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In Vitro Glycosidation Potential Towards Olomoucine-Type Cyclin-Dependent Kinase Inhibitors in Rodent and Primate Microsomes

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Summary

Interspecies differences in glycosidation potential in mammalian tissues represent a factor contributing to ambiguity when endobiotic and/or xenobiotic metabolic pathways are extrapolated from animals to man. Using the TLC/autoradiographic technique, we conducted an *in vitro* investigation involving mouse, rat, monkey, as well as human liver and kidney microsomes to evaluate their glycoconjugation potential towards ^{3}H-labeled, purine-derived selective inhibitors of cyclin-dependent kinases such as olomoucine, bohemine, roscovitine, 6-(2-hydroxybenzyl)amino-2-(1-hydroxymethyl-2-methylpropyl)amino-9-isopropylpurine (compound *A-4*), and 6-(3-hydroxybenzyl)amino-2-[(1(R/S)-hydroxymethyl)propyl]amino-9-isopropylpurine (compound *A-5*) as aglycones. Principally, this study confirmed the aliphatic hydroxyl group of olomoucine-type inhibitors as a relatively suitable target for glucuronide, glucoside, xyloside, galactoside, and/or N-acetylaminoglucoside conjugation. Of the tissues examined, only the mouse microsomes were able to perform glucosidation and galactosidation reactions with the aglycones. On the other hand, monkey microsomes were superior to the mouse microsomes in a variety of glucuronide conjugates produced with compounds A-4 and A-5.

Key words

Glycosidation • Mouse • Rat • Monkey • Human • CDK inhibitors

Introduction

Glycosidations (type II or conjugation reactions in drug metabolism) have been established as playing an important role in the elimination of both endogenous and exogenous compounds in mammals. The reactions include glucuronidation, glucosidation, and N-acetyl-glucosaminidation (Radominska *et al.* 1993, Marschall *et al.* 1994, Bock *et al.* 1999, Radominska-Pandya *et al.* 1999, Tukey and Strassburg 2000). Generally, the

addition of a glycosyl moiety from an activated glycosyl donor to a small molecule (aglycone) in animals, plants, yeast and bacteria can be mediated by a large superfamily of glycosyltransferases (Mackenzie *et al.* 1997).

Recently, olomoucine-type, trisubstituted purine cyclin-dependent kinase (CDK) inhibitors have attracted considerable attention as novel antiproliferative agents (Veselý *et al.* 1994). Moreover, potential clinical applications of these inhibitors are being investigated in different fields: cardiovascular (atherosclerosis, restenosis), nephrology (glomerulonephritis), dermatology (psoriasis), parasitology (unicellular parasites), neurology (Alzheimer´s disease and other neurodegenerative diseases), and viral infections (CMV, HIV, herpes) (Gray *et al.* 1999, Buolamwini 2000, Fischer and Lane 2000, Furet *et al.* 2000, Garcia-Echeverria *et al.* 2000, McDonald and El-Deiry 2000, Rosania and Chang 2000, Sielecki *et al.* 2000). However, ongoing research has revealed a dearth of knowledge concerning the fate of CDK inhibitors at the level of cells, in animals and in man.

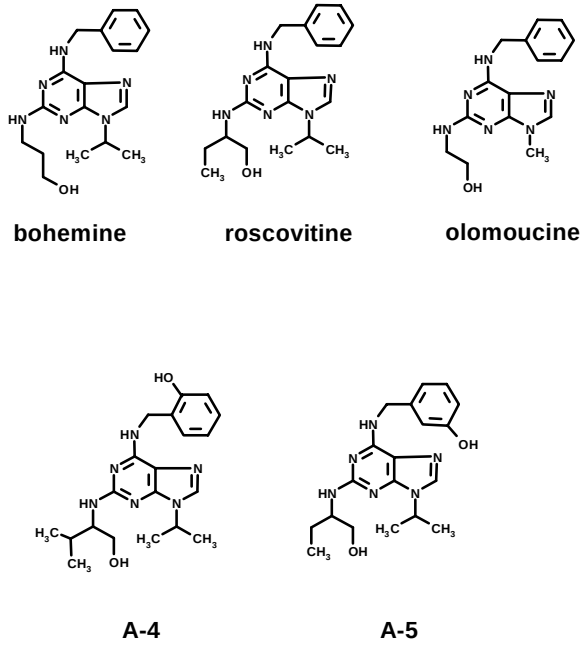

Fig. 1. *Chemical structures of olomoucine-type CDK inhibitors tested as aglycones.*

