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COMMENTARY

INHIBITION OF URATE PRODUCTION BY ALLOPURINOL

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Allopurinol has provided the clinician with an entirely novel approach to the treatment of hyperuricemia. In contrast to the anti-inflammatory and uricosuric agents which traditionally have been applied to the problems of hyperuricemia, allopurinol prevents hyperuricemia by inhibiting the synthesis of urate. Although the overall mode of action by which allopurinol accomplishes this has been well established, the process is by no means simple. In fact, it appears that the complexities of allopurinol's molecular interactions have fostered some misconceptions and misinterpretations. In an attempt to clarify the mechanism of action of allopurinol, the studies of the key properties of this agent and its active metabolite, oxipurinol, have been integrated into the following Commentary.

Mechanism of the inhibition of xanthine oxidase

Although the lowering of urate levels *in vivo* by allopurinol is basically caused by the inhibition of xanthine oxidase, allopurinol should be viewed as an alternate-substrate inhibitor. The tenacious inhibition of xanthine oxidase is a result of the unconventional binding of the oxidized product, oxipurinol (Fig. 1).

The excellent substrate activity of allopurinol is characterized by a binding constant ($1–7 \times 10^{-7}$ M) that is about 5 to 40-fold lower than the K_m of the physiological substrate, xanthine [1], and a V_{max} that is 2 to 6-fold faster than the V_{max} for xanthine [2–4]. However, under conditions where oxygen serves to accept electrons from xanthine oxidase, the rapid rate for the oxidation of allopurinol exponentially decreases ($T_{1/2}$ = 18 sec at 25°) to a rate of <1 per cent the rate observed with xanthine [5]. This deceleration in the rate for the conversion of allopurinol to oxipurinol is caused by a rather unusual type of alternate-product inhibition [3, 6] that has been termed "suicidal" [3]. The identification of oxipurinol as the active inhibitor of its own production as well as the production of urate and the discovery of the intricate mechanism of its inhibition were the results of two independent investigations in 1970 [3, 6].

The clues that greatly aided the understanding of the nature of the inhibition arose from earlier studies with xanthine oxidase. The original study of Elion [1] demonstrated that while allopurinol could slowly (within 5 min at 25°) bring about the "apparent inactivation" of xanthine oxidase in the absence or presence of xanthine, "apparent inactivation" by oxipurinol required the presence of xanthine. In the absence of xanthine, the binding of oxipurinol was considerably weaker. The other studies revealed that the rapid oxidation of allopurinol or xanthine in the presence of allopurinol, as mediated by xanthine oxidase, could be sustained (i.e. avoiding the exponential decline in rate) if an artificial electron acceptor, such as 2,6-dichlorophenolindophenol (DCIP), phenazinemethosulfate or ferricyanide, was substituted for oxygen [2, 4]. The molecular events that were reflected in these observations were dependent upon the oxidation state of one of the elements of the self-contained electron

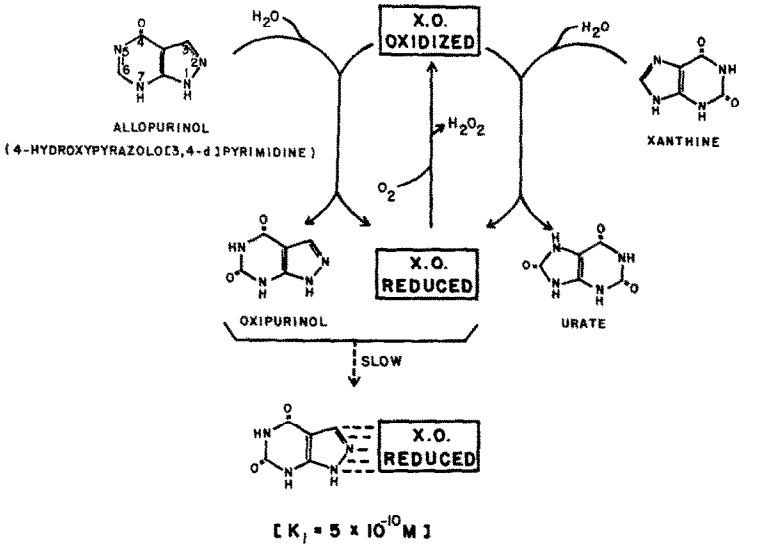

Fig. 1. Production of the tight-binding inhibitor of xanthine oxidase (X.O.).

transport chain of xanthine oxidase. That is, the tight binding of oxipurinol ($K_I = 5 \times 10^{-10}$ M [6]) occurs only when molybdenum (Mo) is in an electronically reduced [3, 6] state; and, conversely, the rapid release of oxipurinol is promoted by the efficient reoxidation of this element by the artificial electron acceptors [2–4].

