MOLECULAR BIOLOGICAL PROBLEMS OF THE CREATION OF DRUGS AND STUDY OF THE MECHANISM OF THEIR ACTION

METABOLISM OF THE ANTITUMOR DRUG THIOPHOSPHAMIDE IN THE RAT

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The antitumor drug thiophosphamide [N,N',N"-tri(ethylene)triamide of thiophosphoric acid, I] is widely used in clinical practice for the treatment of malignant tumors of the ovary, breast cancer, and chronic lympholeukemia [7].

Studies of the kinetics of the absorption of I, its distribution among the organs and tissues, and its excretion [2, 3] are now well known. In a number of studies [4, 13] the stability of I in aqueous, aqueous-salt, and acid solutions was investigated. It was shown that the drug is stable in neutral media; in acid media it is converted to products with opening of the ethylenimine rings. And yet, the biotransformation of I has been insufficiently studied. In [14, 15] the method of thin-layer chromatography was used to identify the N,N',N"-tri(ethylene)triamide of phosphoric acid – TEP (II), which is the product of sulfoxidation of I – together with an unnamed substance in the urine. The presence of a number of other metabolites was noted, but their structure could not be determined. As a rule, these investigations were conducted only by methods of chromatography.

In this work we used a combination of methods of TLC and mass spectrometry, permitting sufficiently reliable establishment of the structure of the isolated products of metabolism. To facilitate the identification of the metabolites we made a preliminary study of the mass spectra of certain conversion products of I in hydrochloric acid solution.

EXPERIMENTAL

The electron impact mass spectra were obtained on a Varian MAT-112 instrument (Federal Republic of Germany); the energy of the ionizing electrons was 70 eV. Experiments in vivo were conducted on a noninbred male rats weighing 120-140 g. The drug was injected intravenously in a dose of 5 mg per rat. Urine was collected each hour for a period of 10 h. Metabolites were isolated from lyophilized urine of rats that had received the drug by three extractions with chloroform, followed by separation by TLC on Silufol plates in the system methanol–chloroform (4:3); the spots were developed with ninhydrin. The preparative elution of the compounds obtained was carried out by the method of descending chromatography, described in [9]. The structure of the conversion products of I in HCl solution and that of the metabolites were established according to the mass spectra.

RESULTS AND DISCUSSION

We studied the conversion of I in HCl solutions of various concentrations (0.1 N, 1 N, and 3 N) at a ratio of 1 mole of the drug to 3 moles of HCl. In all cases hydrochloric acid hydrolysis proceeds with the formation of practically the same products; the rate of conversion depends on the acid concentration.

Figure 1a presents a chromatogram of the conversion products of I in 3 N HCl, when their greatest concentration was observed (37°C for 1 h).

Earlier [8] we studied the electron impact mass spectra of I. It was shown that the elimination of ethylenimine radicals and a sulfur atom from M^+ is the determining pathway of mass spectrometric decomposition. The nature of the fragmentation of I was used to establish the structure of the conversion products of the drug.

A substance identical with the original I in chromatographic mobility and mass spectra was isolated from the zone with R_f 0.60. Compound III – two peaks of M^+, 225, 227 – was

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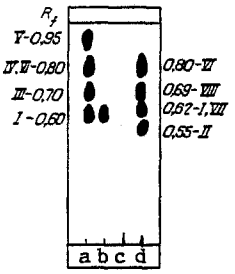


Fig. 1. Chromatogram of a mixture of conversion products of I in HCl solution (a); b) reference standard; preparation of a chloroform extract of urine of the control (c) and experimental (d) groups of rats. Chromatographic system methanol–chloroform (4:3). Development of spots with ninhydrin.

detected in the zone with R_f 0.70. The characteristic intensity ratio (3:1) of the peaks of the molecular ion is an indication that the molecule contains one chlorine atom. The presence of the peaks of the ions $(\overline{CH_2CH_2N})_2P^{-1+}$ m/z 115 and $(\overline{CH_2CH_2N})_2P{\equiv}^{+}S$ m/z 147 in the spectrum, present in the mass spectrum of the drug itself, the doublet $(\overline{CH_2CH_2N})(ClCH_2CH_2NH)P{\equiv}S^{+}$ m/z 183, 185 (3:1), the ion $(\overline{CH_2CH_2N})_2P(S)NHCH_2CH_2^{-1+}$ m/z 190, as well as the increase in the mass by 36 amu in comparison with I indicate the opening of one of the ethylenimine rings with the addition of one molecule of HCl.

