IN VIVO METABOLISM OF 2,6,9-TRISUBSTITUTED PURINE-DERIVED CYCLIN-DEPENDENT KINASE INHIBITOR BOHEMINE IN MICE: GLUCOSIDATION AS THE PRINCIPAL METABOLIC ROUTE

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ABSTRACT:

Synthetic cyclin-dependent kinase inhibitors have recently been referred to as effective antiproliferative agents. This study was conducted to characterize clearance of a 3H-labeled, trisubstituted purine-type inhibitor, 8-[3H]bohemine [6-benzylamino-2-(3-hydroxypropylamino)-9-isopropylpurine], in mice. Radioactivity profiles were analyzed by liquid scintillation counting and by thin layer chromatography followed by autoradiography. Metabolite structures were elucidated by mass spectrometry, NMR, and enzymatic analyses. Bohemine was rapidly and completely metabolized in vivo and disappeared from circulation during the first 60 min following intravenous administration. The metabolites were partially eliminated by the hepatobiliary tract and partly by renal excretion. The terminal hydroxyl group located at the C2 side chain of bohemine made the compound susceptible to main metabolic attacks, i.e., distinct types of conjugation reactions with glycosyl donors as well as an oxidative reaction. Other pathways were of relatively minor significance. Bohemine O-β-D-glucoside was the most abundant metabolite to be excreted. The enzymatic mechanism responsible for bohemine glucosidation in vitro required the presence of a UDP-glucoside donor. Additional glycosidation products were observed after inclusion of UDP-glucuronide, UDP-xyllose, UDP-galactose, or UDP-N-acetylgalactosamine into microsomal incubates. Glycosidations occurred faster in the kidney incubates than in hepatic ones. The second principal bohemine metabolite was a carboxylic acid, 6-benzylamino-2-(2-carboxyethylamino)-9-isopropylpurine. A cytosolic, 4-methylpyrazole-sensitive alcohol dehydrogenase class I was shown to mediate oxidation of the terminal hydroxyl group of bohemine into this acid, which was the only metabolite found in the blood in significant amounts. However, it displayed only weak cyclin-dependent kinase-1-inhibitory activity (IC50 > 100 μM) when compared with that of bohemine (IC50 ~ 1 μM).

During evolution, a highly ordered and conserved array of mechanisms regulating cyclin-dependent kinases (CDKs1), which govern the timing of cell cycle progression and cell division, has developed in eukaryotes. Recently, natural peptide CDK inhibitors have been uncovered and shown to play an important regulatory role in cell differentiation, proliferation, senescence, and programmed death (Chellappan et al., 1998). It has also been demonstrated that the effects of these endogenous inhibitors can be partly mimicked by synthetic reagents for molecular biological, biochemical, and morphological studies of the cell cycle. Importantly, the inhibitors suppress a subset of CDKs related to CDK1. These include cyclin/CDK1, cyclin/CDK2, and cyclin/CDK7 complexes. They appear to play a more critical role in the cell cycle than a subset of CDKs involving cyclin/CDK4 and cyclin/CDK6. Clinical applications of the inhibitors are also being extensively investigated in the treatment of atherosclerosis and vascular diseases, dermatological, nephrological, and neurological diseases, and parasitic and viral infections (Hung et al., 1996; Schow et al., 1997; Gray et al., 1998, 1999; Walker, 1998; Chang et al., 1999; Imbach et al., 1999; Meijer et al., 1999; Sielecki et al., 2000).

1 Abbreviations used are: CDK, cyclin-dependent kinase; ADH, alcohol dehydrogenase; Bq, becquerel; 4-MP, 4-methylpyrazole; TLC, thin layer chromatography; MS, mass spectrometry.

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olomoucine, roscovitine, and purvalanol, paullones, indirubins, and others (Gray et al., 1999; Meijer et al., 1999; Sielecki et al., 2000).