Our previous paper (Chmela *et al.* 2001) reported olomoucine-type CDK inhibitor 6-benzylamino-2-(3-hydroxypropyl)amino-9-isopropylpurine, named as bohemine, as a relatively good sugar acceptor in mice. A number of studies have shown that there exist vast species differences in glycosidation potential in animal tissues and the structural requirements for formation of various glycosides remain to be elucidated (Burchell *et al.* 1995). This problem is basic since glycosyl-transferases can use thousands of endobiotic and xenobiotic compounds as substrates (Mackenzie *et al.* 1997, Crout and Vic 1998, Elhalabi and Rice 1999, Radominska-Pandya *et al.* 1999, Tukey and Strassburg 2000). In particular, glycosidation might influence the *in vivo* metabolic fate of a new potential anticancer CDK inhibitory agent such as 6-benzylamino-2-(1-ethyl-2-hydroxyethylamino)-9-isopropylpurine, designated as roscovitine (Meijer *et al.* 1997), that has entered into clinical trials recently (Fischer and Lane 2000). Thus, we conducted the following *in vitro* investigation involving mouse, rat, monkey, as well as human liver and kidney microsomes with the aim of assessing their glycoconjugation potential towards several purine-derived selective CDK inhibitors whose structure is shown in Figure 1.

Material and Methods

Chemicals

β-Glucosidase, EC 3.2.1.21, β-glucuronidase, EC 3.2.1.31, UDP-glucuronic acid, UDP-glucose, UDP-galactose, UDP-xylose, UDP-glucosamine, UDP-N-acetylglucosamine and D-gluconic acid 1,5-lactone were purchased from Sigma-Aldrich, St. Louis, Missouri; and Silica gel 60 F_{254} TLC sheets were purchased from Merck, Darmstadt, Germany. All other chemicals and reagents used were of analytical or HPLC grade.

Chemical syntheses

6-Benzylamino-2-(3-hydroxypropyl)amino-9-isopropylpurine *(bohemine),* 6-benzylamino-2-(2-hydroxyethyl)amino-9-methylpurine (*olomoucine*), 6-benzylamino-2-[1-hydroxymethyl) propyl]amino-9-isopropylpurine (*roscovitine*), 6-(2-hydroxybenzyl)amino-2-(1-hydroxymethyl-2-methyl-propyl)amino-9-isopropylpurine (compound *A-4*), and 6-(3-hydroxybenzyl)amino-2-[(1(R/S)-hydroxymethyl) propyl]amino-9-isopropylpurine (compound *A-5*) derivatives were synthesized, labeled by ^{3}H at position 8 of their purine rings, and/or modified by diazomethylation as described previously (Havlíček *et al.* 1997, Otyepka *et al.* 2000, Chmela *et al.* 2001). The specific activities of the ^{3}H-labeled compounds used

throughout this study were between 56.2 TBq/mol to 490 TBq/mol.

Animal and human tissues

All the experiments using animal and human tissues 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 mice microsomes were obtained from monitored male NMRI outbred mice of mixed genetic background and of approximately 20-25 g body weight (BioTest, Konárovice, Czech Republic). The rat microsomes were isolated from monitored male Wistar-Han outbred rats of about 200 g body weight (BioTest, Konárovice, Czech Republic). The monkey (*Macaca mulatta*, 2.9 kg body weight) tissues were obtained from two experimental controls of a toxicologic study that had been treated twice by a vehiculum (citrate, ethanol, Cremophore L) and sacrified two weeks after the second application of the vehiculum (BioTest, Konárovice, Czech Republic). The human tissues were obtained from surgery of hepatic (two adult men) or kidney (two adult men) tumors. Only macroscopically normal pieces of the fresh tissue were utilized for the isolation of microsomal fractions.

