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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

recovery was estimated by comparing the assayed value to the known amount of cyanide added to the incubation.

2.3. Spectrophotometric assay for the determination of cyanide generation

For the purpose of constructing a calibration curve, hydrogen cyanide standards were prepared using the following procedure. A few milliliters of 5 M sulfuric acid were added to a bubbler flask containing 5 mL of standard potassium cyanide solution (10 μg/mL). The flask was connected to a midget impinger containing a 2 mM sodium hydroxide solution. Nitrogen was bubbled through the system and into the sodium hydroxide solution for 30 min. An aliquot of the absorbed solution was transferred into a 10 mL volumetric flask. An aliquot (0.3 mL) of a saturated aqueous bromine solution was added to each sample and the mixture was allowed to stand for 1 min to ensure complete bromination. The excess bromine was decolorized using a sodium arsenite solution (1.5%, m/v). An aliquot (0.4 mL) of pyridine reagent (3 mL of concentrated HCl, 18 mL of pyridine and 12 mL of water) was then added followed by 1 mL of anthranilic acid solution (0.1%, m/v). The mixture was allowed to stand for 10 min for complete color development and the resulting solution was diluted to volume (10 mL) using a dilute sodium hydroxide solution to maintain a pH of approximately 7.2. The absorbance was immediately measured at 400 nm using a Beckman DU-600 UV spectrophotometer using disposable methacrylate cuvettes (Sarstedt, Germany) having a 1 cm path length and a standard curve was generated.

Subsequently, compound **I** (45 μM) was incubated with pooled human liver microsomes (1 mg/mL protein) at 37 °C for 2 h in a 25 mL midget bubbler bottle in a final volume of 5 mL. The reaction was initiated with the addition of 1 mM NADPH. The bubbler bottle was fitted with a stopper attached to a sintered glass filter having a porosity of 145–175 μm (Aldrich, Milwaukee, WI). A length of Tygon® tubing (approximately 20 cm) was connected the bubbler to the impinger while the inlet of the bubbler was capped off during incubation. Following the incubation, the inlet was opened to allow nitrogen to bubble though the system for 2 h to displace any metabolically generated cyanide from the incubation matrix. Nitrogen gas exited through the outlet of the bubbler that was attached to a 35-mL midget impinger containing approximately 2 mM NaOH to trap the cyanide. The nitrogen flow was adjusted to optimum levels to prevent excessive frothing. Aliquots of the sodium hydroxide solution were then taken and analyzed for the cyanide content using the above stated colorimetric assay. A control experiment was conducted to check the recovery of cyanide from the microsomal matrix. To a sealed impinger was added a known quantity of KCN followed by a few drops of sulfuric acid. To this mixture 5 mL of microsomal mixture (containing microsomes, phosphate buffer, NADPH, etc.) was injected and incubated for 2 h at 37 °C. Process previously described was followed to displace, trap and derivatize the cyanide from the incubation mixture. Percent recovery was calculated by comparing the amount detected by the analytical assay to the original amount introduced into the impinger.

2.4. Metabolite profiling and identification

The LC–MS system used for the metabolite profiling and identification consisted of a Perkin-Elmer SYS-S200 autosampler (Perkin-Elmer Corp., Norwalk, CT) and a Rheos HPLC pump (Flux Instruments, Switzerland) attached to a BDS Hypersil C18 (2.1 mm × 150 mm, 5 μm) column (Keystone Scientific, Bellfonte, PA). The mobile phase was delivered at 0.2 mL/min and began at 95% solvent A (0.1% formic acid) and increased linearly to 90% solvent B (acetonitrile) in 18 min. The effluent was introduced into an LCQ ion-trap mass spectrometer (Thermoelectron, San Jose, CA) equipped with an electrospray ionization (ESI) source. The instrument was operated at a 5 kV potential in the positive ionization mode and the capillary temperature was maintained at approximately 200 °C.

The metabolism of compound **I** (2 μM) was characterized by incubating the drug with human liver microsomes (1 mg/mL protein) in the presence of NADPH (1 mM) at 37 °C for 60 min. Incubations in the absence of NADPH, or using heat-inactivated (boiled) human liver microsomes, served as controls. Selected incubations were supplemented with GSH (5 mM) to trap electrophilic drug metabolites as glutathione conjugates. The incubations were terminated by the addition of two-volumes of acetonitrile, followed by vortexing and centrifugation to pellet the precipitated protein. The supernatant was evaporated and reconstituted in mobile phase and analyzed by LC–MS/MS.