Recent studies have shown the significant antiproliferative activity of synthetic CDK inhibitors in vivo, which has encouraged investigators to evaluate their potential use as new anticancer agents. During the short period that has elapsed since the unusual binding mode of olomoucine to CDK2 was elucidated (Vesely et al., 1994; Schulze-Gahmen et al., 1995), more than 3000 novel 2,6,9-trisubstituted purine compounds have been synthesized and screened for their effects. Their selectivity for molecular targets has made them invaluable reagents for molecular biological, biochemical, and morphological studies of the cell cycle. Importantly, the inhibitors suppress a subset of CDKs related to CDK1. These include cyclin/CDK1, cyclin/CDK2, and cyclin/CDK7 complexes. They appear to play a more critical role in the cell cycle than a subset of CDKs involving cyclin/CDK4 and cyclin/CDK6. Clinical applications of the inhibitors are also being extensively investigated in the treatment of atherosclerosis and vascular diseases, dermatological, nephrological, and neurological diseases, and parasitic and viral infections (Hung et al., 1996; Schow et al., 1997; Gray et al., 1998, 1999; Walker, 1998; Chang et al., 1999; Imbach et al., 1999; Meijer et al., 1999; Sielecki et al., 2000).
To determine bohemine distribution and excretion, the animals were anesthetized using 0.05 to 0.08 mg of pentobarbital/g of body weight administered intraperitoneally. A stock solution of unlabeled bohemine (11.3 mg/ml) was prepared in 0.07 M HCl, and shortly before use it was further diluted in 0.9% NaCl to a final concentration of 1.13 mg/ml unlabeled drug. \(^{[\text{3}H]}\)Bohemine (74 kBq/g of body weight) was added to 300 ml of this solution. To achieve a dosage of about 13 \(\mu\)g (\(\approx 40\) nmol) of bohemine/g of animal body weight, an appropriate volume of the diluted bohemine solution, proportional to the weight of the animal, was injected into either the tail vein or subcutaneously. Animals were killed 2, 5, 10, 30, 60, or 120 min after drug administration \((n = 3)\). Control short-term i.v. experiments \((5 \text{ and } 10 \text{ min})\) were also performed using transient \((1-\text{to-2-min})\) ether narcosis instead of pentobarbital. Liver, stomach, kidney, gall bladder, two parts of intestine (a proximal \(6-\text{to-8-cm}\) segment and the remaining part of the bowel), spleen, and urinary bladder were removed and weighed. Samples of blood, lungs, brain, bone marrow, retroperitoneal fat, cardiac muscle, and posterior femoral muscle were also removed for further analysis.

**Preparation of Mouse Liver and Kidney Cytosolic and Microsomal Fractions.** Liver and kidney subcellular fractions were prepared by differential centrifugation \((10,000 \text{g for } 15 \text{ min followed by } 100,000 \text{g for } 60 \text{ min using an Avanti 30 Centrifuge and Optima LE-80K Ultracentrifuge, Beckman, Fuller-}
\text{ton, CA})\). A 25 M sucrose solution. The fraction aliquots were stored at \(-80^\circ\text{C}\) until used. Proteins were determined according to Bradford \((1976)\) with bovine serum albumin as a standard.

**In Vivo Studies Using Anesthetized Mice.** All the experiments using animals were authorized by the Institutional Ethical Committee and carried out according to institutional guidelines in compliance with Czech national laws, and in agreement with the Declaration of Helsinki. The in vivo experiments were carried out in male NMRI outbred mice of mixed genetic background and of approximately 20 to 25 g of body weight between 5 and 7 weeks of age (VUF, Konarowice, Czech Republic).

**Experimental Procedures**

**Chemicals.** Alcohol dehydrogenase (ADH), EC 1.1.1.1, from equine liver \((9A589)\); alcohol dehydrogenase, EC 1.1.1.1, from baker’s yeast \((A7011)\); \(\beta\)-NAD \((N5622)\); \(\beta\)-glucuronidase, EC 3.2.1.31, from bovine liver \((G0251)\); sulfatase, EC 3.1.6.1, from Aerobacter aerogenes \((S1629)\); sulfatase, EC 3.1.6.1, from Limpets \((S8629)\); \(\beta\)-glucosidase, EC 3.2.1.21 \((G4511)\); \(\alpha\)-gluco-
sidase, EC 3.2.1.20, recombiant form, from Saccharomyces cerevisiae \((G0660)\); \(\delta\)-gluconic acid 1,5-lactone, UDP-glucuronic acid, UDP-glucose, UDP-galactose, UDP-mannose, UDP-N-acetylglucosamine, UDP-galactosamine, UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, \(\alpha\)-octyl-\(\beta\)-glucoside, \(\alpha\)-octyl-\(\alpha\)-glucoside, ATP, histone (type III-S), 4-methylpyra-
zole hydrochloride \((4-\text{MP})\), disulfiram, and the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and aprotinin were obtained from Sigma-Aldrich \((\text{St. Louis, MO})\). \([\text{3}H]\)ATP was purchased from Amersham Pharmacia Biotech \((\text{Little Chalfont, England, Uppsala, Sweden, and Piscat-
away, NJ})\). Bovine serum albumin \((\text{fraction V})\) was produced by Biovetra \((\text{Ivanovice n.H., Czech Republic})\). Silica gel for column chromatography \((\text{Kieselgel 60, particle size of } 40-63 \mu m)\) and silica gel \((60 F_{254})\) TLC sheets were purchased from Merck \((\text{Darmstadt, Germany})\). All other chemicals and reagents used were of analytical or high-performance liquid chromatography grade.