The members of the electron transport chain, in their oxidized states, consist of Mo (VI), two iron/sulfur centers and FAD. Reducing equivalents flow from the substrate to O_2 via this chain, entering at Mo and exiting at FAD (see Ref. 7 for review). The iron/sulfur centers act as electron reservoirs serving to maintain the efficiency of this process [8]. When oxipurinol binds to the active site of xanthine oxidase, it complexes Mo [5] in the formal oxidation state of IV [3] and thereby renders this component inaccessible to reoxidation by the other components of the chain or by O_2. The artificial electron acceptors can, on the other hand, directly reoxidize the Mo and cause the rapid release of the tightly bound oxipurinol [2–4, 9].

Conditions for inhibition in vitro

The three constituents required to produce the most effective inhibition of xanthine oxidase are: oxipurinol, xanthine oxidase with Mo in the oxidation state of IV (reduced), and time. Oxipurinol, in slight excess, must be incubated with xanthine oxidase. It can either be added directly, or generated from allopurinol [1, 3, 6]. It should be reiterated here that the binding of oxipurinol under these conditions is three orders of magnitude more tenacious than that of allopurinol. In experiments where allopurinol and xanthine oxidase were incubated either anaerobically [3] or in the presence of a chemical reductant [6], only oxipurinol (not allopurinol) was found to be tightly bound to the enzyme.

The Mo of the enzyme may be reduced either by a substrate, such as hypoxanthine, xanthine or allopurinol [1, 3, 5], or by a chemical such as dithionite [6]. Furthermore, since the binding of oxipurinol is a slow process, the enzyme must remain reduced for the required time period. This is accomplished with either excess reductant [1, 3, 6] or with stoichiometric amounts of reductant (i.e. six reducing equivalents/active center) and strict anaerobicity [3]. It is also important to note that the time-dependence for the binding is very sensitive to temperature. The development of the inhibition requires an incubation of less than 1 min at 40°, but over 2 hr at 2° [6].

Whereas violation of the above conditions will result in less than maximal inhibition, adherence to them will produce an enzyme–inhibitor complex with a dissociation constant of 5×10^{-10} M [6].* The slowness of the dissociation of this complex is exemplified by the initial inability of either the dilution of the complex or the addition of substrate to promote its breakdown [6]. This tight-binding property of oxipurinol has been exploited in titration studies with xanthine oxidase. It was of interest that a stoichiometry of less than 1 mole of bound inhibitor/mole of enzyme was obtained [3, 6]. These findings confirmed the earlier evidence [10–12] that a population of non-functional enzyme was present in the preparations of xanthine oxidase. Resolution of functional and non-functional enzyme was later accomplished with an affinity column consisting of a derivative of oxipurinol as the attached ligand [13].

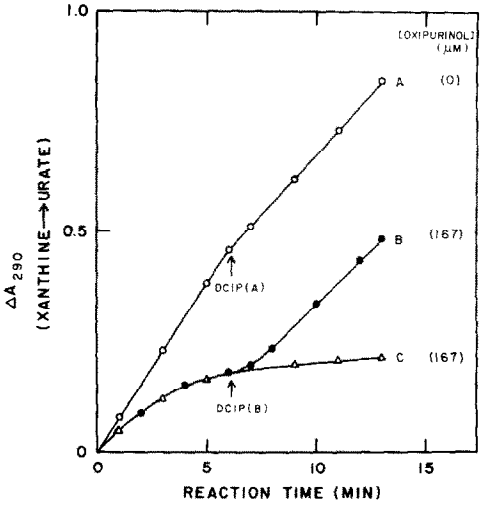

Fig. 2. Time-dependence for the binding of oxipurinol to xanthine oxidase and for the release of oxupurinol as promoted by dichlorophenolindophenol (DCIP). The oxidation of xanthine (167 μM) by bovine milk xanthine oxidase at 25° was monitored spectrophotometrically ($\Delta E_{290} = 7.8$ mM^{-1}, cm^{-1}). Reactions B and C illustrate the slow formation of the inhibitor complex. The dissociation of this complex after the reoxidation of reduced xanthine oxidase (note short lag) by DCIP is illustrated in reaction B. The reduction of DCIP has a $\Delta E_{290} = -0.9$ mM^{-1}, cm^{-1}. This figure was reproduced from Ref. 9.