3. Results

3.1. In vitro metabolism of compound *I*

Compound **I** is structurally similar to analogs whose metabolism has already been published [2,13]. The analogs differed primarily in substitution at the 6-position of the pyrazinone ring and they all underwent metabolic activation to yield metabolites arising from oxidation and rearrangement of the pyrazinone moiety. When **I** was incubated in human liver microsomes supplemented with glutathione, a glutathione adduct (**M1**) and a cys–gly adduct (**M2**) were identified by LC–MS/MS (Fig. 1). Based on the total-ion chromatogram, only 5% of the parent remained after the two hours of incubation which indicated extensive metabolism (data not shown). The major metabolic pathway (based on total ion intensity) appeared to be oxidation of the pyrazinone ring resulting in two metabolites (**M3** and **M4**) which were apparently devoid of the cyano moiety. These metabolites had the same molecular weight, LC–MS/MS fragmentation pattern and HPLC retention time as those of the chloro-containing analog [13]. Another metabolite

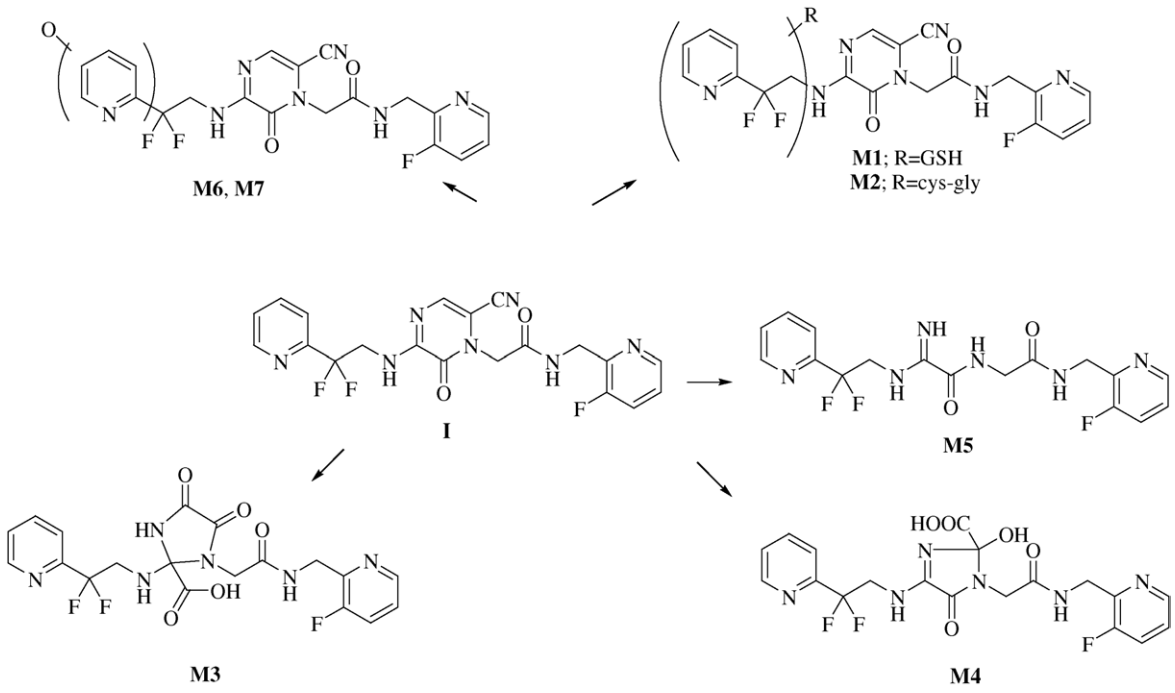

Fig. 1. Proposed microsomal metabolism of compound **I**.

having *m*/*z* 395 (**M5**) is formed via further degradation of the two metabolites **M3** and **M4**. Two other oxidative metabolites, **M6** and **M7**, also were identified. Based on the fragmentation pattern obtained from LC–MS/MS analysis of metabolites **M6** and **M7**, oxidation most likely occurred on the 2,2-difluoro-2-pyridyl ring. Since the in vitro metabolic profile of **I** contained metabolites devoid of the cyano-moiety (**M3** and **M4**), it was deduced that the cyano group was lost as part of the metabolic activation and thus two analytical methods were adapted to quantify the extent of cyanide generation by oxidative bioactivation.