**Radiolabeled Compounds and Chemical Syntheses.** Bohemine was syn-
thesized as described by Havlicek et al. \((1997)\). \(8-[\text{3}H]\)Bohemine was prepared by an isotope exchange reaction. Briefly, a reaction mixture containing bohe-
mine, palladiumoxide/barium sulfate as a catalyst, and dioxane-0.1 M aqueous disodium carbonate \((9:1)\) as a solvent was stirred with a tritium gas \((\text{3} H_2\text{ with about } 60\% \text{ carrier-free radioactivity})\) at \(23^\circ\text{C}\) for 2 h. The solvent was then removed by lyophilization, the residue was dissolved in a mixture of tert-
butanol-H\text{O} \((9:1)\), and the catalyst was removed by centrifugation. The product was purified by TLC on aluminum oxide \(150 F_{254}\) sheets \((\text{Merck})\) with toluene-methanol \((97:3)\) as a mobile phase. The specific activity of the \(\text{[3}H\)bo-
hemine product was 300 TBq/mol, and its radiochemical purity was at least 98\% when checked by TLC on Silicagel \(60 F_{254}\) sheets \((\text{Merck})\) with chloro-
form-methanol \((95:5)\).

Bohemine metabolite M-3, extracted from a TLC spot (see below), was eserstified with diazomethane into its corresponding methyl ester. The structural identification of the eserstified product of this reaction was performed by MS, \(\text{[1}H\text{]NMR, and Fourier-transform infrared spectroscopy, and the product was ascer-
tained as 6-benzylamino-9-isopropyl-2-(2-methoxybenzyethylaminy-
lo)purine (see Results).**

**In Vivo Study Using Anesthetized Mice.** All the experiments using animals were authorized by the Institutional Ethical Committee and carried out according to institutional guidelines in compliance with Czech national laws, and in agreement with the Declaration of Helsinki. The in vivo experiments were carried out in male NMRI outbred mice of mixed genetic background and of approximately 20 to 25 g of body weight between 5 and 7 weeks of age (VUF, Konarowice, Czech Republic).
μl in a HEPES buffer (50 mM, pH 7.4). After 10 min, the incubation was stopped by adding SDS sample buffer and the product resolved using 12.5% SDS-polyacrylamide gel electrophoresis (Mini-Protein II System, Bio-Rad Laboratories, Hercules, CA). The gels were analyzed by densitometry as described previously (Vesely et al., 1994) with the only exception that an Imaging Analyzer BAS 1800 (Fujiﬁlm, Tokyo, Japan) was used to quantify the phosphorylated histone. Experiments were performed in duplicate.

Extraction of Metabolites. For 1H-metabolite analysis and identiﬁcation, blood, tissues, and organs were collected, weighed, and homogenized in a 9-fold volume of ethanol. The stomach, intestine, urinary bladder, and gall bladder were processed with their contents included. The in vivo incubates were stopped and extracted using ethanol in the same manner. The ethanolic supernatants (4000 g, 5 min) were then decanted, concentrated, and used for the isolation and identiﬁcation of metabolites. The sediments were found to contain less than 12% of the total sample dose when measured for radioactivity. Moreover, crude blood and liver samples were subject to distillation, in parallel with the extraction. The evaporated water phase was collected in a condensate receiver. Less than 10% of the 3H radioactivity was found to pass parallel with the extraction. The evaporated water phase was collected in a condensate receiver. The 3H label, conﬁned to the C8 of the bohemine purine ring, was a marker complying with the requirements of this study.

TLC. The ethanolic extracts (supernatants) were concentrated in vacuo under a nitrogen atmosphere at 40°C and chromatographed on Silicagel 60 F254 sheets (Merck). TLC was routinely run in acidic mobile phase chloroform-methanol-acetic acid (90:10:1 v/v; phase A). Acetic acid was replaced by 1 volume of concentrated aqueous ammonia (phase B) to diagnose (by changing TLC mobility of the spots) the acidic/basic character of substances. The spots were visualized by autoradiography. For structure identiﬁcation, the spots were scraped out into tubes, extracted with ethanol, and dried, and chemical structures of the isolated metabolites were elucidated by means of enzymatic analyses, MS, and NMR spectroscopy (see Results).

Liquid Scintillation Analysis. Radioactivity was measured in a Canberra Packard TriCarb 2700 TR Liquid Scintillation Analyzer (Canberra Packard, Groningen, The Netherlands, and Meriden, CT) with an automatic quench correction. Canberra Packard Ultima Gold scintillation ﬂuid, 2 ml per 100-μl aliquots, was used as a scintillation cocktail.

 Autoradiography. Autoradiography of the TLC spots was performed using either 1H-Hyperﬁlm or Kodak BioMax MS ﬁlm with Kodak BioMax Tran- Screen LE at −70°C (Amersham Pharmacia Biotech). The detection limit of both techniques was about 0.01 Bq/cm2 (equivalent to about 0.3 pmol of a labeled substance per spot), provided the duration of the exposure was about 72 h in the ﬁrst case, and about twice as short in the second. The spot intensities were computerized and data processed using GeneGenius (Syngene, Cambridge, UK) and Imaging Analyzer BAS 1800 (Fujiﬁlm); 32P-SDS-polyacrylamide gel electrophoresis gels were evaluated using the Imaging Analyzer BAS 1800 only.