Reversibility of inhibition

Oxipurinol is correctly described as a "titrating," "stoichiometric," or "pseudoirreversible" inhibitor that causes apparent inactivation of xanthine oxidase, but it is not an irreversible inhibitor. There is no evidence for covalent bond formation. In fact, the reversibility of the inhibition has been demonstrated by several different approaches. For example, the inhibition is slowly reversed by prolonged dialysis [1], or rapidly reversed in the presence of certain artificial electron acceptors [2–4, 9]. The rapid reversal, as well as the slow development of the inhibition, is illustrated in Fig. 2. This reversal is due to the direct reoxidation of Mo (IV) and subsequent release of oxipurinol. Reversal will also occur spontaneously ($T_{1/2} = 6$ hr) if the xanthine oxidase–oxipurinol complex is incubated in the absence of a reductant under aerobic conditions [3]. In this case, however, it is not established whether the dissociation of the inhibitor or the reoxidation of the Mo occurs first. Finally, the activity will return at an intermediate rate if the inhibitor complex is incubated with xanthine [6, 9]. The amount of reversal in the presence of substrate is directly related to the duration of catalytic cycling. While the initial reversal is negligible, approximately 50 per cent reversal is observed within 10–15 min. It is conceivable that the superoxide anion ($O_2^{\cdot-}$)

* Comparative studies of the overall potency of inhibitors of xanthine oxidase vs allopurinol should be referenced against this K_I value for oxipurinol.

generated during the catalytic cycling may facilitate the reoxidation of reduced Mo.

Clearly, enzyme complexed with oxipurinol will not catalyze the oxidation of substrates that bind to the xanthine site. However, the remaining portions of the molecule should not be considered inert. Although a paucity of information on this subject is available, it is established that the xanthine oxidase–oxipurinol complex can readily accept reducing equivalents from NADH by direct transfer at the FAD site [3]. Presumably, the oxidation of NADH could, therefore, catalytically proceed with O_2 as the electron acceptor and without displacement of bound oxipurinol.* Consequently, empirical evidence should be obtained before it is assumed that the oxidation or reduction of an unconventional substrate by xanthine oxidase will be prevented by allopurinol or oxipurinol.

Studies in vivo

The efficacy of allopurinol seems to be facilitated by the fact that the active inhibitor is metabolically produced *in vivo*. Studies have shown [14–17] that the oral administration of oxipurinol will bring about the lowering of plasma and urinary urate, but the required dose (600 mg/day) is twice that required with allopurinol. Actually, the plasma levels of oxipurinol achieved from a single 300 mg dose of allopurinol were slightly higher than those obtained after giving 600 mg oxipurinol directly [15]. The disadvantage of the oral administration of oxipurinol instead of allopurinol seems to be related to the relatively poor absorption of oxipurinol [15].

The production *in vivo* of oxipurinol manages to persist throughout the entire course of treatment with allopurinol [16]. A portion of allopurinol is undoubtedly oxidized by xanthine oxidase that is not complexed with oxipurinol. Studies with partially purified enzyme [18] and *in vivo* studies with mice [19] support the hypothesis that the remainder may be oxidized by aldehyde oxidase (EC 1.2.3.1). This enzyme will catalyze the conversion of hypoxanthine to xanthine as well as allopurinol to oxipurinol, but not the conversion of xanthine to urate (see Ref. 20 for review).

There were two case histories of xanthinuric patients, grossly deficient in xanthine oxidase, that are pertinent to this topic. One patient readily converted allopurinol to oxipurinol [21], but the other patient converted only minute amounts [16]. The oxidation of allopurinol by the former patient had been attributed to the catalytic action of IMP dehydrogenase (EC 1.2.1.1.4) subsequent to the formation of allopurinol nucleotide [21]. Recent studies *in vitro*, however, have demonstrated that allopurinol nucleotide is not a substrate for this enzyme (i.e. the velocity was less than 0.003 per cent of that observed with IMP [22]).

An alternate explanation of these data is that aldehyde oxidase is capable of oxidizing allopurinol in humans as it does in mice. Thus, the patient who formed oxipurinol from allopurinol possessed aldehyde oxidase, while the other patient was deficient in aldehyde oxidase as well as xanthine oxidase.

To date, there are reports of five xanthinuric patients. Two of these patients readily convert allopurinol to oxipurinol. A review of these studies is presented in Ref. 7.

It is of interest that oxipurinol, a structural isomer of xanthine, behaves very much like urate with respect to its clearance in the human kidney. Its rate of clearance (approximately 20 ml/min) is only about three times faster than that for urate [15, 16]. Apparently, it is reabsorbed [15, 16], but not secreted [15], at the urate site in the tubules, and is responsive to the uricosuric action of probenecid [15, 16]. This uncommon feature accounts for the extended half-life (18–33 hr) of oxipurinol in human plasma [15, 16], and probably is important to the time-dependence of the formation of the inhibitor complex with xanthine oxidase. Allopurinol, on the other hand, is rapidly cleared by the kidney with a rate approaching the glomerular filtration rate [16].

