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In vitro metabolism of a thrombin inhibitor and quantitation of metabolically generated cyanide

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Abstract

During the metabolic characterization of compound **I**, 2-{6-cyano-3-[(2,2-difluoro-2-pyridin-2-ylethyl)amino]-2-oxopyrazin-1(2H)-yl]-*N*-[(3-fluoropyridin-2-yl)methyl]acetamide, evidence was obtained for extensive oxidative bioactivation of the pyrazinone ring system and some of the resulting metabolites were apparently devoid of the cyano moiety. Two assays, a spectrophotometric and a high-pressure liquid chromatography (HPLC) pre-column derivatization method, were evaluated for their ability to detect and quantify cyanide that is metabolically generated from liver microsomal incubations. When **I** was incubated (45 μM) in the presence of NADPH-fortified human liver microsomes for 2 h, 7.5 μM of cyanide was detected using the spectrophotometric assay and 8.9 μM was measured using the HPLC methodology. Overall, the results from the two assays appeared to agree reasonably well with each other. However, the HPLC assay was the preferred method for the evaluation of cyanide formation in vitro due to its sensitivity, reliability, and ease of use.

Keywords: Cyanide; Metabolism; Bioactivation

1. Introduction

As part of drug discovery efforts, the metabolism of substituted pyrazinone ring containing thrombin inhibitors has been extensively studied. In these studies, the in vitro metabolism of various analogs with different substituents at the 6-position of the pyrazinone ring was evaluated [1]. There was evidence of extensive metabolic activation of an analog with a methyl group at the 6-position of the pyrazinone ring to form a reactive intermediate which underwent substantial covalent protein binding [2]. However, when a chloro group was introduced at the 6-position of the pyrazinone ring, metabolites were formed which resulted in the loss of HCl. Analogously, compound **I** (2-{6-cyano-3-[(2,2-difluoro-2-pyridin-2-ylethyl)amino]-2-oxopyrazin-1(2H)-yl]-*N*-[(3-fluoropyridin-2-yl)methyl]acetamide) has a cyano group at the 6-position of the pyrazinone ring and upon metabolic activation, this compound could potentially form a chemically reactive intermediate that would result in the formation of hydrogen cyanide (HCN) which, in turn, may result in cyanide toxicity.

There are many cyano-containing compounds that are found both in industrial chemicals and natural products. They are widely used in the chemical industry as synthetic intermediates, in the production of synthetic rubber, in fertilizer,

Abbreviations: CAN, acetonitrile; Da, daltons; ESI, electrospray ionization; Compound **I**, 2-{6-cyano-3-[(2,2-difluoro-2-pyridin-2-ylethyl)amino]-2-oxopyrazin-1(2H)-yl]-*N*-[(3-fluoropyridin-2-yl)methyl]acetamide; CID, collision induced dissociation; GSH, glutathione; LC, liquid chromatography; KCN, potassium cyanide; NDA, 2,3-naphthalene dicarboxyaldehyde; HCN, hydrogen cyanide; LOQ, limit-of-quantitation; HPLC, high-pressure liquid chromatography; TIC, total ion chromatogram

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and in the extraction of gold and silver [3]. Cyano-containing substances also are found domestically in the form of rodenticide, tobacco smoke, or in the seeds of common fruits such as apples, peaches, plums, apricots, cherries, and almonds [4–6]. Exposure to cyanide also may come from the administration of drugs. The putative antineoplastic agent amygdalin and the potent antihypertensive drug nitroprusside have been shown to produce measurable levels of cyanide when administered to patients [7]. Cyanide is a chemical asphyxiant which renders the body incapable of utilizing an adequate supply of oxygen, primarily by inhibiting the cytochrome oxidase system [8]. Hydrogen cyanide is highly toxic and minute amounts can produce toxicological effects in humans [9–11]. The minimum lethal oral dose of cyanide in humans is approximately 50 mg, whereas inhalation of 0.13 mg/L will cause death [10]. Chronic low-level exposure to cyanide produces various pathologic signs and symptoms including neuropathy, and ultra-structural changes of heart muscle leading to cardiovascular toxicity [9–11]. Thyroid function also is affected due to enhanced formation of thiocyanate, which can block uptake of iodine by the thyroid gland resulting in a goiter [10,11].