3.2. HPLC assay for quantitation of cyanide generation in liver microsomal incubates

Representative chromatograms of control microsomal incubations (in the absence of compound **I**) and incubates which contained 45 μM of compound **I** are shown in Fig. 2. The specificity of the assay was demonstrated by the lack of endogenous interference peaks in the control incubates at the retention time of the fluorescent cyanide derivative. The assay was found to be linear ($r^2 > 0.99$) over a cyanide concentration range of 5–500 nM and the typical equation describing the standard line was $Y = 3.0774X - 12.176$. Replicate injections ($n = 9$) of a 5 nM cyanide standard demonstrated reasonable accuracy (mean assayed value was 5.23 ± 0.51 nM) and precision (%CV = 9.8%) and was considered as the LOQ for the assay. Recovery experiments, with and without the presence of liver microsomes, were conducted to evaluate the efficiency of the pre-column derivatization procedure. In the presence of liver microsomal protein, approximately 63%

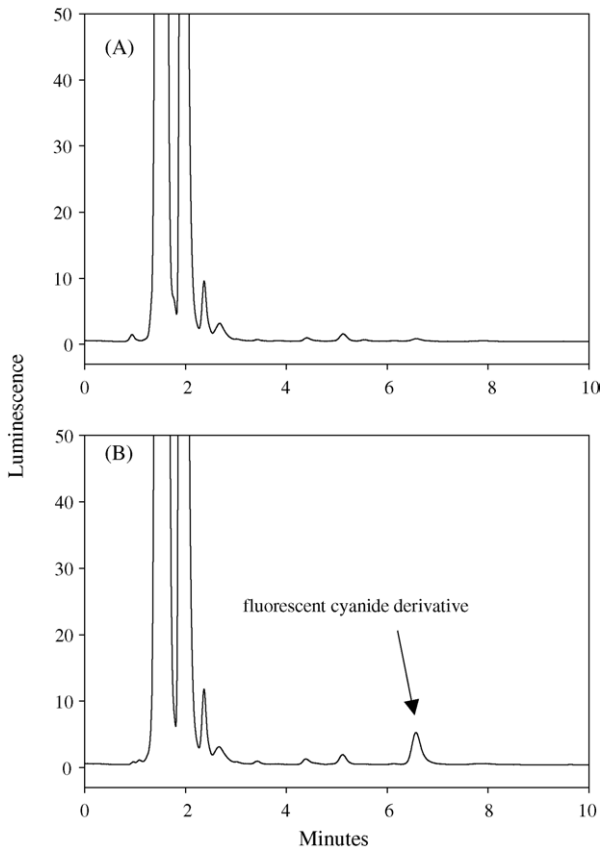

Fig. 2. Representative fluorescence chromatograms of liver microsomal incubations of: (A) control incubates without compound **I** and (B) incubates which contained 45 μM of compound **I** showing the presence of derivatized cyanide.

of the cyanide was recovered, relative to standards devoid of microsomes which suggested that hydrogen cyanide may have been trapped by the components of the microsomal incubation medium or is further metabolized (data not shown). To compensate for this, a correction factor was used to allow for the accurate quantitation of cyanide in the liver microsomal incubates. When **I** (45 μM) was incubated in the presence of human liver microsomes for 2 h, 8.9 ± 3.3 μM of cyanide was detected using the HPLC assay, which represented approximately 20% of the initial molar amount of **I**.

3.3. Spectrophotometric assay for quantitation of cyanide generation in liver microsomal incubates

A standard curve for hydrogen cyanide was linear ($r^2 > 0.99$) over a cyanide concentration range of 0.5–40 μM and the typical equation describing the standard line was $Y = 0.1442X + 6.978$. As part of recovery and trapping efficiency experiments, approximately 50% of the hydrogen cyanide was recovered (data not shown). Similar to the HPLC assay, a correction factor was used to allow for the accurate quantitation of cyanide in the liver microsomal incubates. The spectrophotometric analysis had to be carried out immediately due to the instability of the colorimetric complex. Furthermore, the same derivatization time has to be used for both the standards and the actual in vitro samples making the timing of sample analysis very important. Control incubations in the absence of **I** resulted in absorbance values similar to those of the background values, which was indicative of assay specificity. When **I** (45 μM) was incubated in NADPH-fortified pooled human liver microsomes for 2 h, significant levels of hydrogen cyanide were detected. After correcting for trapping efficiency, hydrogen cyanide was generated (7.5 μM) from the microsomal incubations and accounted for 17% of the initial molar amount of **I**. This result from the spectrophotometric analysis was comparable to the value obtained using the HPLC assay.