Spectroscopic Methods. Electrospray ionization MS and MSn were carried out using an ion trap mass spectrometer LCQ (Finnigan-MAT, San Jose, CA). The analyzed samples were either standards, diluted by 1% acetic acid in methanol, or ethanolic extracts of TLC spots acidiﬁed with 1 μl of acetic acid. The samples were introduced with a ﬂow rate of 2 μl/min to the electrospray interface with a source voltage of 5.6 kV, sheath gas ﬂow of 28, and a capillary temperature of 200°C. The selected ions were fragmented in the ion trap (isolation width, 60; collision energy, 25%). Mass spectra were scanned in a full scan regime as well as in zoom scan and MSn. 1H NMR and 13C NMR spectra were measured on a Varian VX-400 instrument (Palo Alto, CA) at 400 and 100 MHz, respectively. J values are given in Hz.

Calculations. To evaluate the total radioactivity content in the circulatory system of mice, the concentration of radioactivity in blood was multiplied by a factor amounting to 8% of mouse body weight. The relative accumulation of 1H radioactivity in different mouse body tissues was represented as multiples of its blood concentration [y = (Bq/g of wet tissue)/(Bq/ml of blood)]. The relative distribution of metabolites present in the respective tissues was calculated from radiochromatograms as the area of the metabolite/areas of all metabolites + start area + area of parent compound residue). The results were then expressed graphically after being converted to a corresponding fraction of the total administered dose, provided the relative distribution of radioactivity among organs was known from scintillation counting measurements (see Results).

Results

1H-labeled trisubstituted purine bohemine was used to study the metabolism of this selective CDK inhibitor in mice in vivo. First, we focused on the distribution of radioactivity among different mouse body tissues as well as on the identiﬁcation of pathways of radioactivity elimination from the animal body. Next, we concentrated on resolution of tissue extracts and identiﬁcation of major products of bohemine metabolism by TLC, enzymatic analyses, and spectroscopic methods.

In Vivo Experiments. Distribution of 1H Radioactivity in Tissues. Two minutes after [1H]bohemine i.v. injection, the radioactivity concentration in blood amounted to about 2.9% of the total dose/ml of blood while being stored in several organs above concentrations present in the blood. It was elevated in the liver, kidney, in the ﬁrst 8 cm of the proximal intestine, and in gall bladder. Table 1 demonstrates that mean concentrations of 1H radioactivity in the liver and kidney exceeded those in blood about 3- to 5-fold. Later, the radioactivity appeared in more distal parts of the intestine as well as in the urinary bladder. There was no 1H radioactivity accumulation in any of the other tissues examined (see Experimental Procedures), as indicated by comparing radioactivity concentrations in the above tissues and blood. Table 2 shows the time course of the total 1H radioactivity dose distribution among different mouse body tissues. The total radioactivity content in blood, kidney, and liver peaked within the ﬁrst 2 min after i.v. injection and then declined progressively. The radioactivity dropped very steeply in blood, amounting to less than 1/10 of the total dosage within 2 min after its i.v. administration. It is apparent (Table 2) that during the ﬁrst 30 min, a fraction of the radioactivity sequestered in the liver, intestine, kidney, and urinary bladder achieved more than 50% of the total dose. The portion of the total radioactivity dosage excreted into the intestine was approximately the same as that sequestered into the urinary bladder during the ﬁrst 60 min (Table 2).

TABLE 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sampling Time (Minutes)</th>
<th>Relative 1H radioactivity levels (μg/ml)</th>
<th>(mean ± S.D., n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2</td>
<td>1.00 ± 0.26</td>
<td>57.10 ± 2.38</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.93 ± 0.37</td>
<td>2.12 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.45 ± 0.26</td>
<td>0.36 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.75 ± 0.26</td>
<td>0.07 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>30.94 ± 3.64</td>
<td>13.09 ± 3.64</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>5.71 ± 0.45</td>
<td>3.12 ± 0.45</td>
</tr>
<tr>
<td>Kidney</td>
<td>2</td>
<td>0.45 ± 0.26</td>
<td>0.36 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.00 ± 0.26</td>
<td>0.45 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.08 ± 0.38</td>
<td>0.26 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.59 ± 0.68</td>
<td>2.93 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>25.23 ± 0.68</td>
<td>17.09 ± 0.68</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>33.94 ± 0.75</td>
<td>24.23 ± 0.75</td>
</tr>
</tbody>
</table>

* Established by liquid scintillation counting and represented as multiples (y-ratios) of the blood radioactivity concentration following intravenous injection of 74 kBq of [1H]bohemine/g of body weight (see Experimental Procedures). The blood levels (kBq/ml) were as follows: 67.09 ± 7.21 (2 min), 57.10 ± 15.35 (5 min), 52.13 ± 4.59 (10 min), 33.94 ± 5.90 (30 min), 25.23 ± 7.38 (60 min), and 12.04 ± 3.39 (120 min), respectively.
Both peaking and elimination of the radioactivity were somewhat more protracted after [3H]bohemine s.c. injection than after the i.v. administration. About twice as much time (60 min) was necessary to sequester 50% of the total s.c. radioactivity dose in the same target organs. Peak concentrations of [3H] radioactivity (1.0–1.5% of the total dose/ml) were contained in blood between 10 and 120 min following s.c. administration (data not shown).