Incubation of bohemine and its metabolites was performed with microsomes and enzymes. Liver and kidney microsomes were prepared in 0.25 M sucrose solution by differential centrifugation and their protein content was determined as described previously (Chmela *et al.* 2001). Shortly before use, the microsomes were transferred by repeated centrifugation (100 000 *x g*, 60 min) into a phosphate buffer (0.1 M, pH 7.4) supplemented with 5 mM $MgCl_2$. The incubations (0.2 ml, final volume) conducted in the presence of glycosyl donors employed microsomes (0.4 mg protein) and either UDP-glucuronic acid, UDP-glucose, UDP-galactose, UDP-xylose, UDP-glucosamine, or UDP-N-acetyl-glucosamine at concentrations given in the Results section. These were further supplemented with about 300 kBq ^{3}H-labeled compound (final concentrations of 3.0 μM or 26 μM compound; see Results). Incubates of ^{3}H-labeled reaction products extracted from TLC spots (see below) were supplemented with either 2.5 Sigma units β-glucosidase, or 0.67 Sigma units β-glucuronidase in citrate buffer (0.05 M, pH 5.0). The reactions were allowed to proceed in open tubes at 37 °C under continuous mixing and aliquots were removed after 30 min, 2 h and 4 h. Control tubes were supplemented with an appropriate volume of the buffers instead of the glycosyl donors or enzymes. No NADPH or NADPH-generating systems were added to reaction mixtures. The the reactions were performed in duplicates (see Results).

Extraction of metabolites and TLC analysis

For product screening, the aliquots removed as indicated above were extracted with a ninefold volume of ethanol and the ethanolic supernatants (4 000 x *g*, 5 min) and analyzed by TLC with subsequent ^{3}H-autoradiography and densitometry as described previously (Chmela *et al.* 2001). In particular, TLC was routinely run in acidic mobile phase chloroform-methanol-acetic acid 90:10:1 (by vol.; phase A). Acetic acid was replaced by one volume of concentrated aqueous ammonia (phase B) to establish (by changing TLC mobility of the spots) the acidic/basic character of substances. Autoradiography was performed using either ^{3}H-Hyperfilm or Kodak BioMax MS film with Kodak BioMax TranScreen LE at –70 °C (Amersham Pharmacia Biotech, Little Chalfont, England, Uppsala, Sweden, and Piscataway, NJ). Spot intensities were computerized and data processed using GeneGenius (Syngene, Cambridge, UK). Alternatively, the TLC spots were scraped out and subjected to enzymatic analysis with glycosidases.

Phase A/phase B R_F values of parent CDK inhibitors were as follows: bohemine, 0.63/0.75; olomoucine, 0.42/0.43; roscovitine, 0.65/0.79; A-4, 0.65/0.75; A-5, 0.44/0.41. Methylethers of parent compounds A-4 and A-5 prepared by diazomethane treatment (Chmela *et al.* 2001) exhibited R_F values of 0.66/0.78 and 0.64/0.76, respectively.

Results

Having published TLC, MS, and NMR data indicating that bohemine β-glucuronide and bohemine β-glucoside were the main phase II products of bohemine metabolism in mice *in vivo* (Chmela *et al.* 2001), we decided to assess, by a TLC/autoradiography screening, the glycoconjugation potential towards several olomoucine-type CDK inhibitors in various mammals. In the first part of this study, we investigated the ability of mouse, rat, monkey and human liver and kidney microsomes to glycosylate bohemine with an array of active glycosyl donors *in vitro*. In the second part, glucosidation and glucuronidation of bohemine, olomoucine, roscovitine, derivative A-4 and derivative A-5 were tested with mouse and monkey microsomes as representatives of the rodent and primate species, respectively.