4. Discussion

The potential for a drug candidate to liberate cyanide in vivo is a potential safety concern during drug discovery and development. Sometimes, the use of cyanide-generating substances is of medical necessity, as in the case of nitroprusside, a potent antihypertensive. Cyanide formation is a major adverse effect of nitroprusside in humans and toxic blood levels of cyanide limits the infusion rate of this drug [10]. The controversial anti-cancer drug amygdalin has been shown to produce substantial elevations in blood cyanide levels in a small population of patients [7]. As the discovery and development of curative or life-prolonging drugs continues, cyano-containing compounds are being encountered more frequently. If a compound was shown to release substantial amounts of cyanide in vitro, its consideration for drug candidacy may be discontinued early in the discovery process. Thus, the characterization of the amount of cyanide formed and the type of cyanide-generating structures becomes an important part of drug development and research and as such, a reliable, cost-effective, and time efficient in vitro model to assess the potential for cyanide generation by oxidative bioactivation could be a useful tool.

The measurement of cyanide in biological samples by ultraviolet and spectrophotometric methods has been reported previously [14–18]. However, these methods lacked the sensitivity to detect cyanide at the nanomolar level. More recently a chromatographic cyanide assay was developed, using a pre-column fluorescence derivatization technique, and has been successfully used for in vivo toxicological applications [12]. This assay has been adapted to demonstrate its usefulness in detecting metabolically generated cyanide in liver microsomal incubates. The chromatographic assay for the determination of in vitro cyanide generation included four steps: (1) in vitro liver microsomal incubation, (2) derivatization to form a strongly fluorescent derivative, (3) HPLC separation, and (4) fluorescence detection of the cyanide derivative without further sample preparation. Alternatively, a spectrophotometric method utilizing Konig's reaction [15,17,19] was used to detect and quantify the released cyanide in microsomal incubates. Hydrogen cyanide was generated by the reaction of sulfuric acid with potassium cyanide to generate a standard curve. The liberated hydrogen cyanide was efficiently trapped in a sodium hydroxide solution by bubbling nitrogen gas through the solution. The sodium hydroxide solution converted the hydrogen cyanide into sodium cyanide which then reacted with bromine to yield cyanogen bromide. The cyanogen bromide was then treated with pyridine to form glutaconic aldehyde which upon treatment with p-aminobenzoic acid yields a yellow polymethine dye which was monitored spectrophotometrically [19].

When **I** was incubated in NADPH-fortified human liver microsomes supplemented with glutathione, a glutathione adduct (**M1**) and a cys–gly adduct (**M2**) were identified by LC–MS/MS, although the major metabolic pathway appeared to be oxidation of the pyrazinone ring. A mechanism was proposed (Fig. 3) in which the pyrazinone ring undergoes epoxidation followed by ring opening to an unstable intermediate such as a cyanohydrin, which could lose hydrogen cyanide and rearrange to form two metabolites (**M3** and **M4**). Another metabolite (**M5**) was formed via further degradation of **M3** and **M4**. The detailed mechanism of bioactivation and mass spectral fragmentation patterns of a structurally related analog have been previously published [13]. The previous analog, whose metabolites were exhaustively identified by LC–NMR and LC–MS^n, was substituted at the 6-position of the pyrazinone ring with a chloro group (instead of a cyano group as in compound **I**); metabolic activation of the chloro or cyano substituted analogs results in putative loss of HCl or HCN, respectively, and yields identical metabolites. The metabolites of **I** had identical LC-retention times and LC–MS^n fragmentation patterns to the previously