**Tissue Metabolite Profiles.** In the next part of our experiments, we were interested in whether the radioactivity contained in blood and concentrated in mouse organs was the parent compound [3H]bohemine or a product of its metabolism.

A blood [3H] radioactivity profile is given in Fig. 1. It is obvious that after i.v. administration, [3H]bohemine completely disappeared from the circulation in about 60 min. As illustrated, by far the most prevalent portion of [3H] radioactivity in the profile was accounted for by the presence of a spot designated as M-3 (Fig. 1; Table 3). The intensity of this spot declined very slowly over time and persisted in the circulation beyond our observation period. The transient presence of two other spots designated as M-1 and M-2 was also registered in the blood radioactivity profile (Fig. 1; Table 3). The time course of [3H] radioactivity distribution among the principal [3H] radioactivity spots observed in mouse liver, proximal intestine, kidney, and urinary bladder ex vivo extracts can be seen in Fig. 2. It is evident that there were some quantitative differences in radioactivity profiles within these organs. Ten minutes after bohemine i.v. administration, the principal spot accounting for 54% of the radioactivity deposit in liver was the same as that found in blood and designated as M-3 (Fig. 2). On the other hand, in kidney, intestine, and urinary bladder the predominant spot was M-2. This made up for 39, 25, and 11% of the radioactivity pools present in the respective organ extracts 10 min after the administration, but only about 5% of the radioactivity found in the liver extract. A small amount of bohemine was detected in the proximal intestine, whereas there was no bohemine in the urinary bladder (Fig. 2). Furthermore, in the liver and kidney, the rather steep curves of parent bohemine and M-2 spots contrast with a relatively slow decline in M-3 intensity. Moreover, between 10 and 30 min following i.v. administration, the intensity of the M-3 spot outweighed that of M-2 in the kidney because of much faster disappearance of M-2.

To obviate potential interference between pentobarbital and bohemine metabolism, we used animals transiently narcotized with ether, instead of pentobarbital (see Experimental Procedures). However, in these short-term experiments, no apparent differences could be demonstrated between metabolite profiles in ex vivo organ extracts obtained from animals anesthetized by pentobarbital or by ether (data not shown).

**Structure Elucidation of the Principal Metabolites Obtained in the in Vivo Experiments.** When the polar spot M-1 remaining in the close vicinity of the start (see Table 3 for Rf values) was extracted and incubated with the enzyme β-glucuronidase, it yielded a single [3H]-labeled product with chromatographic mobility identical to that of bohemine. No products were detected after incubation of the M-1 spot extract with sulfatas or glucosidases as described under Experimental Procedures. Soft ionization MS of the M-1 spot extract indicated the presence of a compound with an M, of 516 (m/z = 517 [M + 1]+), which corroborated the structure of bohemine glucuronide. Consequent fragmentation in an ion trap led to a neutral loss of 176 that could be explained by elimination of a glucuronide moiety given that subsequent fragmentation in an ion trap led to a neutral loss of 162 that could be explained by elimination of a hexose moiety since, again, fragmentation of the rest of the molecule (an ion of m/z = 341 [M + 1]+) was indistinguishable from that of bohemine (Table 3). The treatment of

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**TABLE 2**

**Relative distribution of [3H] radioactivity among different mouse body organs (mean ± S.D., n = 3)**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sampling Time (Minutes)</th>
<th>Relative Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Blood</td>
<td>6.8 ± 0.73</td>
<td>5.79 ± 1.56</td>
</tr>
<tr>
<td>Liver</td>
<td>22.39 ± 4.66</td>
<td>20.65 ± 4.86</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.31 ± 0.60</td>
<td>5.97 ± 2.11</td>
</tr>
<tr>
<td>Proximal gut</td>
<td>2.96 ± 0.79</td>
<td>6.76 ± 0.63</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.22 ± 0.15</td>
<td>3.92 ± 1.73</td>
</tr>
</tbody>
</table>

* Radioactivity contents in individual organs were established by liquid scintillation measurements and expressed as percentages of the total administered dose—intravenous injection of 74 kBq of [3H]bohemine/g of body weight (see Experimental Procedures).

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The radioactivity distribution was estimated from percentages of areas appor- tioned to the individual spots after their TLC and autoradiography evaluation. Radioactivity in individual spots was converted to a corresponding fraction of the total administered dose, provided the amount of radioactivity in blood was estimated using liquid scintillation measurement data.

