PRODUCT INHIBITION OF AMINOPYRINE N-DEMETHYLATION IN ISOLATED HEPATOCYTES

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The product inhibition of aminopyrine (AM) N-demethylation by 4-monomethylaminoantipyrine (MAA) was examined *in vitro* in preparations of isolated rat hepatocytes using gas chromatograph-mass spectrometer (GC-MS). The kinetic study for AM N-demethylation, which was determined by MAA formation, was studied at concentrations from 0.2 to 4.0 mM with or without various concentrations of D₃-MAA. D₃-MAA inhibited AM N-demethylation non-competitively at concentrations of 0.2 to 1.0 mM of AM ($K_i = 1.81$ mM), though it did competitively at concentrations of 1.0 to 4.0 mM of AM ($K_i = 0.81$ mM). From the present observation, it was suggested that the inhibition type of AM N-demethylation by D₃-MAA was dependent on the substrate (AM) concentration.

Keywords — product inhibition; isolated hepatocyte; aminopyrine; GC-MS; mass fragmentography; stable isotope

Aminopyrine (AM) has been widely used as an analgesic and antipyretic drug or a model drug for the investigation of hepatic microsomal mixed function oxidase *in vitro*¹⁻⁶⁾ and *in vivo*.⁷⁻¹¹⁾ The major metabolic route is two sequential N-demethylation to give 4-monomethylaminoantipyrine (MAA) and 4-aminoantipyrine (AA). MAA undergoes oxidation to form 4-formylaminoantipyrine (FAA), which was confirmed by our laboratory.^{6,7)}

As for the N-demethylation of AM, Gram *et al.*¹²⁾ reported that excess AM appeared to inhibit its own dealkylation to AA. Recently, Bast and Noordhoek suggested the product inhibition of AM N-demethylation by its major metabolite, MAA in the *in vivo*¹³⁾ and *in vitro*¹⁴⁾ experiments using rats.

The rate of AM N-demethylation in microsomes is affected markedly by co-factor, *i. e.* a factor in soluble fraction of rat liver,¹⁵⁾ ethylenediaminetetraacetic acid (EDTA),¹⁵⁾ nicotinamide,¹⁶⁾ and semicarbazide.¹⁷⁾ Isolated

hepatocyte system has been available for *in vitro* studies of drug metabolism because its use has the advantage of eliminating the influences of co-factor and many extrahepatic variables.

In the present study, the kinetic study for product inhibition of AM N-demethylation by MAA was performed in the isolated hepatocyte system by using deuterium labeled MAA as an inhibitor (D₃-MAA) and internal standard (D₆-MAA) for mass fragmentography.

MATERIALS AND METHODS

Chemicals — Reagent-grade aminopyrine (AM) and 1-phenyl-3-methyl-5-pyrazolone were purchased from Aldrich Chemical Company, Inc. (Milwaukee, USA). *N*-2-Hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (Hepes), EDTA, bovine serum albumine (fraction V) and dimethyl-D₆-sulfate (99 atom% D) were all from Sigma Chemical Company (ST. Lois, USA). Collagenase was obtained from Boehringer Mannheim GmbH, West Germany. All other chemi-

TABLE I. Structure of Substrate (AM), Metabolite (MAA), Inhibitor (D_3 -MAA) and Internal Standard (D_6 -MAA)

		R ₁	R ₂	R ₃	R ₄	m/z
Substrate	I	CH ₃	CH ₃	CH ₃	CH ₃	231
Product	II	CH ₃	H	CH ₃	CH ₃	217
Inhibitor	III	CH ₃	H	CH ₃	CD ₃	220
Internal standard	IV	CH ₃	H	CD ₃	CD ₃	223

separated by centrifugation, and an aliquot of 5 ml of the supernatant was extracted twice with 20 ml of chloroform. The combined extract was used for metabolite measurement. MAA formed from AM was determined by the method of Goromaru *et al.*¹⁹⁾ using gas chromatograph-mass spectrometer (GC-MS).

RESULTS AND DISCUSSION

The kinetic study for the product inhibition of AM N-demethylation (MAA formation) by MAA was performed in isolated hepatocytes using deuterium labeled MAA (D_3 -MAA) as an inhibitor. The cytochrome P-450 monooxygenase system catalyzes the AM N-demethylation to MAA and formaldehyde. Fig. 1 is a representative mass fragmentogram of the internal standard (D_6 -MAA) and product MAA obtained after aerobic incubation of AM with isolated hepatocytes. The isotope effect must be considered in studies of the metabolism of compounds. In the case of D_3 -MAA, it was confirmed by Goromaru *et al.*¹⁹⁾ that the metabolic behavior of D_3 -MAA was similar to that of MAA. Mass fragmentography makes it possible to detect MAA (m/z 217) and D_6 -MAA (m/z 223) selectively by changing the accelerating voltage continually between 3 kV (for m/z 217) and 2.96 kV (for m/z 223) after m/z 217 is adjusted at 3 kV. Under the condition described above, D_3 -MAA added as an inhibitor does not interfere in the detection of MAA. Thus,

mass fragmentography permitted to examine product inhibition directly using isotope labeled product. Fig. 2 shows the standard curve for MAA. A linear standard curve for MAA was observed (r