From the zone with R_f 0.80 we isolated compound IV, in the mass spectrum of which the group of the molecular ion M^+ 261, 263, 265 (9:6:1) is observed, corresponding to a content of two atoms of chlorine. Characteristic decomposition with the formation of $[M-Cl]^+$ m/z 226, 228 (3:1), $(ClCH_2CH_2NH)_2{\equiv}^{+}S$ m/z 219, 221, 223 (9:6:1), $(\overline{CH_2CH_2N})(ClCH_2CH_2NH)P{\equiv}^{+}S$ m/z 183, 185 (3:1) (Fig. 2) is evidence of the addition of two molecules of HCl at the ethylenimine ring. When the eluate of the zone with R_f 0.95 was evaporated, substance V with mass 297 (three atoms of ^{35}Cl), corresponding to the addition of three molecules of HCl and the ethylenimine ring, was isolated. Just as in the cases discussed above, fragmentation of this compound occurs with elimination of a chlorine atom $[M-Cl]^+$ m/z 262, 264, 266 (9:6:1) and cleavage of the P–N bond: $(ClCH_2CH_2NH)_2P{\equiv}^{+}S$ m/z 219, 221, 223 (9:6:1).

Together with the conversion products of I discussed, still another substance in addition to IV could be detected in the zone with R_f 0.80, by temperature fractionation of the sample in an ionization chamber: VI, the mass spectrum of which corresponded to a content of three atoms of chlorine in the molecule – M^+ 281 (three atoms of ^{35}Cl). However, the behavior of this compound under electron impact differed from that of the preceding β-chloroethylamide derivatives. The most intense peak in the mass spectrum of VI belongs to an ion with two atoms of chlorine m/z 232, 234, 236 (9:6:1). In this case the eliminating particle is CH_2Cl, which we had practically never observed before. According to the data of the high-resolution mass spectra, the gross formula $C_5H_{13}PN_3O^{35}Cl_2$ (found: 232.0168; calculated 232.0173) corresponds to the ion with m/z 232 (two atoms of ^{35}Cl), i.e., instead of a sulfur atom the ion contains an atom of oxygen $(ClCH_2CH_2NH)_2P(O)HN^{+}{=}CH_2$. According to the DADI spectra, further breakdown of this ion occurs with the formation of the ion $(ClCH_2CH_2NH)_2P{\equiv}^{+}O$ m/z 203, 205, 207 (9:6:1) with cleavage of the P–N bond. The data obtained suggest that the substance VI isolated is a trichloro-derivative of TEP (see Fig. 2, VI).

Thus, in hydrochloric acid under these conditions I adds HCl at the ethylenimine rings: I → III → IV → V. The formation of VI is evidently due to the previously noted [13] partial conversion of I to II in acid solutions, followed by opening of the ethylenimine ring. The intermediate mono- and dichloro-derivatives of II were not observed, which is evidently due to the high rate of their conversion to VI. In addition to the products enumerated above, we observed the formation of a glassy mass insoluble in acids and alkalies, probably consisting of polymerization products.

