Study of Enzyme Inhibitory Activities by Dipyridamole[1]

Tadataka NORO,*[,a] Mariko EHARA,[b] and Akira UENO[a]

School of Pharmaceutical Sciences, University of Shizuoka,[a] 395 Yada, Shizuoka 422, Japan and Boehringer Ingelheim Co., Ltd.,[b] 103 Takada, Yato, Kawanishi, Hyogo 666-01, Japan. *Received October 9, 1989*

Inhibitory activities of Dipyridamole (DPM, 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine) against xanthine oxidase (XO), carbonic anhydorase (CA) and monoamine oxidase (MAO) were studied, *in vitro*. DPM did not inhibit XO and CA, but it strongly inhibited MAO. The type of inhibition by DPM against MAO with respect to benzylamine as a substrate was uncompetitive.

Keywords dipyridamole; inhibitor; xanthine oxidase; carbonic anhydrase; monoamine oxidase; xanthine; *p*-nitrophenyl acetate; benzylamine; uncompetitive inhibition

Introduction

Dipyridamole (DPM)[2] is 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine, and it has been clinically used as a coronary vasodilator,[3] an inhibitor of platelet aggregation,[4] and a drug for the decrease of urinary protein.[5] Some examples of the effect of DPM on enzymes so far reported are inhibition on adenosine deaminase[6] and cyclic adenosine 3′,5′-monophosphate phosphodiesterase,[4a] and stimulation of adenylate cyclase.[4a] We took the interest in the effects of DPM on other enzymes. In this paper we report the inhibitory effects of DPM on xanthine oxidase (XO, EC 1.2.3.2), carbonic anhydrase (CA, EC 4.2.1.1) and monoamine oxidase (MAO, EC 1.4.3.4).

Materials and Methods

Materials DPM was offered by Boehringer Ingelheim Co., Ltd. Xanthine oxidase from cow's milk was obtained from Boehringer Mannheim Co., Ltd. Carbonic anhydrase from bovine erythrocytes and monoamine oxidase from bovine plasma were obtained from Sigma Chemical Company. Xanthine was obtained from ICN Pharmaceuticals Inc. Tween 80 was obtained from Wako Pure Chemical Industries Ltd. Benzylamine and *p*-nitrophenyl acetate were obtained from Tokyo Kasei Kogyo Co., Ltd. Potassium dihydrogenphosphate, disodium hydrogenphosphate and 2-amino-2-hydroxymethyl-1,3-propanediol were obtained from Kanto Chemical Co., Inc.

Substrates The substrate of XO, 0.15 mM xanthine in water, the substrate of CA, 1.5 mM *p*-nitrophenyl acetate (*p*-nitrophenyl acetate (136.0 mg) to be dissolved in acetone (10.0 ml) and to be added to water to 500 ml), and the substrate of MAO, 10 mM benzylamine sulfate (benzylamine (0.54 g) to be dissolved in 2 N H_2SO_4 (2.5 ml), and to be added to water to 500 ml) were prepared immediately before use.

Enzyme Solutions Enzyme solution of XO containing about 0.07 units per ml in 1/15 M phosphate buffer (pH 7.5), enzyme solution of CA containing about 200 units per ml in 15 mM Tris–sulfate buffer (pH 7.6), and enzyme solution of MAO containing about 0.02 units per ml in 0.2 M potassium phosphate buffer (pH 7.4), were prepared immediately before use.

Test Solution DPM was dissolved in ethanol and Tween 80, and diluted with water to give final concentrations of 2% (w/v) for ethanol and 1% (w/v) for Tween 80 in the test solution. At these concentrations of ethanol and Tween 80, enzyme activities were little affected.

Assay of Enzyme Activities The spectrophotometric measurements were carried out with a Hitachi U-1100 spectrophotometer. A blank was prepared in the same way as the assay of each enzyme activity, but the enzyme solution was added to the assay mixture after adding 1 N HCl.

a) XO The XO activities with xanthine as a substrate were measured spectrophotometrically by the method previously described[7] and with the following modification. The test solution (1.0 ml), 1/15 M phosphate buffer (pH 7.5) (2.9 ml) and enzyme solution of XO (0.1 ml) were mixed and preincubated at 37 °C for 15 min. After this, the substrate of XO (2.0 ml) was added to the assay mixture, and the whole was incubated at 37 °C for 30 min. Then, 1 N HCl (1.0 ml) was added to stop the reaction. The absorbance of the reaction mixture was measured spectrophotometrically at 290 nm.

b) CA The CA activities with *p*-nitrophenyl acetate as a substrate were measured spectrophotometrically by the method of Umemura *et al.*[8] with the following modification. The test solution (1.0 ml), 15 mM Tris–sulfate buffer (pH 7.6) (2.9 ml) and the enzyme solution of CA (0.1 ml) were mixed and preincubated at 37 °C for 15 min. After this, the substrate of CA (2.0 ml) was added to the assay mixture, and the whole was incubated at 37 °C for 40 min. Then, 1 N HCl (1.0 ml) was added to stop the reaction. The absorbance of the reaction mixture was measured spectrophotometrically at 348 nm.

c) MAO The MAO activities with benzylamine sulfate as a substrate were measured spectrophotometrically by the method previously described[9] and with the following modification. The test solution (1.0 ml), 0.2 M potassium phosphate buffer (pH 7.4) (3.9 ml) and enzyme solution of MAO (0.1 ml) were mixed, and preincubated at 37 °C for 15 min. After this, the substrate of MAO (1.0 ml) was added to the assay mixture, and the whole was incubated at 37 °C for 120 min. Then, 1 N HCl (1.0 ml) was added to stop the reaction. The absorbance of the reaction mixture was measured spectrophotometrically at 250 nm.

