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SHORT COMMUNICATIONS

5-Hydroxypyrazinamide, a human metabolite of pyrazinamide

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The *in vivo* metabolic pathways of pyrazinamide have been studied in humans [1]. Two of the three major metabolites of pyrazinamide have already been identified as pyrazinoic acid and 5-hydroxypyrazinoic acid [2]. We identified the third metabolite as 5-hydroxypyrazinamide in this study.

Methods

Wavelength scanning was performed using a U-3200 Spectrophotometer (Hitachi Ltd., Tokyo, Japan). The high performance liquid chromatographic system consisted of an LC-5A high performance liquid chromatograph (Shimadzu Co., Kyoto, Japan), an SPD-2A UV spectrophotometric detector (Shimadzu Co.) and a C-R1B chromatopac recorder (Shimadzu Co.). The column was a μ-Bondapak C18 (30 cm × 3.9 mm) (Waters Associates Milford, MA, U.S.A.). The mobile phase consisted of 0.02 M KH_2PO_4 with a pH of 2.2 and a flow rate of 1.8 ml/min. The detection wavelength was 254 nm. Mass spectra were obtained as previously described [3]. Urine was collected from two healthy men for 24 hr after they had taken 3 g of pyrazinamide and it was stored at −20°. Pyrazinamide and its metabolites in the urine were determined as previously described [4]. The unidentified metabolite described in the Results and Discussion was lyophilized for identification.

Chemicals. Pyrazinamide was provided by the Sankyo Co., Ltd. (Tokyo, Japan). 5-Hydroxypyrazinamide was prepared from pyrazinamide as previously described [3]. Xanthine oxidase from buttermilk was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were obtained from the Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Results and discussion

Pyrazinamide, the unidentified metabolite (Compound II), 5-hydroxypyrazinoic acid and pyrazinoic acid were eluted in this order by the chromatographic procedure as previously described [4]. Table 1 shows that both the unidentified metabolite and 5-hydroxypyrazinamide have the same maximum absorption spectrum at 256 nm and the same retention time at pH 2.2, pH 3.65 and pH 5.1 of the mobile phase, as determined by high performance liquid chromatography described in Methods (data not shown except for those at pH 2.2). Mass spectral analysis of the unidentified metabolite and 5-hydroxypyrazinamide showed the same fragmentation pattern: a molecular ion at 139 m/z, loss of NH_2 (ion at 123 m/z), and loss of $CONH_2$ (ion at 95 m/z). This fragmentation pattern was identical with the one for 5-hydroxypyrazinamide described by Pitre *et al.* [5]. During the investigation of the *in vitro* metabolism of pyrazinamide, Pitre *et al.* [5] found that 5-hydroxypyrazinamide was produced from pyrazinamide by xanthine oxidase in the rat subcellular fraction. This metabolite has never been identified *in vivo* before but was detected as the unidentified metabolite (compound II) in urine by Auscher *et al.* [4]. We confirmed the same metabolite on chromatography and identified it as 5-hydroxypyrazinamide. Weiner and Tinker [2] suggested that the first metabolic attack *in vivo* on pyrazinamide is deamidation, resulting in the production of pyrazinoic acid which is oxidized subsequently to 5-hydroxypyrazinoic acid by xanthine oxidase. Our results suggest the operation of an alternative pathway by which pyrazinamide is directly oxidized by xanthine oxidase, followed by microsomal deamidation in the liver to form 5-hydroxypyrazinoic acid. This pathway *in vivo* was the same as that described in the *in vitro* studies [5]. The identification of the unknown metabolite (compound II) as 5-hydroxypyrazinamide led us to question why a large amount of 5-hydroxypyrazinamide was found in the urine of patients with xanthinuria who were regarded as lacking xanthine oxidase activity [4]. Presumably, aldehyde oxidase is responsible for the direct oxidation of pyrazinamide as it appears to be for the formation of oxypurinol from allopurinol in a xanthinuric individual [6].

In summary, two major metabolites of pyrazinamide in humans are pyrazinoic acid and 5-hydroxypyrazinoic acid. We report here the identification of the third major urinary metabolite, 5-hydroxypyrazinamide, which was identified by u.v. absorption, high performance liquid chromatography, and mass spectral analysis.

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Table 1. Wavelength of maximum absorbance and retention time

Compounds	Maximum absorbance (nm) (pH 2.0)	Retention time (pH 2.2, flow rate 1.8 ml/min)
Pyrazinamide	269	9.48
Pyrazinoic acid	268	8.36
5-Hydroxypyrazinoic acid	255	5.17
Unidentified compound (Compound II)	256	5.88
5-Hydroxypyrazinamide	256	5.88

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Effects on dihydrofolate reductase of methotrexate metabolites and intracellular folates formed following methotrexate exposure of human breast cancer cells