In a study of the metabolism of I in the rat organism, hourly collection of urine showed that the drug and its metabolites begin to be excreted 2 h after intravenous injection (monitoring according to TLC). The bulk of the drug and its derivatives are excreted within an interval of 4-6 h. On the chromatogram of a chloroform extract of the control

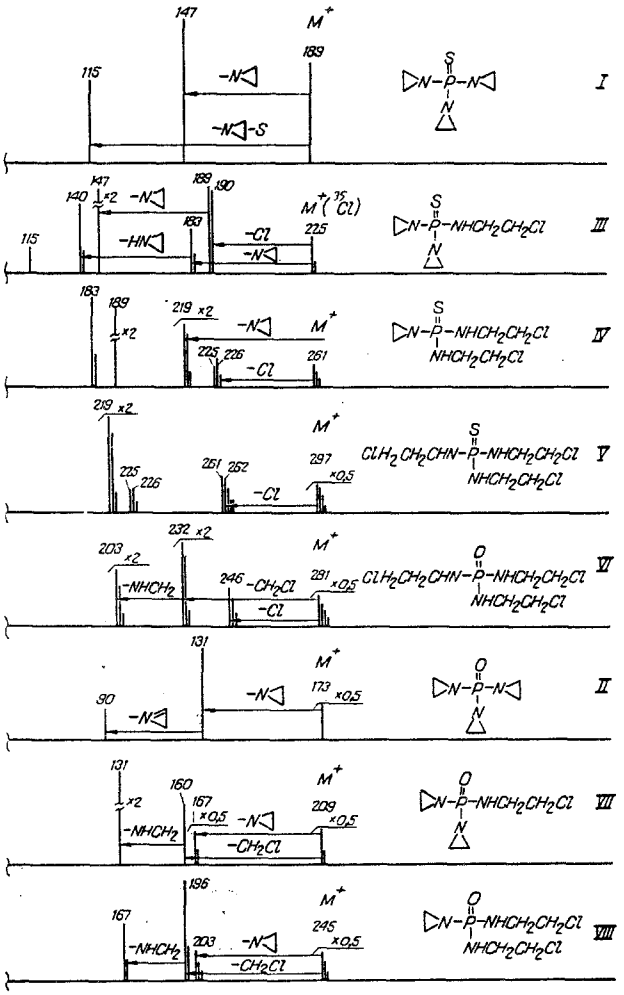


Fig. 2. Mass spectra of conversion products of I in HCl solution and metabolites of I in the rat organism.

(Fig. 1c) and experimental (Fig. 1d) urine specimens 4 h after injection, the composition of the metabolites of I can be compared with the assortment of compounds formed from I in hydrochloric acid solution. A substance with M^+ 173 was isolated from the zone with R_f 0.55. The mass spectra contain an intense peak of the ion with m/z 131. Earlier [8] in a study of the mass spectra of the drug dipin, the structure of which contains a phosphoroethylenamide fragment $P(=O)(\overline{NCH_2CH_2})_2$, it was shown that the ion $\overset{+}{O} \equiv P(\overline{NCH_2CH_2})_2$ corresponds to the intense peak with m/z 131. Since in our case there is an elimination of 42 amu from M^+, which is characteristic of the ethylenimine radical, forming m/z 131, evidently we are dealing with the sulfoxidation product – TEP (Fig. 2, II).

$$S=P(\overline{NCH_2CH_2})_3 \xrightarrow{[O]} O=P(\overline{NCH_2CH_2})_3$$

Actually, data on the chromatograph mobility and mass spectra of the substance isolated are in full agreement with the analogous characteristics of a preparation of TEP.*

Two substances were isolated from the zone with R_f 0.62 in temperature fractionation. The mass spectrum of the first completely coincided with the spectrum of the unchanged drug I; the second (VII) had M^+ 209, 211. An analysis of the group of peaks of M^+, the presence of m/z 131, as well as the 36 amu increase in mass in comparison with II, indicate that it is a monochloro-derivative of TEP (Fig. 2, VII) – the product of the addition of HCl at the ethylenimine ring. The spectrum also contains a fragment $[M-CH_2Cl]^+$ m/z 160, formed in cleavage of the β_{C-C} bond relative to the nitrogen atom.