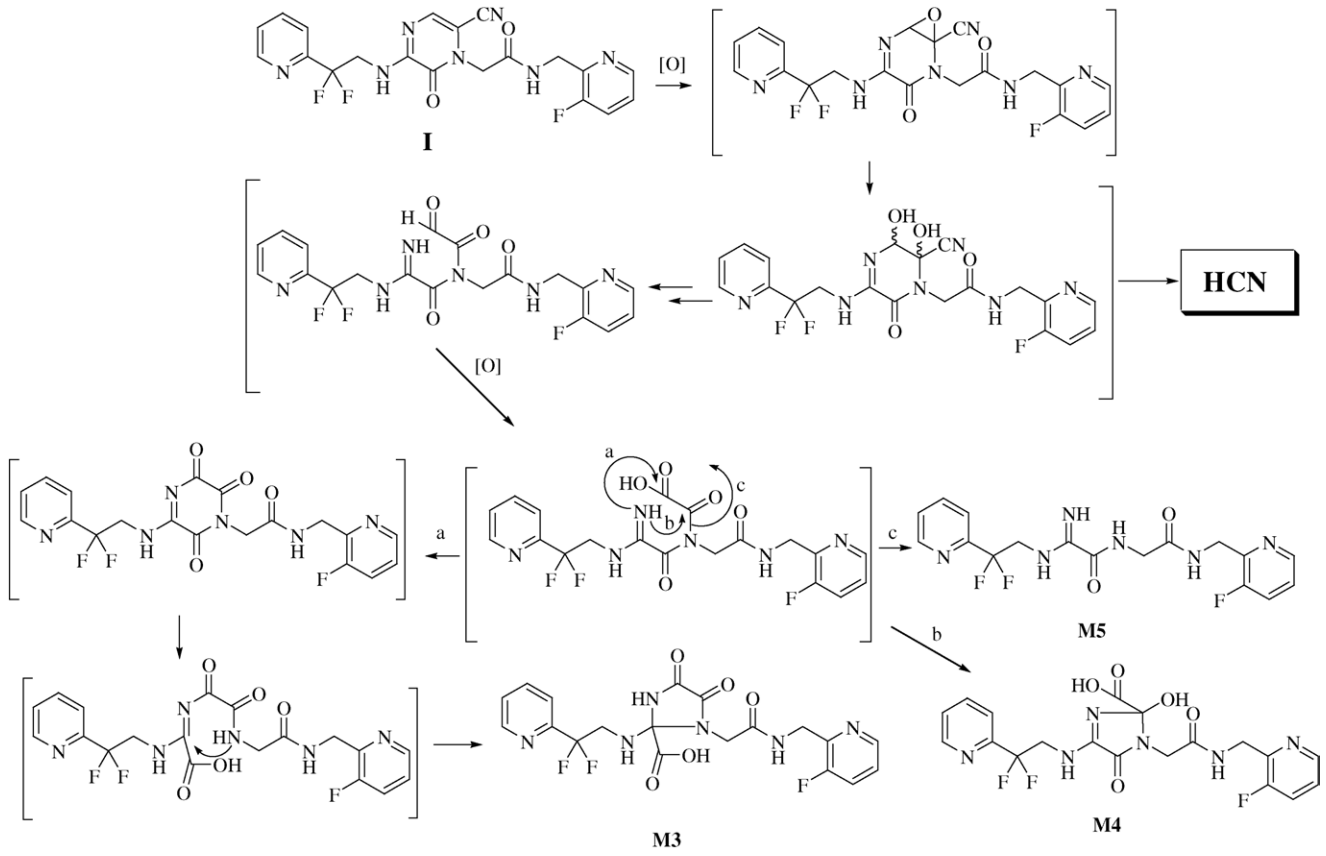

Fig. 3. Proposed mechanism for bioactivation of the pyrazinone ring of compound **I** and release of hydrogen cyanide.

characterized metabolites of the chloro analog. Based on the mechanistic scheme proposed for the biotransformation of **I** (Fig. 3), it was anticipated that hydrogen cyanide would be released upon oxidative bioactivation. Both the spectrophotometric and HPLC assays showed that **I** underwent extensive bioactivation in NADPH-fortified human liver microsomes to produce cyanide. The results from the two different methodologies agreed well with each other and suggested that on a molar basis, approximately 17–20% of the initial amount of **I** was bioactivated to a metabolite which was capable of releasing hydrogen cyanide in vitro.

The HPLC-fluorescence analysis of trace levels of cyanide in the microsomal incubates required that care be taken to prevent sample contamination and interference. The original published method for the HPLC cyanide assay described the use of methanol to prepare the NDA derivatization reagent [12]. When the HPLC assay was first applied (as per the published procedure) to liver microsomal incubates, a large interfering peak was present in the chromatograms of samples which contained no compound. Further investigation suggested the cause of the interference may have been the methanol used to prepare the NDA solution. Several different grades and lots of methanol were examined, all of which yielded a significant interference peak but when ethanol (anhydrous, 200 proof) was used to prepare the NDA solution, the interfering peak largely disappeared. The reduction of the interfering peak was extremely important as it allowed for a lowered limit-of-quantitation (5 nM) and did not compromise the overall assay precision.