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the M-2 spot extract with the enzyme β-glucosidase produced a single radioactive spot with the same mobility as authentic reference [3H]bohemine. To gain deeper insight into what the configuration of a metabolite contained in an M-3 spot extract was tentatively identified as an acid based on its dissimilar TLC mobilities in chromatographic phases (Table 3). In accord with that conjecture, a soft ionization MS analysis suggested a structure generated by oxidation of the 2-(3-hydroxypropylamino) group of bohemine to a 2-(2-carboxyethylamino) group (m/z = 355 [M + 1]+). Consequent methylesterification of the M-3 spot extract led to a single product with a chromatographic mobility characterized by the m/z values of 0.85 and 0.91 in phases A and B, respectively. These m/z values were entirely distinct from those of M-3 (Table 3). Further analysis of the methylesterified product by MS², 1H NMR, and IR ascertained it as 6-benzylamino-9-isopropyl-2-(2-methoxybenzylamino)purine (m/z = 369 [M + 1]+; Table 3). Thus, the M-3 spot metabolite, representing the principal portion of the radioactivity contained in blood and liver and persisting in the mouse body beyond our observation period (Figs. 1 and 2) was a carboxylic acid, 6-benzylamino-2-(2-carboxyethylamino)-9-isopropylpurine. This conclusion was further corroborated by a subsequent analysis of a product of the bohemine reaction with enzyme ADH (see below).

**Incubation of Bohemine in Microsomal Incubates Supplemented with Activated Glycosides.** Having obtained data indicating
that bohemine $\beta$-glucuronide and bohemine $\beta$-glucoside were the main phase II products of bohemine metabolism in mice in vivo, we incubated $[3\text{H}]$bohemine with mouse liver or kidney microsomes in the presence of either UDP-glucuronic acid, UDP-glucose, or a lipophilic glucose donor, $n$-octyl-glucoside. In both types of microsomes, the activity of a UDP-glucose-consuming system governed the kinetics (Fig. 4). Only small amounts of the corresponding product were formed in the presence of UDP-glucuronic acid donor (Fig. 4), whereas the artificial donor $n$-octyl-glucoside was ineffective in our assays (not shown). Subsequent TLC, MS, and enzymatic analyses showed that the UDP-glucuronide reaction product was identical to spot M-1 bohemine $\beta$-glucuronide, and the UDP-glucoside reaction product was identical to spot M-2 bohemine $\beta$-glucoside. Bohemine was then incubated in microsomes supplemented with various activated glycosides, such as UDP-galactose, UDP-mannose, UDP-xylose, UDP-glucosamine, UDP-galactosamine, UDP-$N$-acytelyglucosamine, UDP-$N$-acytelgalactosamine, or $n$-octyl-$\alpha$-glucoside, with the aim to further explore the glycosidation potential of the microsomes. As is apparent from Fig. 4, UDP-xylose was as effective a sugar donor as UDP-glucose, both in the kidney and in liver microsomes. UDP-Galactose and UDP-$N$-acetylgalactosamine were also remarkably good donors (Fig. 4). With each of these active glycosides, the kidney microsomes exhibited significantly higher specific glycosyltransferase activity toward bohemine than did liver microsomes (Fig. 4). The remaining compounds were inactive as donors.

The in vitro UDP-xylose reaction product was tentatively identified as bohemine xyloside based on soft ionization MS analysis ($m/z = 473 [M + 1]^{+}$); $R_f$ values of this product were different from those

![Fig. 2. Relative distribution of $^3$H radioactivity (mean ± S.D., n = 3) among the bohemine and metabolite spots M-1 to M-3 in liver, proximal intestine, kidney, and urinary bladder at different intervals following intravenous injection of 74 kBq of $[^3$H]bohemine/g of mouse body weight.](image-url)
of the product of UDP-glucose reaction (Table 3). Similarly, the UDP-N-acetylglusosamine reaction product was tentatively identified as bohemine N-acetylaminoglucoside (m/z 544 [M + 1]; Table 3). On the contrary, neither MS nor TLC mobility characteristics of the UDP-galactoside reaction product were sufficient to differentiate it from those of bohemine glucoside (Table 3).

The M-3 Spot Metabolite Is Produced in Cytosolic Incubates—Bohemine Is a Substrate for ADH. When monitored by TLC, [3H]bohemine incubation with either a liver or kidney cytosolic fraction in the presence of NAD coenzyme resulted in detection of a single metabolite. The TLC mobility, diazomethylation, and consequent MS/1H NMR analyses identified this product as metabolite M-3 (see above). This in vitro formation of the metabolite was inhibited by a 5 μM classical ADH inhibitor, 4-MP, while a 100 μM aldehyde dehydrogenase inhibitor, disulfiram, exerted no significant effects (data not shown). Bohemine was then incubated with commercial equine liver ADH. It was found to change bohemine into M-3. This reaction was also sensitive to 4-MP and resistant to disulfiram. Consequently, it can be concluded that bohemine is a substrate for a cytosolic, 4-MP-sensitive ADH class I that is able to catalyze its oxidation into the corresponding carboxylic acid, 6-benzylamino-2-(2-carboxyethylamino)-9-isopropylpurine. By contrast, no products were detected in incubates of bohemine with yeast ADH (data not shown).