FIG. 1. Mass Fragmentograms of Product MAA (m/z 217), Inhibitor D_3 -MAA (m/z 220), and Internal Standard D_6 -MAA (m/z 223)

Conditions: 1.5% OV-17 on Shimalite W (80–100 mesh), 3 mm (i.d.) $\times$ 1 m, glass column, column temp: 170 °C, He gas flow: 30 ml/min, ionizing current: 300 μ A, ionizing energy: 23 eV, separator and ion chamber temp: 250 °C.

FIG. 2. *Standard Curves of MAA* FIG. 3. *Lineweaver-Burk Plots of Isolated Hepatocyte N-Demethylation of AM in the Absence (Control) or Presence (A, B, C) of Inhibitor (D₃-MAA)*

Isolated hepatocytes were incubated in the presence of 0.2 to 4.0 mM of AM for 5 min. The rate of AM N-demethylation was assessed by MAA formation / 10⁶ cells per min. Each point represents mean of four experiments.

Control: without D₃-MAA, A: 2.0 mM D₃-MAA, B: 1.0 mM D₃-MAA, C: 0.5 mM D₃-MAA

= 0.99) by plotting the peak height ratio of m/z 217 to 223 against known concentrations of MAA. The effect of D₃-MAA on the AM N-demethylation in isolated hepatocytes is shown in Fig. 3. The Lineweaver-Burk plots of the rate of MAA formation were all non-linear after incubation with and without D₃-MAA. Isolated hepatocytes without D₃-MAA (the control) gave a low K_m of 0.61 ± 0.21 mM at low AM concentrations and a high K_m of 1.83 ± 0.09 mM at high AM concentrations with corresponding V_{max} values of 3.03 ± 0.21 and 6.25 ± 1.21 nmol MAA 10⁶ cells⁻¹ min⁻¹, respectively. As for the effect of D₃-MAA on AM N-demethylation, at low concentrations from 0.2 to 1.0 mM of AM, D₃-MAA inhibited MAA formation from AM non-competitively, though it did competitively at high concentrations from 1.0 to 4.0 mM of AM. At high concentrations of substrate (AM), K_i value of 0.81 mM can be calculated by plotting the K_m versus inhibitor (D₃-MAA) concentration (Fig. 4). On the other hand, at low concentrations of substrate, K_i value of 1.91 mM was obtained by plotting the inverse of velocity ($1/V$) versus D₃-MAA concentration (Fig. 5). From each K_i value, it was suggested that AM N-

FIG. 4. *Plots for K_m at High Substrate Range versus Inhibitor Concentration*

demethylation is inhibited more strongly by product MAA at high concentrations of AM than at low concentrations. The *in vitro* finding using isolated hepatocytes is closely correlated with the report from Gram *et al.*¹²⁾ that excess AM seems to inhibit its own dealkylation to AA *via* MAA.

As described in the preface, Bast and Noordhoek¹⁴⁾ performed the similar study for the product inhibition during the hepatic microsomal N-demethylation of AM in rats. According to their report, the *in vitro* microsomal N-demethylation of (¹⁴C)-AM, measured by H¹⁴CHO formation, was competitively inhibited by MAA ($K_i = 0.81$ mM). The K_i value was equal to our K_i value at high concentrations of AM. However, they could not determine the K_i value at low concentrations of AM. As for the difference, a number of factors may be responsible: (1) differences in analytical method (¹⁴C-formaldehyde *versus* MAA detection) (2) differences in the enzyme source (microsome *versus* hepatocytes). However, it seems to be the most probable that they mislead the plots at low concentrations of AM. This is the reason why they could obtain neither non-linear plots nor K_i value at low concentrations of AM. In the

literature, non-linear plots for AM N-demethylation were reported by many researchers under the same experimental conditions as theirs.^{2,3,20)} Such non-linear plots suggest the presence of different types of AM N-demethylase as pointed out by Pederson and Aust.^{2,3)} According to their reports, the low K_m component in AM N-demethylase takes part in AM metabolism at low concentrations of AM, though the high K_m component does at high concentrations of AM. The diversity of AM N-demethylase was also observed in the effect of D₃-MAA on AM N-demethylation.

The enzymatic reaction is dependent on the two processes. The first is the binding of substrate to enzyme and the second is the release of product from substrate-enzyme complex. From the present study, it was suggested that the high K_m component was inhibited by MAA at the first process of enzymatic reaction, though the low K_m component was done at second process. The detailed mechanism for the product inhibition of AM is still unknown at this stage. However, the mechanism is worth further exploration.