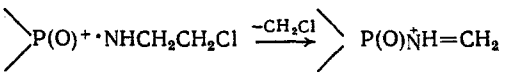
In the zone with R_f 0.69 we detected the compound M^+ 245, 247, 249 (VIII) – the product of the addition of two molecules of HCl. Mass spectrometric decomposition is analogous to that of VII, but with a 36 amu shift of the masses of the fragments.

*We synthesized a preparation of TEP from $POCl_3$ and ethylenimine according to the procedure of [12].

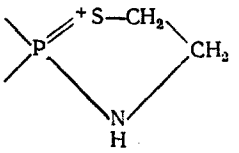
From the zone with R_f 0.90 we isolated a substance with molecular weight 281 (three atoms of ^{35}Cl), identical in mass spectrum with the compound VI.

According to the data of R_f and the mass spectra, compounds VI-VIII are identical with the conversion products of the substance TEP in HCl solution.

An examination of the mass spectra of the metabolites that are chloro-derivatives of TEP (compounds VI, VII, VIII), and the analogous chloro-derivatives of thiophosphamide (compounds III, IV, V) shows that although there is a definite similarity, an essential difference is observed in the ease of elimination of a chlorine atom and a CH_2Cl fragment from the molecular ion. The profitability of formation of the ion $[M-CH_2Cl]^+$ in the case of derivatives of TEP is evidence of a substantial localization of charge on the nitrogen atom of the chloroethylamine group in the molecular ion of these compounds:



In the case of amides of thiophosphoric acid, such a pathway of the decomposition of M^+ is not characteristic. The charge is evidently localized on the sulfur atom, and the profitability of the elimination of Cl is due to the formation of a stable five-membered cyclic fragment A:



A

The absence of an oxygen-containing stable ion of type A in the case of TEP derivatives is evidently explained by the higher ionization potential of oxygen in comparison with the potential of sulfur, which also leads to an essentially different localization of charge in these compounds.

It is noteworthy that in [1] the authors explain the increase in the antitumor activity and the decrease in the toxicity of thiophosphamide in comparison with TEP through the formation of a more stable carbocation on account of the greater nucleophilicity of the sulfur atom in comparison with the oxygen atom. The formation of a stable five-membered ring A was proposed as a stabilizing factor.

Thus, the mass spectrometric data cited are in good agreement with the proposed hypothesis.

A comparison of the data obtained on the metabolism of thiophosphamide and other compounds containing phosphoroethylenamide groups shows that, in contrast to phosphamide [5], fopurin [6], and dipin [10], in the case of thiophosphamide the metabolites excreted with the urine do not include products formed by the addition of HCl to the thiophosphamide molecule. Together with the unchanged drug, TEP – the product of sulfoxidation of thiophosphamide – and β-chloroethylamido-derivatives of TEP are detected in the urine of rats. The absence of chloro-derivatives of thiophosphamide is probably due either to the inability of the drug itself, like TEP, to penetrate into the walls of the stomach and interact with the hydrochloric acid of the gastric juice, which, as has been shown for phosphamide [5] and dipin [11], plays the main role in the formation of β-chloroethylamide derivatives, or to the fact that the rate of sulfoxidation is significantly greater than the rate of penetration of thiophosphamide into the stomach. This question will require separate investigation.

LITERATURE CITED

1. G. N. Bogdanov and N. M. Émanuél', Urgent Problems of Experimental Chemotherapy of Tumors [in Russian], Chernogolovka (1982), pp. 22-25.
2. V. S. Mosienko and V. M. Pivnyuk, Vopr. Onkol., 14, No. 5, 73-76 (1968).
3. V. M. Pivnyuk, Urgent Problems of Experimental Chemotherapy of Tumors [in Russian], Chernogolovka (1982), pp. 78-79.
4. T. L. Pyatigorskaya, O. Yu. Zhilkova, V. S. Shilkovskii, et al., Khim.-farm. Zh., No. 10, 1235-1241 (1985).