CDK Inhibition by Bohemine Metabolite M-3. The only metabolite that was found to circulate in blood in significant amounts was

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**Fig. 3. Bohemine O-β-d-glucoside (numbering assignment used).**

**Fig. 4. Incubation of [3H]bohemine with mouse liver and kidney microsomes in the presence of activated glycosides.**

Incubation interval: 30 min (UDP-glucoside and UDP-xyloside) or 120 min (UDP-glucuronide, UDP-galactoside, and UDP-N-acetylamino-glucoside). Incubations were monitored by TLC 3H autoradiography. Data represent means from densitometric evaluation of duplicate experiments.
bohemine carboxylic acid (the M-3 spot metabolite). This was characterized by rather slow elimination and prolonged persistence in the mouse body (Figs. 1 and 2). For practical reasons, it was important to ascertain whether this metabolite could mimic CDK-inhibitory, anti-proliferative effects of the metabolically unstable parent compound. However, the carboxylic acid derivative failed to inhibit CDK1-cyclin B substantially in vitro, even when tested in a concentration range up to 100 μM, while bohemine displayed an IC₅₀ value for CDK1-cyclin B complex of 1 μM (see Experimental Procedures; data not shown).

**Discussion**

Currently, investigations on novel purine-derived CDK inhibitors are being conducted to evaluate their potential therapeutic effects. As this trend is likely to continue, there is a need to establish the relationship between the chemical structure of the inhibitors and their pharmacokinetic properties in vivo.

Using [³H]bohemine-injected male mice, we identified the liver and kidney as the organs responsible for clearance of this selective CDK inhibitor. Principal bohemine metabolites were isolated from mouse body fluids and tissues and their chemical structures were elucidated. Our data indicate that the predominant bohemine metabolite to be eliminated was a bohemine glucoside conjugate (an M-2 spot metabolite).

Generally, the most common xenobiotic conjugates to be encountered in mammals are glucuronic acid derivatives. If glucuronidation is possible, the remaining types of glycosidations are considered to be of minor importance in animals, although they are common in plants and invertebrates (Tang, 1990). However, novel pathways for O-glucoside conjugate formation that play a role in the metabolism of foreign and/or endogenous compounds in mammals have been identified recently, namely O-β-D-glucosidation (Gessner et al., 1973), O-α-D-glucosidation (Kamimura et al., 1988), and O-β-D-N-acetylglucosaminidation (Marschall et al., 1989). Our study demonstrated that the terminal hydroxyl group of bohemine was extensively conjugated with a hexoside moiety different from glucuronide. As a matter of fact, the TLC and MS characteristics obtained in our experiments did not discriminate between hexoside epimers. Nevertheless, the enzymatic analysis performed according to Matern et al. (1984) and Tang (1990) suggested the bohemine O-β-D-glucoside as the most probable candidate structure for metabolite M-2. This was confirmed by ¹H NMR and ¹³C NMR measurements that established the presence of O-β-glucoside anomer in the metabolite. Nakano et al. (1986a,b) were likely the first to describe O-β-glucosidation of non-acidic hydroxyl groups in mammals (in dogs). Boberg et al. (1998) uncovered similar preferential formation of O-β-D-glucoside conjugate, as compared with production of glucuronide, using cerivastatin as a foreign aglycone substrate in dogs. Interestingly, the same detoxification reaction was absent in mice and rats (Boberg et al., 1998).

O-β-Glucosidation of the nonacidal primary hydroxyl group of bohemine as observed in our experiments might, therefore, be a reaction of similar significance in mice.

The glucoside conjugate may be synthesized by either a UDP-sugar-dependent microsomal glucosidation system or a nucleotide-independent, i.e., lipid-soluble sugar-utilizing system (Matern et al., 1984; Matern and Matern, 1990). Our microsomal incubates synthesized bohemine β-glucoside when supplemented with UDP-glucose, whereas those with n-ctyl-β-glucoside remained inactive. Thus, the favored enzymatic mechanism responsible for the bohemine glucoside formation in mice was sugar nucleotide-dependent. Formation of additional products was observed after inclusion of UDP-xylene, UDP-galactose, and UDP-N-acetylglucosamine into incubates. MS data strongly suggest that the products of UDP-xylene reaction and UDP-N-acetylgalactosamine reaction were the corresponding glycosides bohemine xyloside and bohemine N-acetylamino glucoside, respectively. The glycosylation reactions were significantly faster in mouse kidney microsomes than in the liver fraction. No conclusions can be drawn at this time concerning the nature of the enzyme systems catalyzing these reactions and their physiological significance. It is noteworthy that the presence of UDP-xyllose- and UDP-galactose-dependent transglycosylation reactions has been demonstrated in human liver microsomes (Radominska et al., 1993). Moreover, N-acetyl glucosaminidation has been established as an important conjugation pathway in humans (Marschall et al., 1992).

