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

NEW XANTHINE OXIDASE INHIBITORS FROM THE CLASS OF PYRAZOLO[3,4-d]-PYRIMIDINES AND PYRAZOLO[3,4-b]PYRIDINES.

I. MECHANISM OF THE ACTION OF ALLOPURINOL AND ITS ANALOGS

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UDC 615.254.1:547.828].015:577.152.342

Enzymes that oxidize xanthine — xanthine oxidase (XO) (EC 1.2.3.2) and xanthine dehydrogenase (XD) (EC 1.2.3.7) — belong to the class of molybdenum-containing hydroxylases and contain not only two molybdenum atoms, but also eight atoms of nonheme iron and two molecules of flavin adenine nucleotide (FAD), in their prosthetic group. XO and XD catalyze the oxidative dehydroxylation of substrates according to the following reaction:

$$RH \xrightarrow[OH^-]{-2e;\ -H^+} ROH,$$

where RH is the oxidation substrate. The enzymes are widely represented in living organisms of various degrees of organization (from bacteria up to mammals). They represent key enzymes of purine catabolism. Numerous functions are ascribed to the xanthine-oxidizing enzymes: As a result of their low specificity they are capable of catalyzing the oxidation of a number of foreign substances for the organism, including those taken in as drugs, purines, pteridines, pyrimidines, and other nitrogen-containing heterocycles, thereby accomplishing their detoxification and promoting their elimination from the organism [1].

In pharmacological practice, 4-hydroxypyrazolo[3,4-d]pyrimidine (AP; synonyms: allopurinol, milurite, xyloric) is widely used as an inhibitor of XO and XD. Allopurinol prevents the oxidation of xanthine and hypoxanthine to uric acid in primary hyperuricemia (for example, in gout), as well as in secondary hyperuricemia, which arises, in particular, as a result of the treatment of tumor diseases by irradiation or with cytostatics, during prolonged starvation, in the presence of reduced kidney function, and in a number of other cases [1,2]. In connection with this, the interest in the search for analogs of AP and for an elucidation of the molecular mechanism of their action is understandable. This communication presents data on the kinetics of the inhibition of XO with the aid of AP and proposes a scheme describing the mechanism of inhibition.

EXPERIMENTAL

Xanthine oxidase was obtained from cow's milk [3]. The activity of the enzyme was determined on a Specord UV-VIS spectrophotometer (German Democratic Republic) in 0.05 M phosphate buffer, pH 8.0, containing 10^{-4} M EDTA, at 25°C, according to the conversion of xanthine to uric acid at 295 nm. Experiments on the inhibition of XO using AP were conducted without preliminary incubation, beginning the reaction by adding the enzyme.

The AP was purified by recrystallization from water; the preparation was homogeneous according to the data of high-resolution liquid chromatography.

RESULTS AND DISCUSSION

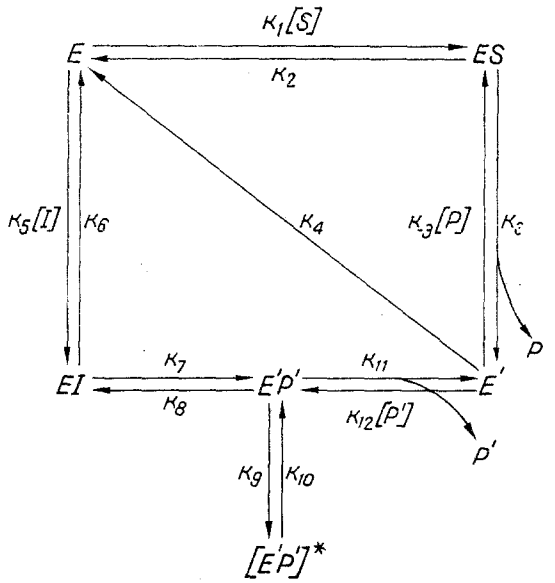
In the presence of XO, AP is hydroxylated at the 6-position, forming 4,6-dihydroxypyrazolo[3,4-d]pyrimidine (hydroxypurinol, alloxanthine) (AX). For XO it has been shown [1] that the enzyme interacts with xanthine according to a two-cycle mechanism: First there is a separation of the product from the reduced enzyme, and then the enzyme is reoxidized.

Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 16, No. 6, pp. 650-655, June, 1982. Original article submitted November 3, 1981.

0091-150X/82/1606-0417$07.50

Evidently AP, just like xanthine, is oxidized according to a two-cycle mechanism. In the oxidation of AP in the presence of XO, the rate of accumulation of AX drops exponentially [4], which, as was shown earlier [5], is a consequence of the formation of a strong complex of AX with the reduced form of XO. The effective dissociation constant of this complex at room temperature and pH values close to the physiological is $5.4 \cdot 10^{-10}$ M. Since the time of reaching of a maximum degree of inhibition does not depend on the AP concentration within a broad range of concentrations, this means that the limiting step of inhibition is a first (or pseudofirst) order reaction. The slow dissociation of the strong complex of the reduced enzyme with AX is achieved by reoxidation in the presence of oxygen, additions of high concentrations of xanthine or artificial acceptors — phenazine methosulfate or ferricyanide [6].

On the basis of the data cited we can suggest the following scheme of inhibition of XO — a reaction in the presence of AP or its analogs:



where E is the oxidized form of XO; E' is the reduced form of XO; S is xanthine; P is uric acid; I is allopurinol; P' is alloxanthine; EI is a reversible complex of XO with AP; E'P' is a reversible complex of reduced XO with AX; [E'P']* is a strong complex of reduced XO with alloxanthine.

In the calculation of the scheme the following assumptions were made:

$$k_{-3}[P] \ll k_3[ES]; \quad k_8[E'P'] \ll k_7[EI].$$

The concentrations [S], [E], and [O_2] were considered constant. The reaction rate

$$v = k_3[ES]. \tag{1}$$

With these assumptions, the expression for the reaction rate is obtained from the solution of the system of equations:

$$\frac{d[E'P']}{dt} = k_7[EI] - k_{11}[E'P'] + k_{12}[E'][P'] = 0; \tag{2}$$

$$\frac{d[E']}{dt} = k_3[ES] - k_4[E'] + k_{11}[E'P'] - k_{12}[E'][P'] = 0; \tag{3}$$

$$\frac{d[ES]}{dt} = k_1[E][S] - k_2[ES] - k_3[ES] = 0; \tag{4}$$

$$\frac{d[EI]}{dt} = k_5[E][I] - k_6[EI] - k_7[EI] = 0; \tag{5}$$

$$[E_0] = [E] + [ES] + [E'] + [EI] + [E'P'] + [E'P']^*; \tag{6}$$

$$\frac{d[E'P']^*}{dt} = k_9[E'P'] - k_{10}[E'P']^*. \tag{7}$$

For [E'P'] we obtain:

$$[E'P'] = \frac{\frac{k_7[E][I]}{K_i} + \frac{k_{12}}{k_4}\left[\frac{k_3[E][S]}{K_m} + \frac{k_7[E][I]}{K_i}\right]\cdot[P']}{k_{11}}. \tag{8}$$

For the constants included in Eq. (8) the following values are cited in the literature: $K_i \sim 10^{-5}$ M [7]; $k_3 = 10^3$ min^{-1} [1]; $k_4 = 1.2\cdot10^3$ min^{-1}[1]; $k_{11} = 160$ min^{-1} [4]; $K_m = 3\cdot10^{-6}$ M. Let us assume that k_7, as the rate constant of intramolecular electron transfer, is more than 10^3 min^{-1} [1]. The value $k_{12} = 2\cdot10^6$ M$^{-1}\cdot$min^{-1} was estimated according to the value of the dissociation constant of the complex [E'P'], $0.8\cdot10^{-6}$ M [8], and the value of k_{11}. According to Eq. (4), the fraction of AP converted to AX ([P']/[I]) does not depend on the initial concentration [I] and does not exceed 20%. Considering all these data, in the expression for [E'P'] we can neglect the term containing [P'], and then:

$$[E'P'] = \frac{k_7[E][I]}{K_i \cdot k_{11}}. \tag{9}$$

Solving Eq. (7) considering Eq. (9) permits the expression of the reaction rate v_i in terms of v_z, the initial rate, and v_s, the steady-state reaction rate, which is established when equilibrium is reached with respect to all steps of the process:

$$v_i = v_s + (v_z - v_s)\cdot e^{-k't}, \tag{10}$$

where

$$k' = \frac{k_{10}\left(1 + \frac{[I]}{K_i}\right) + \frac{k_{10}[S]}{K_m}\left(1 + \frac{k_3}{k_4}\right) + \frac{k_{10}k_7}{K_i}\left(\frac{1}{k_{11}} + \frac{1}{k_4}\right)}{1 + \frac{[S]}{K_m}\left(1 + \frac{k_3}{k_4}\right) + \frac{[I]}{K_i} + \frac{k_7[I]}{K_i}\left(\frac{1}{k_{11}} + \frac{1}{k_4}\right)}. \tag{11}$$

Considering that $k_3 \approx k_4$, $k_4 \gg k_{11}$, $k_9 \gg k_{10}$ [5, 8], we obtain

$$k' = \frac{k_{10}\left(1 + \frac{[I]}{K_i} + \frac{2[S]}{K_m}\right) + \frac{k_7 k_9[I]}{k_{11}\cdot k_i}}{1 + \frac{[I]}{K_i} + \frac{2[S]}{K_m} + \frac{k_7[I]}{k_{11}\cdot K_i}}; \tag{12}$$

$$v_s = \frac{V\frac{[S]}{K_m}}{1 + \frac{[S]}{K_m}\left(1 + \frac{k_3}{k_4}\right) + \frac{[I]}{K_i}\left[1 + \frac{k_9\cdot k_7}{k_{10}\cdot k_{11}} + \frac{k_7(k_4 + k_{11})}{k_4\cdot k_{11}}\right]}; \tag{13}$$

$$v_z = \frac{V\frac{[S]}{K_m}}{1 + \frac{[S]}{K_m}\left(1 + \frac{k_3}{k_4}\right) + \frac{[I]}{K_i}\left(1 + \frac{k_7}{k_4} + \frac{k_7}{k_{11}}\right)}, \tag{14}$$

where

$$K_m = \frac{k_2 + k_3}{k_1}; \quad K_i = \frac{k_6 + k_7}{k_5}.$$
$$V = k_3[E_0] \tag{15}$$

To determine the value of K_i we conducted experiments at comparable concentrations of xanthine and AP and high concentrations of XO, so that exhaustion of the substrate occurred rather rapidly, before a strong complex (E'P')* had time to be formed. For this series of experiments:

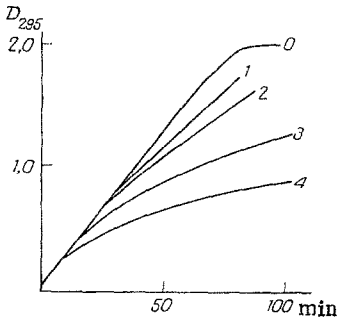


Fig. 1

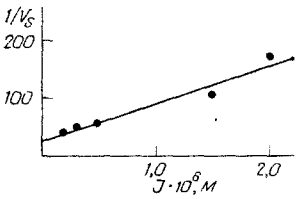


Fig. 2

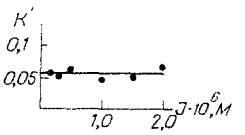


Fig. 3

Fig. 1. Kinetic curves of the formation of uric acid at various AP concentrations. 1) 0; 2) $2 \cdot 10^{-7}$ m; 3) $5 \cdot 10^{-7}$ M; 4) $1.5 \cdot 10^{-6}$ M; 5) $2.0 \cdot 10^{-6}$ M. Initial xanthine concentration $2 \cdot 10^{-4}$ M, XO $2 \cdot 10^{-4}$ M. 0.05 M phosphate buffer, 10^{-4} M EDTA, pH 8.0, 25°C.