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REFERENCES

- 1) B. N. LaDu, L. Gaudette, N. Trousof, and B. B. Brodie: Enzymatic dealkylation of aminopyrine (pyramidon) and other alkylamine, *J. Biol. Chem.*, **214**, 741–751 (1955).
- 2) T. C. Pederson and S. D. Aust: Aminopyrine demethylase kinetic evidence for multiple microsomal activities, *Biochem. Pharmacol.*, **19**, 2221–2230 (1970).
- 3) S. D. Aust and J. B. Stevens: Aminopyrine demethylase-Multiplicity shown by dieldrin and DDT inhibition, *Biochem. Pharmacol.*, **20**, 1061–1069 (1971).
- 4) A. Bast and J. Noordhoek: Evaluation and comparison of colorimetric, radiometric and high performance liquid chromatographic assays for aminopyrine N-demethylation by rat liver microsomes, *J. Pharm. Pharmacol.*, **33**, 14–18 (1981).
- 5) K. Inoue, K. Fujimori, M. Mizokami, M. Sunouchi, A. Takanaka, and Y. Omori: Simultaneous determination

FIG. 5. Plots for $1/V$ at Low Substrate Range *versus* Inhibitor Concentration

A: 0.2 mM AM, B: 0.5 mM AM, C: 1.0 mM AM.

- of aminopyrine hydroxylation and aminopyrine N-demethylation in liver microsomes by high performance liquid chromatography, *J. Chromatogr.*, **274**, 201–208 (1983).
- 6) K. Matsuyama, A. Noda, S. Goto, T. Tanaka, and S. Iguchi: Influence of phenobarbital and 3-methylcholanthrene on the metabolism of aminopyrine in isolated hepatocyte system, *J. Pharmacobio-Dyn.*, **6**, 821–828 (1983).
 - 7) S. Iguchi, T. Goromaru, and A. Noda: A new metabolite of aminopyrine (aminophenazone) in man, 4-formylaminoantipyrine, *Chem. Pharm. Bull.*, **23**, 932–934 (1975).
 - 8) T. Goromaru, A. Noda, K. Matsuyama, and S. Iguchi: Metabolism and excretion of aminopyrine in man, *Chem. Pharm. Bull.*, **24**, 1376–1383 (1976).
 - 9) G. F. Lockwood and J. B. Houston: Influence of phenobarbitone on disposition of aminopyrine and its metabolites in rat, *J. Pharm. Pharmacol.*, **32**, 619–623 (1980).
 - 10) J. B. Houston, G. F. Lockwood, and G. Taylor: Aminopyrine demethylation kinetics, use of metabolite exhalation rates as an index of enhanced mixed-function oxidase activity *in vivo*, *Drug Metab. Dispos.*, **9**, 449–455 (1981).
 - 11) K. Matsuyama, S. Takenaka, A. Noda, and S. Iguchi: Effect of temperature on the metabolism of aminopyrine, *Chem. Pharm. Bull.*, **31**, 1404–1407 (1983).
 - 12) T. E. Gram, J. T. Wilson, and J. R. Fouts: Some characteristics of hepatic microsomal system which metabolize aminopyrine in the rat and rabbit, *J. Pharmacol. Exp. Ther.*, **159**, 172–181 (1968).
 - 13) A. Bast, L. W. C. Scheefhals, and J. Noordhoek: Dose-dependent kinetics of aminopyrine metabolism in the rat caused by product inhibition and determined by capillary GLC, *Pharmacology*, **25**, 130–137 (1982).
 - 14) A. Bast and J. Noordhoek: Product inhibition during the hepatic microsomal N-demethylation of aminopyrine in the rat, *Biochem. Pharmacol.*, **30**, 19–24 (1981).
 - 15) T. Kamataki, N. Ozawa, M. Kitada, H. Kitagawa, and R. Sato: The occurrence of an inhibitor of lipid peroxidation in rat liver soluble fraction and its effect on microsomal drug oxidations, *Biochem. Pharmacol.*, **23**, 2485–2490 (1974).
 - 16) J. B. Schenkman, J. A. Ball, and R. W. Estabrook: On the use of nicotinamide in assays for microsomal mixed-function oxidase activity, *Biochem. Pharmacol.*, **16**, 1071–1081 (1967).
 - 17) T. Matsubara, A. Touchi, and Y. Tochino: Hepatic aminopyrine N-demethylase system: Further studies of assay procedure, *Jpn. J. Pharmacol.*, **27**, 127–136 (1977).
 - 18) P. Moldeus and S. Orrenius: Isolation and use of liver cells, *Methods Enzymol.*, **52**, 60–71 (1978).
 - 19) T. Goromaru, K. Matsuyama, A. Noda, and S. Iguchi: The measurement of plasma concentration of aminopyrine and its metabolite in man, *Chem. Pharm. Bull.*, **26**, 33–37 (1978).
 - 20) A. N. Kotake: Direct measurement of aminopyrine N-demethylase and antipyrine hydroxylase activity in a monolayer rat primary isolated hepatocyte system, *Biochem. Pharmacol.*, **30**, 2473–2479 (1981).