Interspecies differences in microsomal glycosidations of bohemine

Table 1 demonstrates that all the species examined utilized UDP-glucuronic acid as a potent donor. Interestingly, the most active microsomes consuming this donor were those obtained from the monkey kidney followed by the mice kidney probe. This contrasted with the absence of glucuronidation activity in rat kidney microsomes and with the relatively moderate to low activity in human microsomes. The second most potent donor was UDP-xylose. Besides being effective in mice kidney probes, it was also extensively utilized by rat liver microsomes. On the other hand, mice kidney and liver were the only tissues that employed UDP-glucose and UDP-galactose, the kidney being considerably more effective. Indeed, the mice tissues were unique in the number of donor substrates that they could utilize to generate conjugates. N-acetylglucosamine functioned both in the mouse tissue and rat liver incubates (Table 1). Interspecies differences in microsomal glucosidation and glucuronidation of various aglycones. In Table 2, glucosidation and glucuronidation potential towards five olomoucine-type CDK inhibitors bohemine, roscovitine, olomoucine, A-4, and A-5 in mouse and monkey microsomes were compared. As apparent, bohemine was the best sugar acceptor among the inhibitors having the aliphatic hydroxyl group in their molecule followed by roscovitine in both species. However, out of the inhibitors having both aliphatic and aromatic hydroxyl groups, A-4 seemed to be a better substrate than A-5 since the former compound accepted both glucosyl and glucuronyl groups in mouse microsomes and produced glucuronides in both types of monkey microsomes. Moreover, monkey liver microsomes produced three types of glucuronides from aglycone A-4 and two types from A-5, whereas mouse microsomes synthesized only one glucuronide from each compound A-4 or A-5 (Table 3). Olomoucine was almost inactive as a sugar acceptor.

Table 1. Interspecies differences in microsomal glycosidation of bohemine (3 μM) by liver and kidney microsomes (2 mg microsomal protein/ml) in the presence of UDP-glycosyl donors (5 mM).

	Microsomes	Glucoside (%)			Xyloside (%)			Glucuronide (%)			Galactoside (%)			N-acetylaminoglucoside (%)		
		30'	120'	240'	30'	120'	240'	30'	120'	240'	30'	120'	240'	30'	120'	240'
Mouse	Liver	11	94	100	23	100	100	5	13	45	2	6	10	3	7	15
	Kidney	98	100	100	100	100	100	9	21	25	15	48	50	19	51	60
Rat	Liver	N. D.			26	100	100	11	27	52	N. D.			2	6	14
	Kidney	N. D.			N. D.			N. D.			N. D.			N. D.		
Monkey	Liver	N. D.			N. D.			28	87	100	N. D.			N. D.		
	Kidney	N. D.			N. D.			43	92	100	N. D.			N. D.		
Man	Liver	N. D.			N. D.	< 1	3	2	9	19	N. D.			N. D.		
	Kidney	< 1	3	< 1	N. D.			< 1	4	7	N. D.			N. D.		

Data represent mean percentage of individual ^{3}H-labeled bohemine transformed into glycosides in 30, 120 and 240 min time intervals (duplicate experiments). N. D. - not detected

Analysis of glycosidation products

The reaction products of UDP-glucose, UDP-xylose and UDP-N-acetylglucosamine donors with olomoucine-type inhibitors clearly exhibited dissimilar RF values in mobile TLC phases (Table 3) indicating that they were different entities. However, no distinctive features were found between the glucose and galactose reaction products. Furthermore, the existence of tentative

glucuronide conjugates were confirmed by β-glucuronidase digestion, while glucosidation and galactosidation products were decomposed by β-glucosidase, all of them giving back the ^{3}H-labeled parent inhibitor aglycones. Products of reactions with UDP-xylose and UDP-N-acetylglucosamine were resistant against both glycosidases (data not shown). The glucuronides were also subject to methylesterification reactions (Table 3). Importantly, in monkey liver microsomes where derivatives A-4 and A-5 gave three and two types of glucuronides, respectively, several types of the corresponding methylesterified products appeared (Table 3).