Calculation of the Enzyme Activity **a) XO**[10] unit/mg = $\Delta A_{290\,nm} \times$ ml reaction mixture (7.0 ml)/12.3 × mg enzyme × min (30 min). The molar absorption coefficient for uric acid had been determined in our laboratories to be 12.3×10^3.

b) CA[11] unit/mg = $\Delta A_{348\,nm} \times$ ml reaction mixture (7.0 ml) × 1000/5.4 × mg enzyme × min (40 min). The molar absorption coefficient for *p*-nitrophenol had been determined in our laboratories to be 5.4×10^3.

c) MAO[12] unit/g = $\Delta A_{250\,nm} \times$ ml reaction mixture (7.0 ml)/12.5 × g enzyme × min (120 min). The molar absorption coefficient for benzaldehyde had been determined in our laboratories to be 12.5×10^3.

Estimation of Enzyme Inhibitory Activity Enzyme inhibitory activity was expressed as the percentage of enzyme inhibition in the above assay system, calculated as $(1 - B/A) \times 100$, where A is the activity of the enzyme without test material and B is the activity of the enzyme with test material.

Lineweaver–Burk Plots The Lineweaver–Burk plots[13] for MAO with benzylamine as a substrate were taken under our assay conditions in the absence and in the presence of DPM.

Monitor of DPM by Thin Layer Chromatography (TLC) Silica gel 60 F_{254} (Merck) was employed for TLC, and solvent systems were $CHCl_3$: MeOH = 4:1 (v/v) (*Rf* value of DPM was 0.65), and benzene: acetone = 1:1 (v/v) (*Rf* value of DPM was 0.55). DPM was monitored from the assay mixture.

Results and Discussion

Inhibitory activities of DPM on XO, CA and MAO *in vitro* to be compared with active compounds in our laboratories, are shown in Table I. XO was not inhibited by DPM under concentrations less than 2.0×10^{-4} M. DPM which was monitored by TLC, was not changed by XO in our assay condition. On the other hand, Aoyagi *et al.*[14] reported that DPM inhibited the process to produce methylguanidine from creatinine, and they suggested that DPM might be acting as the radical scavenger for active oxygen, hydroxy radical (·OH). It is known that XO produces the

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TABLE I. Enzyme Inhibitory Activity of Dipyridamole and Related Compounds

Compounds	IC_{50} (M)		
	XO	CA	MAO
Dipyridamol	$>2.0\times10^{-4}$	$>2.0\times10^{-4}$	1.0×10^{-5}
Luteolin[7]	5.9×10^{-7}	—	—
Gallagyldilactone[8]	—	2.2×10^{-7}	—
Ethyl *p*-methoxycinnamate[9]	—	—	6.8×10^{-5}

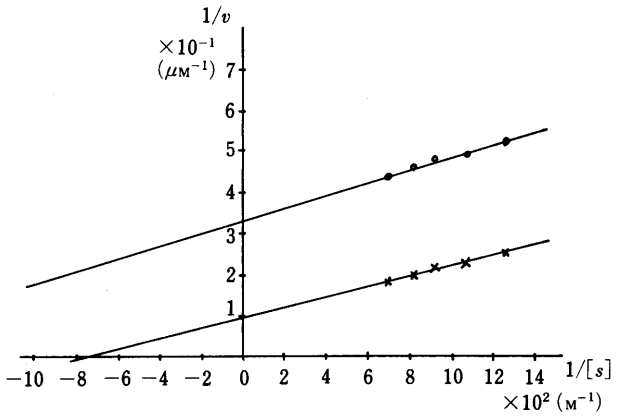

Fig. 1. Inhibitory Effects of DPM on MAO

Lineweaver–Burk plots in the absence (0 M, ---×---) and in the presence (2.0×10^{-5} M, ---○---) of DPM with benzylamine as the substrate. *v*: μM substrate metabolized/g enzyme/min. *s*: substrate.

active oxygen, super oxide anion radical ($\cdot O_2^-$) in the reaction system.[15] However, in our experiment, DPM did not act as the radical scavenger for the active oxygen by XO.

CA was not inhibited by DPM under concentrations less than 2.0×10^{-4} M, and DPM which was monitored on TLC, was not changed by CA in our assay condition, too.

MAO was strongly inhibited by DPM, and IC_{50} (the concentration of 50 percent inhibition) was 1.0×10^{-5} M. On the other hand, DPM which was monitored on TLC, did not show any change by MAO in our assay condition.

Kinetic analysis of inhibition of MAO by DPM is shown by Lineweaver–Burk plots[13] in Fig. 1. The substrate is benzylamine sulfate. MAO is inhibited uncompetitively in our assay condition. K_i value is 1.1×10^{-5} M. DPM does not pass through the blood brain barrier,[16] so it might not work as a psychotropic drug, but further study for the mechanism of MAO inhibition by DPM *in vivo* should be done.

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