiazine derivatives on the enzymes of folic metabolism, and the data obtained concerning the inhibiting activity of compounds have been correlated with their structure.

EXPERIMENTAL

Assays of the pyrimidothiazine derivatives were carried out by the method described previously [4] with certain modifications of the enzyme which was isolated from rat liver. Male rats of weight 120-140 g were killed by decapitation, the livers were removed, and homogenized in the cold in 0.05 M potassium-phosphate buffer at pH 7.4 in the ratio 1:4. The homogenate was centrifuged in a refrigerated centrifuge at 16,000 rpm for 15 min, and the supernatant liquid was precipitated with ammonium sulfate at 70% saturation. After centrifuging (15 min at 16,000 rpm) the precipitate was dissolved in buffer and dialyzed for 2 h against the same buffer. The dialyzate which contained DFR was used for evaluating the inhibiting action of the pyrimidothiazine derivatives. This enzyme preparation, which is fairly stable, withstands storage at −18 to −20°C for 1 month. The dependence of the activity of the obtained DFR on the pH of the medium has been studied by us. The data are presented in Fig. 1. The greatest activity is shown at pH 5.7.

In order to determine the inhibiting activity of the pyrimidothiazine derivatives in regard to DFR the enzyme preparation was incubated in the presence of the substances being tested.

Included in the composition of the incubation mixture were 0.05 M pH 5.7 citrate buffer (1.1 ml); manganese chloride tetrahydrate (0.1 μmole in 0.1 ml [19.8 mg in 1 ml]); nicotinamide (0.073 μmole in 0.1 ml [90 mg in 1 ml]); folic acid (0.5 μmole in 0.1 ml [4.25 mg in 2 ml of 1% sodium-bicarbonate solution]); NADP·H (0.45 μmole in 0.1 ml [3.8 mg in 1 ml]); the compound being tested was at a concentration of $1 \cdot 10^{-5}$ to $1 \cdot 10^{-7}$ mole/liter (10-0.1 mole); enzyme preparation (0.2 ml [protein 2-5 mg]). The total volume was 1.8 ml.

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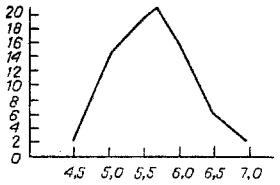


Fig. 1

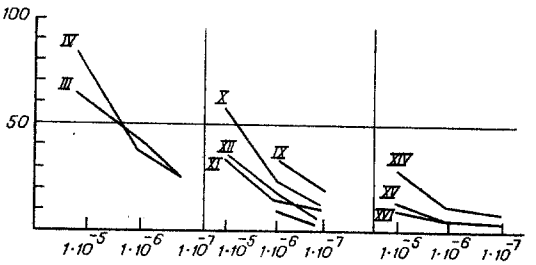


Fig. 2

Fig. 1. Dependence of activity of DFR on pH of medium. Along the abscissa is pH of medium; along the ordinate is the activity of DFR (in extinction units).

Fig. 2. Effect of pyrimido(4,5-b)(1,4)thiazine on the activity of DFR. Along the abscissa is the concentration of the compound (in moles/liter); along the ordinate is the depression of DFR activity (in %).

The incubation was carried out at 37° for 2 h; after this up to 1.8 ml of 10% trichloroacetic acid was added to all samples, and the precipitate was separated by centrifuging.

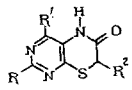
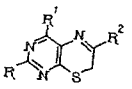
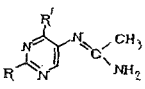
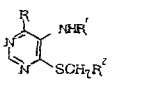
The activity of the DFR was determined by a colorimetric method based on the fact that the tetrahydrofolic acid which forms as a result of the enzymic reaction decomposes in acid medium with the release of p-aminobenzoylglutamic acid. The latter is diazotized, the diazonium salt coupled with naphthylethylene diamine, and the obtained colored solution analyzed colorimetrically. If under the influence of the compound the enzymic reaction is retarded, then the quantity of p-aminobenzoylglutamic acid is correspondingly reduced.

For the azo-coupling reaction 0.8 ml of centrifugate was taken and 0.8 ml of 0.1% aqueous sodium-nitrate solution, 0.8 ml of 0.5% aqueous ammonium-sulfamate solution, and 0.8 ml of a 0.1% aqueous naphthylethylene-diamine solution were added to it. Within 10 min the sample was analyzed colorimetrically on an FÉK with a green filter. By comparing the results of the control and experiment from this colorimetry the action of the compounds on DFR was judged. The pyrimidothiazine derivatives were also studied on DFR which was chromatographed on DEAE-cellulose. In this case the inhibiting action of the compounds under study was determined by a spectrophotometric method from the consumption of NADP·H as the hydrogen source in the enzymic reaction [5]. Aminopterin in a quantity of $4.5 \cdot 10^{-7}$ mole which produced 100% inhibition of the enzyme was used in this as a standard. The experiment with the purified enzyme was carried out at pH 6.8-7.4.

Three groups of compounds (see Table 1) have been studied by us through these methods. The pyrimido(4,5-b)(1,4)thiazin-6-ones (I-VIII) belong to the first group, and are crystalline substances of white (II, III, V-VIII), yellow (I), and cream (IV) color, poorly soluble in water and highly soluble in aqueous alkalies. The 6-aminopyrimidothiazines (IX-XIII) are included in the second group. These compounds are white crystalline substances and form hydrochlorides which are highly-soluble in water. The compounds with the opened thiazine ring (XIV-XVIII) are combined in the third group. These are white crystalline substances soluble in water. In the experiments, compounds I-VIII were used as aqueous solutions of their potassium salts, compounds IX-XIII as aqueous solutions of their hydrochlorides, compounds XIV and XV as aqueous solutions, and XVI and XVII as solutions in acid buffer.