Fig. 2. Dependence of the steady-state rate of formation of uric acid on the initial AP concentration. Initial xanthine concentration $2 \cdot 10^{-4}$ M, XO $2 \cdot 10^{-9}$ M. 0.05 M phosphate buffer, 10^{-4} M EDTA, pH 8.0, 25°C.

Fig. 3. Dependence of the value of k' [Eq. (12)] on the AP concentration.

$$v_i = \frac{V}{1 + \frac{K_m}{[S]} + \frac{[I]}{K_i} \cdot \frac{K_m}{[S]}}. \qquad (16)$$

The value of K_i was determined from the function in Dixon coordinates [9] ($1/v_i$ versus I). This dependence is not linear within a wide range of AP concentrations. Evidently at high AP concentrations the formation of a strong complex (E'P')* begins to have an effect; therefore, to calculate K_i we used only the initial linear portion of the curve. For the quantity K_i we obtained a value of $1 \cdot 10^{-6}$ M, which is close to the value for competitive inhibition of XO with the aid of AP, determined earlier [7].

To confirm the scheme of inhibition cited above, we performed a kinetic analysis under steady-state conditions with respect to all stages of the process, proposed in [10] to differentiate the mechanisms of action of tightly bound inhibitors. The steady-state character of the system was achieved by conducting the reaction at low concentrations of AP and XO, and high concentrations of xanthine. In this case exhaustion of the substrate does not occur for a long time, and equilibrium of the formation of the complex (E'P')* has time to be established during the reaction.

Since the affinity of AP for XO is close to the affinity of xanthine for XO ($K_m = 3 \cdot 10^{-6}$, $K_i = 10^{-6}$), while the constant of decomposition of the complex [E'P'] is less than k_3, by conducting the reaction in the presence of a low AP concentration and a high xanthine concentration, we did not obtain any decrease in the initial reaction rate, but observed only a progressive inhibition, associated with the formation of a strong complex [E'P']*.

Figure 1 presents kinetic curves of the accumulation of uric acid in experiments without preliminary incubation of XO with AP, where inhibition does not affect the initial reaction rate, then the degree of inhibition progressively increases, and 10 minutes after the beginning of the reaction a steady-state rate of accumulation of uric acid is established. The equation for this curve is obtained by integration of Eq. (10):

$$P = v_s \cdot t - \frac{1}{k'}(v_s - v_i)(1 - e^{-k't}). \qquad (17)$$

TABLE 1. Values of k Calculated according to Eq. (20) according to the Data of Various Experiments on the Inhibition of XO by High Concentrations of AP

k', min^{-1}	[I] $M \cdot 10^5$	Conditions of reaction	Literature Source
1.1	$2.7 \cdot 10^{-5}$	Aerobic oxidation of xanthine in the presence of AP ([S] = $7 \cdot 10^{-5}$ M)	Our data
2.3	8.0-25	Aerobic oxidation of AP	[4]
1.4	16.7	Aerobic oxidation of AP recorded according to nonenzymatic reduction of cytochrome c	[5]
1.6	16.7	Slow phase of reduction of FAD in anaerobic reduction of XO by AP, ascribed by the authors to the reaction of complex formation	[6]

In accordance with Eq. (13), a linear dependence of v_s on the AP concentration with a slope of $6 \cdot 10^7$ is observed (Fig. 2).

The values of k' were calculated according to the kinetic curves (see Fig. 1), using the values of the segments π_0 intercepted by the linear portions of the kinetic curves (portions with a steady-state rate), continued to intersection with the y-axis, in accordance with Eq. (17).

$$\pi_0 = \frac{v_z - v_s}{k'}. \tag{18}$$

As can be seen from Fig. 3, within the investigated range of concentrations the value of k' does not depend on the AP concentration and is equal to 0.06 min^{-1}. At AP concentrations from $2 \cdot 10^{-6}$ to $2 \cdot 10^{-7}$ M and xanthine concentrations $2 \cdot 10^{-4}$ M, $[I]/K_i \ll [S]/K_m$. Then

$$k' = \frac{\frac{k_{10} \cdot 2[S]}{K_m} + \frac{k_7 k_9 [I]}{k_{11} \cdot K_i}}{\frac{2[S]}{K_m} + \frac{k_7 [I]}{k_{11} \cdot K_i}}. \tag{19}$$