Table 2. Differences in glycosidation potential towards CDK inhibitors in mouse and monkey microsomes.

	Microsomes	Glucosides (%)					Glucuronides (%)				
		Bohemine	Roscovitine	Olomoucine	A-4	A-5	Bohemine	Roscovitine	Olomoucine	A-4	A-5
Mouse	Liver	100	2	N. D.	3	N. D.	21	N. D.	N. D.	52	96
	Kidney	100	9	N. D.	10	N. D.	7	N. D.	N. D.	100	98
Monkey	Liver	N. D.	N. D.	N. D.	N. D.	N. D.	61	13	4	73	31
	Kidney	N. D.	N. D.	N. D.	N. D.	N. D.	52	< 1	N. D.	6	N. D.

Incubations of CDK inhibitors with microsomes (2 mg microsomal protein/ml) in the presence of UDP-glucose or UDP-glucuronic acid (5 mM) were run for 2 h. The concentration of individual CDK inhibitors was adjusted by the addition of the non-labeled CDK inhibitor to a uniform level of 26 μM (i. e., the concentration of the substrate with the lowest specific radioactivity) in all probes. Data represent mean percentage of individual ^{3}H-labeled inhibitors transformed into glycosides (duplicate experiments) N. D. - not detected.

Table 3. TLC R_F values of various glycosylation products of 8-[^{3}H]-labeled CDK inhibitors.

Products	R_F values (A/B [a])				
	Bohemine	Roscovitine	Olomoucine	A-4	A-5
Glucoside	0.10/0.10	0.17/0.10	N. D.	0.14/0.06	N. D.
Xyloside	0.19/0.16	N. D.	N. D.	N. D.	N. D.
Glucuronide	0.09/0.00	0.11/0.00	0.03/0.00	0.11/0.00[b] 0.08/0.00[c] 0.13/0.01[c]	0.00/0.00[b] 0.03/0.00[c]
Methylesterified glucuronide	0.31/0.19	0.32/0.19	0.19/0.08	0.22/0.16[d] 0.31/0.19[e] 0.32/0.19[e]	0.12/0.10[d] 0.18/012[e]
Galactoside	0.10/0.10	N. D.	N. D.	N. D.	N. D.
N-acetylaminoglucoside	0.05/0.04	0.08/0.05	N. D.	N. D.	N. D.

[a]) A - R_F values in phase A (chloroform-methanol-acetic acid 90:10:1); B - R_F values in phase B (chloroform-methanol-ammonia 90:10:1) (Silica gel 60 F_{254} TLC sheets); [b]) mouse liver and kidney and monkey kidney microsomes; [c]) monkey liver microsomes; [d]) product of methylesterification of b); [e]) product of methylesterification of c). N. D. - not detected.

Discussion

In this study we screened *in vitro* glycosidation potential towards olomoucine-type CDK inhibitors in two rodent and two primate species. Other authors have acknowledged TLC and autoradiographic techniques used throughout this study as effective methods for determining glycosidation of a wide range of substrates (Bansal and Gessner 1980). HPLC techniques are more expensive and time consuming in general (Burchell *et al.* 1995).

Interpreting the results of this study, we have assumed that products of the glycosylation reactions performed by animal microsomes were appropriate

glycosides of individual CDK inhibitors tested by us. This premise was made by analogy, considering the explicit results of the previous study demonstrating the presence of the glycosides by using MS and NMR methods (Chmela *et al.* 2001). Three lines of evidence further substantiate this. First, as to bohemine, there was a clear congruity between RF values of its glycosidation products in different microsomes and standards obtained by Chmela *et al.* (2001). Second, the formation of different glycosidation products was strongly corroborated by their distinct enzymatic digestion, performed according to Matern *et al.* (1984) and Tang (1990) using β-glucuronidase or β-glucosidase, that resulted in ^{3}H-labeled parent aglycone recovery (see Results and Chmela *et al.* 2001). Third, the presence of a carboxylic group presumably introduced by glucuronidation into various inhibitor aglycones was checked by its succesful methylesterification. Nevertheless, our assumption remains open to definitive confirmation by direct techniques.