The principal product of phase I bohemine metabolism found in mice ex vivo extracts was a metabolite formed by oxidation of the bohemine terminal hydroxyl group into a carboxylic one (an M-3 spot metabolite). This metabolite predominated in blood and liver but, surprisingly, represented only a minor fraction of the radioactivity found in intestine, kidney, and in urinary bladder during the first 60 min after the i.v. injection. M-3 is likely secreted by hepatocytes into both the intestinal lumen (via the hepatobiliary system) and the bloodstream (via sinusoidal poles). Alternatively, the metabolite in question might undergo extensive reabsorption in the proximal intestine. Both assumptions are in accord with the presence and persistence of significant amounts of M-3 in blood, liver, and kidney.

Oxidation of the primary hydroxyl group of bohemine into the carboxylic acid might have been catalyzed by a variety of enzyme systems. Here we showed that a 4-MP-sensitive member of the mammalian ADH family of oxidoreductases was responsible for the formation of at least a portion of metabolite M-3. It is worth recalling that medium-chain, dimeric, mostly cytosolic ADHs that are widely distributed in vertebrates as well as in various tissues of individual mammalian species divide into classes and isoforms according to their different subunit composition (Riveros-Rosas et al., 1997). The horse liver ADH, used in our in vitro experiments, consists of two subunits, E and/or S, determining the horse liver enzyme as a high-affinity/low-capacity mammalian ADH class I (Jönnal and Höög, 1995). Other authors have shown that horse liver ADH catalyzes NAD⁺-dependent alcohol oxidation into aldehydes as well as dismutation of aldehydes into their corresponding alcohols and acids (Abeles and Lee, 1960; Henahan and Oppenheimer, 1993; Svensson et al., 1996). In accord with those findings, we observed that horse liver ADH also oxidized bohemine into its corresponding carboxylic acid, at least in vitro. On the other hand, we were unable to identify any products of the bohemine reaction with yeast ADH. This is in agreement with data reporting yeast NAD⁺-dependent ADH to lack aldehyde dehydrogenase properties (Dickinson and Monger, 1973).

The participation of ADH class I in bohemine transformations in mice is strongly suggested by an inhibitory effect of low (5 μM) concentration of a classic ADH inhibitor, 4-MP (Eklund et al., 1990; Kemper and Elfarra, 1996), on the bohemine acid production observed in cytosolic incubates. The mouse cytosolic form of ADH class I has been designated as A₂ (Algar et al., 1983). As expected, the mouse liver cytosol displayed much higher NAD⁺-dependent bohemine dehydrogenase activity than did the kidney cytosol. Furthermore, disulfiram, a potent inhibitor of a cytosolic aldehyde dehydrogenase (Kitson, 1975; Eckfeldt and Yonetani, 1976; Hempel et al., 1984), did not exert significant effects on bohemine oxidation in the incubates, which argues against the importance of this enzyme in cytosolic, NAD⁺-dependent bohemine oxidation.

Naturally, the present data do not exclude a role for other ADH classes and/or other oxidoreductases in oxidation of bohemine in mice in vivo. The most striking result of this study was a lack of metabolites detected in the organ extracts that would unequivocally imply a role in the metabolism of bohemine.
We are indebted to Jarmila Potomkova and Alexander Oulton who kindly provided us with baculoviral constructs. Last but not least, Jirí Bartek (Copenhagen, Denmark) and David P. Lane (Dundee, UK), biology, Academy of Sciences, Czech Republic, for NMR measurements. We acknowledge Zuzana Čelová for their excellent technical assistance. We also acknowledge that the rapidity of bohemine elimination from the mouse body, consequently underscoring our recognition of the powerful potential of mouse organs to detoxify and excrete bohemine.

This aside, we do not exclude the possibility that several metabolites were produced that remained unanalyzed by us. Highly polar minor products might be formed that did not move on TLC plates. Analysis of such spots was hampered by their immobility and by small quantities of single metabolites present in them that could not be identified and/or quantified individually. More recently, we have explored this puzzle using in vitro models such as subcellular fractions, liver cell suspensions, and precision-cut organ tissue slices, enabling us to acquire minor metabolites in sufficient amounts. Our results will be communicated in this regard in a forthcoming paper (M. Ryoka, J. Veselý, Z. Chmela, H. Havlíček, K. Lemr, K. Cervenková, B. Cerny, K. Fukušová, J. Hanuš, M. Belejova, Z. Cervinkova, H. Lotková, J. Lukés, and K. Michalíková, manuscript in preparation).

It can be concluded from this study that the tissue availability of bohemine in mice was grossly reduced shortly after its application, owing to fast clearance of bohemine. Significantly, bohemine carboxylic acid in mice was grossly reduced shortly after its application, facilitating the design of new potent purine CDK inhibitors and their inhibitors in cellular differentiation and development, in Cyclin Dependent Kinase (CDK) Inhibitors (Vogt PK and Reed SI eds) pp 57–103, Springer-Verlag, Berlin.

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