In addition to the inhibition of xanthine oxidase, there exist other factors that probably contribute to the mode of action by which allopurinol inhibits the production of urate. Subsequent to the inhibition of xanthine oxidase, there is a dramatic increase in the reutilization of hypoxanthine and xanthine by conversion to their respective nucleotide monophosphates. In turn, these nucleotides may serve as feedback inhibitors of the *de novo* pathway for the biosynthesis of purines (see Refs. 23 and 24 for reviews). Therefore, in addition to the individual contributions of this enhunced reutilization and subsequent feedback inhibition to the decrease of urate production, there is a multiple lowering of the substrate pressure that hypoxanthine and xanthine exert on inhibited xanthine oxidase. Consequently, these effects may provide additional assistance to the inhibition by oxipurinol.

SUMMARY

The mechanism by which allopurinol decreases urate production involves the inhibition of xanthine oxidase. The active inhibitor, oxipurinol, is metabolically produced by the oxidation of allopurinol as catalyzed by xanthine oxidase and/or aldehyde oxidase. Oxipurinol slowly forms a reversible complex with electronically reduced xanthine oxidase which has a dissociation constant of 5×10^{-10} M. Since, compared to oxipurinol, allopurinol is more efficiently absorbed from the gastrointestinal tract, the production *in vivo* of oxipurinol is more effective than the direct oral administration. Reabsorption of oxipurinol at the urate site of the renal tubules greatly prolongs its biological half-life. The inhibition of xanthine oxidase results in the enhancement of both the reutilization of hypoxanthine and xanthine by conversion of the bases to their nucleotides and the subsequent feedback inhibition of the purine biosynthetic pathway by these nucleotides. These last two processes may provide additional assistance to the inhibition by oxipurinol, since they cause a multiple lowering of the substrate pressure on inhibited xanthine oxidase.

* Catalytic oxidation of NADH by oxipurinol-complexed xanthine oxidase has been demonstrated with ferricyanide serving as the electron acceptor [3]. These results are unfortunately equivocal because ferricyanide will directly reoxidize the Mo (IV) and thereby release the inhibitor.

REFERENCES

1. G. B. Elion, *Ann. rheum. Dis.* **25,** 608 (1966).
2. T. Spector and D. G. Johns, *Biochem. biophys. Res. Commun.* **32,** 1039 (1968).
3. V. Massey, H. Komai, G. Palmer and G. B. Elion, *J. biol. Chem.* **245,** 2837 (1970).
4. D. G. Johns, *Biochem. biophys. Res. Commun.* **31,** 197 (1968).
5. T. Spector and D. G. Johns, *Biochem. biophys. Res. Commun.* **38,** 583 (1969).
6. T. Spector and D. G. Johns, *J. biol. Chem.* **245,** 5079 (1970).
7. R. C. Bray, *The Enzymes* (Ed. P. D. Boyer), Vol. XII, pp. 303–87. Academic Press, New York (1975).
8. J. S. Olson, D. P. Ballou, G. Palmer and V. Massey, *J. biol. Chem.* **249,** 4363 (1974).
9. T. Spector, *Ph.D. Thesis*, Yale University, New Haven (1970).
10. O. H. Lowry, O. A. Bessey and E. J. Crawford, *J. biol. Chem.* **180,** 399 (1949).
11. D. B. Morell, *Biochem. J.* **51,** 657 (1952).
12. R. C. Bray, R. Petterson and A. Ehrenberg, *Biochem. J.* **81,** 178 (1961).
13. D. Edmondson, V. Massey, G. Palmer, L. M. Beacham, III and G. B. Elion, *J. biol. Chem.* **247,** 1597 (1972).
14. R. W. Rundles, *Ann. rheum. Dis.* **25,** 615 (1966).
15. G. B. Elion, T. F. Yü, A. B. Gutman and G. H. Hitchings, *Am. J. Med.* **44,** 69 (1968).
16. G. B. Elion, A. Kovensky, G. H. Hitchings, E. Metz and R. W. Rundles, *Biochem. Pharmac.* **15,** 836 (1966).
17. R. A. Chalmers, H. Krömer, K. Hannelore, J. T. Scott and R. W. E. Watts, *Clin. Sci.* **35,** 353 (1968).
18. D. G. Johns, T. Spector and R. K. Robins, *Biochem. Pharmac.* **18,** 2371 (1969).
19. K. Huh, H. Iwata and I. Yamaoto, *Folia pharmac. jap.* **71,** 175 (1975).
20. T. A. Krenitsky, S. M. Neil, G. B. Elion and G. H. Hitchings, *Archs Biochem. Biophys.* **150,** 585 (1972).
21. R. A. Chalmers, R. Parker, H. A. Simmonds, W. Snedden and R. E. Watts, *Biochem. J.* **112,** 527 (1969).
22. R. L. Miller and D. L. Adamczyk, *Biochem. Pharmac.* **25,** 883 (1976).
23. G. H. Hitchings, *Ann. rheum. Dis.* **25,** 601 (1966).
24. R. W. Rundles, J. B. Wyngaarden, G. H. Hitchings and G. B. Elion, *A. Rev. Pharmac.* **9,** 345 (1969).