Since exposure to cyanide, even at low levels, has serious toxicological consequences, the assessment of its formation from cyano-containing drug candidates is important. In order to quantify cyanide formed via bioactivation of **I** in human liver microsomes, two methods for measuring cyanide were adapted and evaluated. These two different techniques for evaluating the potential of metabolic cyanide generation in vitro will be compared and discussed.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of high-pressure liquid chromatography (HPLC) or analytical grade. Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA). Compound **I** and two of its metabolites (**M1** and **M4**) were prepared at Merck Research Labs (West Point, PA). Glucose 6-phosphate, $NADP^+$, NADPH, glucose 6-phosphate dehydrogenase, taurine and GSH (reduced) were obtained from Sigma Chemical Co. (St. Louis, MO). 2,3-Naphthalene dicarboxyaldehyde (NDA), potassium cyanide, pyridine, sodium arsenite, anthranilic acid and bromine were purchased from Aldrich (Milwaukee, WI). All other chemicals were obtained from commercial sources and were of the highest purity available. Pooled human liver microsomes were purchased from Human Biologics Inc. (Scottsdale, AZ).

The buffer for the microsomal incubations consisted of 100 mM potassium phosphate and 6 mM $MgCl_2$ (pH 7.4). The NDA reagent was prepared by combining an ethanolic solution of NDA (2 mM) with a borate-phosphate buffer (27 mM sodium borate and 47 mM potassium phosphate, pH 8.0) in a 1:1 ratio. The taurine solution (50 mM) was prepared in borate-phosphate buffer (pH 8.0). Both the NDA and taurine solutions were freshly prepared, and the incubation buffer could be stored at 5 °C for up to a month.

2.2. HPLC assay for cyanide generation

The HPLC assay for the determination of cyanide concentration was modified based upon previously published work [12]. The HPLC system included a Hewlett Packard HP 1100 ChemStation with quaternary pump, autosampler, and fluorescence detector (Wilmington, Delaware, USA). A Waters Symmetry C18 column (15 cm × 3.9 mm, 5 μm)(Milford, MA, USA) was used. The mobile phase consisted of a mixture of acetonitrile and 0.1% TFA in water (28:72, v/v, pre-mixed), and was delivered isocratically at a flow rate of 1 mL/min. The fluorescence detector was set at an excitation wavelength of 418 nm and an emission wavelength of 460 nm. The injection volume was 20 μL and the total assay time was 8 min. The elution time for the fluorescent cyanide derivative was approximately 5 min with no interference peak nearby.

A stock solution of potassium cyanide was prepared in 0.1N potassium hydroxide and then was serially diluted in deionized water to make a 10-point calibration curve over the concentration range of 5–500 nM. An aliquot (800 μL) from each standard and microsomal incubate was prepared for analysis by the addition of 100 μL of 50 mM taurine buffer and 100 μL of 2 mM NDA in ethanol: borate buffer (1:1). The samples were vortex mixed and covered with aluminum foil to prevent exposure to direct light and incubated at room temperature for 15 min. The samples then were centrifuged and the supernatant was analyzed by HPLC. The linearity of each standard curve was confirmed by plotting the analyte peak area versus nominal concentration and performing linear regression analysis. The accuracy and precision of the method were evaluated using replicate injections of standards. The limit-of-quantitation (LOQ) was defined as the lowest standard concentration which afforded a reasonable degree of precision (<15% coefficient-of-variation) and accuracy (<20% error from nominal concentration). Moreover, the specificity of the assay was assessed using control samples of liver microsomal protein without cyanide.

Compound **I** (45 μM) was incubated ($n = 5$) with pooled human liver microsomes (1 mg/mL protein) in a shaking water bath at 37 °C for 2 h. The reactions were initiated with the addition of 1 mM NADPH. Following incubation, the reactions were terminated by the addition of 100 μL of 50 mM taurine in the buffer and 1.5 mL of 2 mM NDA in ethanol: borate buffer (1:1) and the samples were processed for analysis using the same procedure used for the standards (described above). Subsequently, control experiments were conducted to check the recovery of cyanide from the microsomal incubation. A known quantity of KCN was added to the microsomal mixture (microsomal protein, phosphate buffer, NADPH) and incubated at 37 °C for 2 h and then assayed for cyanide concentration (as described above). The percent