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Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase; EC 1.5.1.3) is the primary intracellular enzyme responsible for maintaining a pool of reduced folates. These folates serve as coenzymes in a number of one-carbon transfer reactions for the intracellular production of purines, thymidylate, and certain amino acids. The mechanism of action of methotrexate (MTX), a clinically important drug in the treatment of a variety of neoplasms, appears to be multifaceted. MTX is capable of potent inhibition of mammalian DHFR with a binding affinity estimated to lie between 1 nM and 1 pM [1]. Exposure of human breast cancer cells to MTX has been shown to lead to a rapid intracellular accumulation of dihydrofolate (FH_2) with the subsequent appearance of formyl-dihydrofolate (formyl-FH_2) [2]. The latter compound is presumed to be formed via a direct formylation of FH_2 as the amount of these two compounds follows an inverse relationship with a constant sum. This compound has been shown to be a weak substrate for chicken liver DHFR [3]. Its effect on other folate-requiring enzymes has not been reported. MTX undergoes the process of intracellular polyglutamation in breast cells with up to a total of five glutamate residues appended to the para-aminobenzoate moiety of the parent compound. This process imparts several peculiar properties to the MTX polyglutamates (PGs) that parallel those of the naturally occurring folate PGs and include an intracellular half-life that is inversely proportional to the PG tail length and a higher affinity (and, therefore, greater inhibitory potential) for many of the folate-requiring enzymes, including AICAR transformylase and thymidylate synthase [4, 5].

Several investigators have shown that MTX is metabolized in human liver to its 7-hydroxy form [6–8]. This form has also been found to undergo intracellular polyglutamation to metabolites that have been reported to have an enhanced affinity for DHFR [9, 10].

It is the purpose of this report to measure the interactions and potential inhibitory effects on human DHFR—the target enzyme for the parent compound—of the substances that accumulate in a cell following MTX exposure.

Materials and methods

Methotrexate and methotrexate-Glu_{1-4}, as well as folic acid-Glu_5, were obtained from the Drug Synthesis and Development Branch of the National Cancer Institute (Bethesda, MD). 7-Hydroxy-MTX and 7-hydroxy-MTX-Glu_4 were gifts from Drs. I. David Goldman and Richard Seither (Medical College of Virginia, Richmond, VA). Folic acid and sodium dithionite were purchased from the Sigma Chemical Co. (St. Louis, MO). 10-Formyl-FH_2 and 10-formyl-FH_2-Glu_5 were prepared by chemically formylating folic acid or folic acid-Glu_5 using the method of Blakely [11] followed by reduction to the final product using sodium dithionite [3].

Human DHFR (sp. act. 27.3 μmol/min/mg at 37°) was purified from cultured MCF-7 breast cancer cells as previously described [12].

Measurement of DHFR catalyzing activity. The catalytic activity of human DHFR was followed spectrophotometrically according to the method of Bertino and Fischer [13]. Each 1-ml reaction cuvette contained 1 pmol of enzyme, 2 μmol of NADPH, and various concentrations of substrate/inhibitors in 160 mM KCl and 160 mM Tris, pH 7.4. After a 10-min temperature equilibration period (37°), the reaction was initiated with the addition of 0.1 μmol of FH_2. Reactions were followed for 10 min, and the reaction velocities, were determined using an extinction coefficient for the reaction of 12×10^3. Formyl FH_2 was assayed as a substrate for DHFR using an identical method but using an extinction coefficient for the reaction of 21×10^3 at 270 nm (unpublished observations). All kinetic constants were calculated using standard Lineweaver–Burk plots. MTX and MTX-PGs were treated as slow tight-binding inhibitors. The calculation of kinetic constants was simplified by preincubation of the enzyme with the inhibitor to measure the effects of the inhibitors on the catalytic reaction at a time of steady-state interaction with the enzyme, i.e. after the time-dependent slow-binding phase of the inhibitor–enzyme interaction.

Results

Substrate kinetics. Table 1 illustrates the kinetic measurements for FH_2 mono- and pentaglutamates when used as substrates for the reaction catalyzed by human reductase. The Michaelis–Menten constants (K_m) for each substrate in either parent or polyglutamated form were equivalent (1.1. to 1.5 μM).

Inhibition of human DHFR. The inhibition of DHFR by MTX, MTX-PGs, and its metabolites 7-OH-MTX and 7-OH-MTX-Glu_4 is shown in Table 2. For each inhibitor, the K_i value was determined using both the mono- and polyglutamated FH_2 as the substrate for the reaction. Formyl-FH_2 and 7-OH-MTX acted as competitive inhibitors. MTX and MTX-PGs were considered tight-binding inhibitors. In parallel with the effect of polyglutamation on the affinity of the folate co-substrates for DHFR, the polyglutamation of MTX was found to have little effect on the inhibitory potential of this compound. With respect to FH_2-monoglutamate, the higher MTX-PGs were less than 2-fold more potent than the parent inhibitor. This minimal increase in potency of the MTX-PGs was not apparent when FH_2-Glu_5 was used as the substrate for the reaction. By contrast, the inhibitory potential of 7-OH-MTX ($K_i = 2.6–7.4 \times 10^{-8}$ M) was increased by the tetraglutamate such that this compound was 10-fold more potent than the parent metabolite when competing with the monoglutamated FH_2 substrate; like MTX, this increment in