Principally, this study has verified (Table 1) that the aliphatic hydroxyl group of olomoucine-type inhibitors can be extensively conjugated with a glycoside moiety such as glucuronide in all examined species. The results have also suggested that O-β-D-glucoside conjugation, which was confined to microsomes of mouse origin, may not participate in clearance of the inhibitors in rat, monkey, or man. From this it is obvious that the finding of rapid transformation and clearance of bohemine from the mouse body (Chmela *et al.* 2001) cannot be simply extrapolated to other species including man and needs further systematic investigation. It is of relevance that the aliphatic hydroxyl group of the inhibitors is readily prone to oxidation attacks as well (Chmela *et al.* 2001).

It is known that UDP-sugar-dependent glucuronosyltransferase (UGT) isoforms are manifest with mutually distinct but overlapping substrate specificities (Radominska-Pandya *et al.* 1999, Tukey and Strassburg 2000). It had been postulated that some UGTs might be able to utilize various UDP-sugars, as distinct from UDP-glucuronide, to form glycosides (Vanstapel and Blanckaert 1987). However, glucosidation of both endo- and exogenous compounds, a reaction believed to represent a minor pathway if glucuronidation is possible in mammals (Tang 1990), has been shown to be catalyzed by either a specific UDP-glucose-dependent or a sugar nucleotide-independent glucosyltransferase (Matern *et al.* 1984,1990b, Radominska *et al.* 1993). Furthermore, UDP-sugar-dependent N-glucosaminyl-transferase has been identified as an enzyme responsible for N-acetylglucosamidination of small lipophilic agents, both in the liver and kidney (Marschall *et al.* 1987,1989, 1992, Matern *et al.* 1990a). Our results (Table 2) are in accordance with the notion of Marschall *et al.* (1994) suggesting that N-acetylglucosaminidation may be a more selective reaction than glucuronidation and glucosidation. Additionally, formation of galactosides and xylosides has been detected in *in vitro* experiments (Radominska *et al.* 1993, Vanstapel and Blanckaert 1987).

It is still premature to speculate about the physiological significance of individual glycosidation reactions described above as well as about enzyme isoforms participating in them. It is interesting that both organs, i.e. liver and kidney, that substantially influence glucose homeostasis in mammals are also able to channel a significant portion of glycosides into detoxification conjugation reactions. Other authors have explored some interspecies differences in glycosidation capacity. Although many foreign compounds having acidic (e. g. phenolic or carboxylic) groups are in general excreted as a glucuronide (or a sulfate), some of them such as 4-nitrophenol and others, undergo glucosidation (Gessner *et al.* 1973). Also relevant to our results are the papers of Nakano *et al.* (1986a,b) who were the first to report O-β-glucosidation of non-acidic (aliphatic) hydroxyl groups in mammals. Boberg *et al.* (1998) have demonstrated preferential formation of O-β-D-glucoside conjugate, as compared to production of glucuronide, in dogs, though the same detoxification reaction was absent in rodents.

A few comments could be said concerning the future design of new purine-derived CDK inhibitors. Both mice and monkey microsomes preferentially glycosidated bohemine over roscovitine and olomoucine (Table 2). Olomoucine was the least potent glycosyl acceptor. Roscovitine was demonstrated to be an inferior substrate to bohemine, probably due to sterical hindrance exhibited by the branched side chain at its C2 atom. This corroborates roscovitine as the most applicable candidate for a clinical examination.

On the other hand, derivatives A-4 and A-5 each possess two hydroxyl groups – an aliphatic and a phenolic one. It remains to be elucidated, by a more direct examination, which of A-4 and/or A-5 glucuronides obtained by us (Tables 2 and 3) were aliphatic or phenolic ones and which of them were mono- or diglucuronides. The possibility of N-glucuronide formation should also be examined.

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