Independence of the value of k' from [I] can be achieved when

$$\frac{k_7 k_9 [I]}{k_{11} \cdot K_i} \ll \frac{k_{10} \cdot 2[S]}{K_m} \text{ and } \frac{k_7 [I]}{k_{11} \cdot K_i} \ll \frac{2[S]}{K_m}.$$

In this case the value obtained for k' should be close to k_{10}. The other limiting value of k' (considering that $k_7/k_{11} \gg 1$) according to Eq. (12) should be reached when $[I]/K_i \geq 2[S]/K_m$, then $k' = k_9$. In this case $v_s \ll v_z$, and for v_i we shall have:

$$v_i = v_z \cdot e^{-k't}. \tag{20}$$

The values of k', calculated according to Eq. (20) on the basis of the experimental results and the data of various authors, are cited in Table 1, from which it is evident that $k_9 \approx 1.6$ min^{-1}.

In [8] a kinetic scheme was proposed to describe the kinetics of the inhibition of XO with the aid of AX; the scheme also considers the formation of a reversible and strong complex of reduced XO with AX. The values of the constants of the transition [E'P'] $\rightleftarrows$ [E'P']*

differ from those calculated according to our data by a factor of approximately 5. However, the ratios of these constants are close ($3.7 \cdot 10^{-2}$ and $2.5 \cdot 10^{-2}$).

In contrast to the scheme cited in [8], the scheme considered in the present work permits a description of the behavior not only of inhibitors of the AX type, which form complexes only with reduced XO, but also of inhibitors of a broad class, which are simultaneously substrates of XO. Therefore, the proposed scheme can be used for a quantitative estimation of the effectiveness of various XO inhibitors, capable of forming two types of complexes with the enzyme: rapid reversible and slow strong.

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NEW XANTHINE OXIDASE INHIBITORS FROM THE CLASSES OF PYRAZOLO[3,4d]-PYRIMIDINES AND PYRAZOLO[3,4-b]PYRIDINES.

II. COMPARATIVE EVALUATION OF EFFECTIVENESS

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UDC 615.254.1:547.828].015

In a previous communication [1] a mechanism was proposed for the inhibition of xanthine oxidase (XO) [EC 1.2.3.2] by the tightly bound inhibitor 4-hydroxypyrazolo[3,4-d]pyrimidine (allopurinol, I), which is finding wide use in medicine as a hypouricemic agent. This work presents results of a study of the inhibiting activity of new potential XO inhibitors from the classes of pyrazolo[3,4-d]pyrimidines (PP) and pyrazolo[3,4b]pyridines — analogs of allopurinol.

EXPERIMENTAL

The details of the experiment with XO are cited in [1]. The activity of the inhibitors was estimated in experiments without preliminary incubation of the enzyme with the inhibitor according to the decrease in the initial rate of the enzymatic reaction and were characterized by the values of I_{50} and K_i. I_{50} is the inhibitor concentration that decreases the initial rate of the enzymatic reaction by a factor of 2. The values of K_i, the dissociation constants of the rapidly formed reversible complexes of XO with inhibitors, just as in [1], were calculated according to the functions in Dixon coordinates ($1/v_i$ versus I) [2]. The determination of the effective dissociation constants of strong complexes K'_i was performed according to [3]. In these experiments XO was preliminarily reduced with xanthine or dithionite under anaerobic conditions in 0.05 M phosphate buffer, pH 7.8, containing 10^{-4} M EDTA at 0°C. The reduced enzyme was incubated with the inhibitor under the same conditions for 15 min, then aerated for 15 min and the residual activity determined at 25°C. The values

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Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 16, No. 6, pp. 655-660, June, 1982. Original article submitted November 3, 1981.

0091-150X/82/1606-0422$07.50