5. T. S. Safonova, L. F. Linberg, Zh. F. Presnova, et al., Dokl. Akad. Nauk SSSR, 219, No. 5, 1271-1272 (1974).
6. T. S. Safonova and V. A. Chernov, Antitumor Drugs [in Russian], Moscow (1984), pp. 3-10.
7. V. A. Chernov, Therapeutic and Diagnostic Agents Used in Oncologic Practice [in Russian], Moscow (1982), p. 37.
8. V. V. Chistyakov, O. S. Anisimova, Yu. N. Sheinker, and T. S. Safonova, Khim.-farm. Zh., No. 11, 1290-1294 (1984).
9. V. V. Chistyakov, O. S. Anisimova, and Yu. N. Sheinker, Analytical Methods of Testing in the Development and Production of Synthetic Drugs [in Russian], Moscow (1985), pp. 13-19.
10. V. V. Chistyakov, O. S. Anisimova, N. P. Solov'eva, et al., Khim.-farm. Zh., No. 4, 398-402 (1987).
11. V. V. Chistyakov, O. S. Anisimova, G. A. Losev, et al., Ibid., No. 9 (1988).
12. H. Bestian, Justus Liebigs Ann. Chem., 568, 210-244 (1950).
13. I. Maxwell, D. S. Kaushik, and C. G. Butter, Biochem. Pharmacol., 23, 168-170 (1974).
14. L. B. Mellett and L. A. Woods, Cancer Res., 20, 524-532 (1960).
15. L. B. Mellett, P. E. Hodson, and L. A. Woods, J. Lab. Clin. Med., 60, 818-825 (1962).

INFLUENCE OF BONAPHTHONE ON THE SYNTHESIS OF VIRUS-SPECIFIC PROTEINS OF HERPES SIMPLEX VIRUS, TYPE 1

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Bonaphthone (6-bromo-1,2-naphthoquinone, I) – an antiherpes drug synthesized at the S. I. Ordzhonikidze All-Union Chemicopharmaceutical Scientific Research Institute – is widely used clinically for the treatment of herpetic infection of the eyes, herpes of the skin and genitals and herpetic stomatitis [2]. We studied the antiviral activity of I with respect to herpes simplex virus in comparison with the foreign antiherpes drug acyclovir (II) and phosphonacetic acid (III). As can be seen from Table 1, I is a highly active drug, just as active as the known antiherpes drugs. The 50% inhibiting concentration of I is close to that of II and significantly lower than that of III.

Moreover, we established that I inhibits the reproduction of herpes simplex virus types 1 and 2 (HSV 1 and HSV 2) in primary-trypsinized and continuously cultured cell lines; II and acycloguanosine possess more pronounced activity in the indicated lines [4].

In chemical structure I differs from all the known antiherpetic drugs; consequently, it can be assumed that it will possess an original mechanism of action on HSV reproduction, differing from that of the other antiherpetic drugs.

In this communication we present the results of our study of the influence of the drug I on the synthesis of virus-specific and cell proteins on a biochemical model.

MATERIALS AND METHODS

Viruses. We used the strain L_2 (HSV 1), isolated at the D. I. Ivanovskii Institute of Virology, AMS USSR [5], and strain F (HSV 2), obtained by the Institute from Dr. B. Roizman (USA). The virus we reproduced in a culture of Vero cells and a primary culture of chick embryo fibroblasts (CEF). The cells were cultured in Eagle's medium with 10% bovine serum. Medium 199 was used as the maintenance medium after infection with the virus. The infectiousness of the virus in titration in cell culture according to cytopathic effect was 10^9 TCD_{50}/ml.

Analysis of Virus-Specific Proteins in Polyacrylamide Gel (PAAG). A 2-3-day Vero monolayer culture was infected with HSV strains L_2 and F. I in the form of a suspension in nutrient medium was introduced 1 h after the beginning of adsorption at 34°C. A ^{14}C-labeled

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