About 60 compounds were studied with DFR, some of the data obtained being given in Table 1. As is evident from Table 1 a number of the pyrimidothiazine derivatives possess a high inhibiting ability and at a concentration of $1 \cdot 10^{-6}$ mole/liter suppress the activity of the enzyme by 60-95%. This is foremost for 2-amino-4,6-dioxo-, 4,6-dioxo-, and 4-alkoxy-6-oxopyrimidothiazinone (I-III), which are straight thio analogs of the simplest pteridines (for example, xanthopterin) and contain in positions 2 and 4 of the pyrimidine ring substituents also occurring in folic acid. The most active compound from this group, 4,6-dioxo-7-carbethoxypyrimidothiazinone (IV), suppresses the activity of the enzyme by 90% at a concentration of $1 \cdot 10^{-6}$ mole/liter. The presence of a CH_3S group in position 4 leads to a significant lowering of activity (VIII). The inhibiting action is retained for the pyrimidothiazines having an amino group in position 6 of the pyrimidothiazine bicycle (IX-XIII). 4-Methylamino-6-aminopyrimidothiazine (X) possesses the most activity in this group, inhibiting the activity of the enzymes by 60% at a concentration of $1 \cdot 10^{-5}$ mole/liter. If CH_3S, CH_3, NH_2, and $N(CH_3)_2$ groups are located in position 4 of the molecule, then the inhibiting action of the substance is reduced.

TABLE 1. Effect of Pyrimido(4,5-b)(1,4)thiazine Derivatives on the Activity of Dihydrofolate Reductase (DFR)

General formula	Compound	R	R^1	R^2	Suppression of DFR, %: enzyme preparation, $1 \cdot 10^{-6}$ moles/liter	enzyme preparation, $1 \cdot 10^{-8}$ moles/liter	DFR chromatographed on DEAE-cellulose: concentration of compound (in moles/liter)	DFR chromatographed on DEAE-cellulose: suppression (in %)
	Aminopterin	—	—	—	—	70—100	$4,5 \cdot 10^{-7}$	100
	I	NH_2	OH	H	—	70	$1,01 \cdot 10^{-7}$	100
	II	H	OH	H	—	60	$1,01 \cdot 10^{-7}$	50
	III	H	OCH_3	H	—	60	$1,01 \cdot 10^{-7}$	100
	IV	H	OH	$CO_2C_2H_5$	—	95	$0,78 \cdot 10^{-7}$	60—100
	V	H	OCH_3	$CO_2C_2H_5$	—	60	$0,78 \cdot 10^{-7}$	100
	VI	H	OCH_3	$CH_2CO_2C_2H_5$	—	55	$0,69 \cdot 10^{-7}$	70
	VII	OH	OH	H	—	47	$1,01 \cdot 10^{-7}$	100
	VIII	H	SCH_3	H	15	0	—	—
	IX	H	SCH_3	NH_2	—	20	$0,8 \cdot 10^{-7}$	100
	X	H	$NHCH_3$	NH_2	60	25	$0,7 \cdot 10^{-7}$	100
	XI	H	$N(CH_3)_2$	NH_2	35	15	$0,7 \cdot 10^{-7}$	100
	XII	H	NH_2	NH_2	35	15	$0,78 \cdot 10^{-7}$	50
	XIII	NH_2	CH_3	NH_2	—	20	$0,86 \cdot 10^{-7}$	40—50
	XIV	H	NH_2	—	30	10	$1,3 \cdot 10^{-7}$	60
	XV	H	$N(CH_3)_2$	—	15	5	—	—
	XVI	OCH_3	$COCH_3$	$CO_2C_2H_5$	—	0	—	—
	XVII	OCH_3	$COCONH_2$	$CONH_2$	—	30	—	—
	XVIII	NH_2	H	CN	10	5	—	—

It is interesting to note that the compounds from which the sulfur atom is removed (XIV, XV) are less active in comparison with the analogous pyrimidothiazine derivatives. Opening the thiazine ring, while retaining in the molecule the exocyclic nitrogen and sulfur atoms (compounds XVI, XVII, and XVIII), leads to a loss of activity with respect to DFR.

Certain of the substances from the three groups specified above were studied at three concentrations. The data obtained during this are depicted graphically in Fig. 2. At the concentrations studied the pyrimidothiazinones (II and IV) and 6-aminopyrimidothiazine (X) possessed the greatest activity and the substances with the opened thiazine ring (XIV, XV, and XVI), the least. The pyrimidothiazine derivatives were also studied on DFR chromatographed on DEAE cellulose. The pyrimidothiazinones (I, III-VII) and the 6-aminopyrimidothiazines (IX-XI) also showed a high inhibiting activity in this case, while the pyrimidylacetamidine (XIV) exerted a weak action on this enzyme.

Thus, the results of the studies carried out have shown that the changes introduced into the pteridine molecule have led in the compounds studied to the appearance of inhibiting properties with respect to DFR. These data have indicated a similar mechanism of action for pyrimidothiazines, aminopterin, and methotrexate. Furthermore, the pyrimidothiazine derivatives were studied in the enzyme system aminopterin enzyme (EAP). This enzyme is capable of reducing aminopterin to dihydroaminopterin and tetrahydroaminopterin and thereby eliminating its inhibiting action in respect of DFR and the antitumor activity [6]. It was found that 6-aminopyrimidothiazines inhibit not only DFR, but also the EAP which inactivates aminopterin. The data obtained afford a basis for considering pyrimidothiazine derivatives as a new interesting type of folic-acid antagonist.

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