The spectrophotometric assay also was able to reliably detect the production of metabolically generated cyanide, but had several disadvantages relative to the HPLC method. The assay required that the generated cyanide be trapped and the experimental setup (glassware with flow-through nitrogen source) required to trap the cyanide produced from the microsomal incubates was relatively complex. As such, the spectrophotometric methodology was not amenable to a high-throughput format. The incubations for the HPLC assay can be conducted in standard 96-well plates and assayed with minimal sample workup. Secondly, the spectrophotometric assay was significantly less sensitive than the HPLC assay; the LOQ was 500 nM for the spectrophotometric assay as compared to 5 nM for the HPLC assay. The reason for the lack of sensitivity may be attributable to the general insensitivity of the yellow polymethine dye colorimetric method. Moreover, the samples generated for the HPLC assay were stable for up to 1-week when stored refrigerated (results not shown), whereas the colorimetric assay needed to be completed rapidly once initiated.

In conclusion, two analytical methods to quantify cyanide release upon in vitro microsomal bioactivation have been developed. These methods provide a valuable, cost-effective

tool to quickly and effectively screen compounds in the early stages of drug discovery. Assays such as these can be used to improve the probability of success of compounds entering drug development by selecting against compounds which may produce cyanide in vivo following oxidative bioactivation. Because of its simplicity, accuracy, reliability, and sensitivity, the HPLC-fluorescence assay has been the method of choice for the evaluation of in vitro cyanide release by drug candidates which contain metabolically labile cyano functional groups.

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References

[1] C.S. Burgey, K.A. Robinson, T.A. Lyle, P.E.J. Sanderson, S.D. Lewis, B.J. Lucas, J.A. Krueger, R. Singh, C. Miller-Stein, R.B. White, B. Wong, E.A. Lyle, P.D. Williams, C.A. Coburn, B.D. Dorsey, M.T. Stranieri, M.A. Holahan, G.R. Sitko, J.J. Cook, D.R. McMasters, C.M. McDonough, W.M. Sanders, A.A. Wallace, F.C. Clayton, D. Bohn, Y.M. Leonard, T.J. Detwiler Jr., J.J. Lynch Jr., Y. Yan, Z. Chen, L. Kuo, S.J. Gardell, J.A. Shafer, J.P. Vacca, J. Med. Chem. 46 (2003) 461–473.
[2] R. Singh, M.V. Silva-Elipe, P.G. Pearson, B.H. Arison, B.K. Wong, R. White, X. Yu, C.S. Burgey, J.H. Lin, T.A. Baillie, Chem. Res. Toxicol. 16 (2003) 198–207.
[3] M.T. O'Berg, J. Occup. Med. 22 (1981) 245–252.
[4] T.M. Vogt, S.I. Selvin, G. Widdowson, S. Hulley, Am. J. Public Health 67 (1977) 545–549.
[5] E.E. Lasch, R.E. Shawa, Pediatrics 68 (1981) 5–7.
[6] T.A. Shragg, West J. Med. 136 (1982) 65–69.
[7] C.G. Moetral, J. Am. Med. Assoc. 245 (1981) 591–594.
[8] B. Vennesland, E. Conn, C.J. Knowles, A.J. Westley, F. Wissing, Cyanide in Biology, Academic Press, New York, 1981.
[9] S.N. Vogel, T.R. Sultan, Clin. Toxicol. 18 (1981) 367–383.
[10] M.A. Holland, L.M. Kozlowski, Clin. Pharm. 5 (1986) 737–741.
[11] K.W. Kulig, B. Ballantyne, Am. Fam. Physician 48 (1993) 107–114.
[12] A. Sano, N. Takimoto, S. Takitani, J. Chromatogr. B 582 (1992) 131–135.
[13] R. Subramanian, C.C. Lin, J.Z. Ho, S.M. Pitzenberger, M.V. Silva-Elipe, C.R. Gibson, M.P. Braun, X. Yu, J.L. Yergey, R. Singh, Drug Metab. Dispos. 31 (2003) 1437–1447.
[14] A.R. Pettigrew, G.S. Fell, Clin. Chem. 19 (1973) 466–471.
[15] D.J. Johnson, H.L. Williams, Anal. Lett. 18 (1985) 855–869.
[16] P. Lundquist, H. Rosling, B. Sorbo, Clin. Chem. 31 (1985) 591–595.
[17] P. Kaur, S. Upadhyay, V.K. Gupta, Analyst 112 (1987) 1681–1683.
[18] P. Lundquist, B. Sorbo, Clin. Chem. 35 (1989) 617–619.
[19] E. Casassas, M. Peydro, L. Puignou, Anal. Lett. 22 (1989) 729–740.