

IN VITRO METABOLISM OF THE PHOSPHATIDYLINOSITOL 3-KINASE INHIBITOR, WORTMANNIN, BY CARBONYL REDUCTASE

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ABSTRACT:

The phosphatidylinositol 3-kinase inhibitor, wortmannin, is extensively used in molecular signaling studies and has been proposed as a potential antineoplastic agent. The failure to detect wortmannin in mouse plasma after i.v. administration prompted in vitro studies of wortmannin metabolism. Wortmannin was incubated with mouse tissue homogenates, homogenate fractions, or purified, recombinant human carbonyl reductase in the presence of specified cofactors and inhibitors. Reaction products were characterized and quantified with liquid chromatography (LC)/mass spectrometry. Reaction rates were characterized using Michaelis-Menten kinetics. Wortmannin was metabolized to a material 2 atomic mass units greater than wortmannin. Liver homogenate had the highest metabolic activity. Some metabolism occurred in kidney and lung homogenates. Very little metabolism occurred in brain or red blood cell homogenates. Liver S9 fraction and cytosol metabolized wortmannin in the presence of NADPH and, to a much

lesser extent, in the presence of NADH. Microsomal metabolism of wortmannin was minimal. Purified, recombinant human carbonyl reductase metabolized wortmannin. Quercetin, a carbonyl reductase inhibitor, greatly decreased wortmannin metabolism by S9, cytosol, and carbonyl reductase. The K_M for wortmannin metabolism by purified, recombinant human carbonyl reductase was $119 \pm 9 \mu\text{M}$, and the V_{max} was $58 \pm 9 \text{ nmol/min/mg}$ of protein. LC-tandem mass spectrometry spectra indicated that carbonyl reductase metabolized wortmannin to 17-OH-wortmannin. Wortmannin reduction by carbonyl reductase may partly explain why wortmannin is not detected in plasma after being administered to mice. Metabolism of wortmannin to 17-OH-wortmannin has mechanistic, and possibly toxicologic, implications because 17-OH-wortmannin is 10-fold more potent an inhibitor of phosphatidylinositol 3-kinase than is wortmannin.

Identification and characterization of molecules and signaling pathways involved in crucial cellular growth control and survival processes has fostered interest in utilizing these molecules and pathways as targets for anticancer drug development (Murray and Norbury, 2000; Noonberg and Benz, 2000; Demetri, 2001; Fabbro et al., 2002; Shoemaker et al., 2002). Phosphatidylinositol 3-kinase (PI3-kinase¹) plays an important role in cellular growth control and signaling pathways (Nakanishi et al., 1995; Carpenter and Cantley, 1996a,b; Ward et al., 1996; Turchi and Ho, 2000; Wymann et al., 2000; Balla, 2001; Vanhaesebroeck et al., 2001) and has been suggested as a potential molecular target for developing antitumor

drugs (Cardenas et al., 1998; Dong et al., 1999; Berrie, 2001; Stein, 2001). The PI3-kinase inhibitor, wortmannin, is extensively used in molecular signaling studies (Cuenda and Alessi, 2000; Hazeki et al., 1996; Ui et al., 1995) and has been proposed as a potential antineoplastic agent. As part of a preclinical evaluation of wortmannin performed in anticipation of subsequent clinical studies, we studied the pharmacokinetics of wortmannin in female CD₂F₁ mice that had been given 5 mg/kg i.v. doses of wortmannin. Using high-performance liquid chromatography, we were unable to detect wortmannin in the plasma of mice euthanized at 2 or 5 min after dosing, which were the earliest times sampled. Therefore, we developed a more sensitive LC/MS method to detect wortmannin and possible metabolites and applied it to two in vitro situations. We initially characterized the pH-dependent decomposition of wortmannin in tissue culture media and other aqueous environments (Holleran et al., 2003). We have now investigated the metabolism of wortmannin under in vitro conditions that have shown wortmannin to be stable (Holleran et al., 2003) and that have been demonstrated to be suitable for the enzyme demonstrated to be responsible for wortmannin metabolism (Felsted et al., 1977).

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¹ Abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; amu, atomic mass unit(s).

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Materials and Methods

Reagents. NADPH, NADH, FAD, bovine serum albumin, quercetin, EDTA, lysozyme, sodium dodecyl sulfate, isopropyl β -D-thiogalactopyranoside, phenylmethanesulfonyl fluoride, streptomycin sulfate, ampicillin, glutathione-agarose, dithiothreitol, and glutathione were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate-buffered saline, Tris-HCl, ethyl acetate, acetonitrile, methanol, glacial acetic acid, and formic acid were obtained from Fisher Chemicals (Fairlawn, NJ). Medical grade nitrogen and liquid nitrogen were purchased from Valley National Gases (West Mifflin, PA).

Wortmannin and NSC 722135 (pyrrolidino-wortmannin), the wortmannin analog used as the internal standard in LC/MS assays, were supplied by the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD).

Mice. Specific-pathogen-free, adult, female CD₂F₁ mice were obtained from the Animal Program administered by the Biological Testing Branch of the National Cancer Institute. To minimize exogenous infection, mice were maintained in microisolator cages in separate rooms and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Preparation of Mouse Tissue Homogenates and Hepatic Fractions. Mice were euthanized by carbon dioxide asphyxiation and exsanguinated by cardiac puncture, after which their livers, lungs, kidneys, and brains were rapidly removed. Gallbladders were removed with care to prevent contamination of the liver with bile. Livers, lungs, kidneys, and brains were placed immediately onto dry ice and then stored at -70°C until used.

Blood was centrifuged at 13,000g for 5 min. The resulting red blood cell pellet was washed twice with iced phosphate-buffered saline, resuspended to the original blood volume with phosphate-buffered saline, and stored at -70°C until used.

Tissues were washed with 0.15 M KCl, minced, and placed into a glass Potter-Elvehjem homogenizer with a Teflon pestle. Tissues were homogenized in 3 volumes of 0.1 M potassium phosphate buffer (pH 7.4) and transferred to 15-ml polypropylene tubes. Nuclei and cellular debris were sedimented by centrifugation for 10 min at 2500g and 4°C . In some cases, the resulting supernatant fractions were stored at -70°C until used. Mouse hepatic fractions were prepared as follows. Portions of the mouse liver 2500g supernatant were transferred into clean 15-ml Corex tubes and centrifuged for 15 min at 9600g and 4°C to sediment mitochondria and produce a 9600g supernatant (S9 fraction). Microsomal and cytosolic fractions were prepared by centrifugation of the S9 fraction for 10 min at 29,500g and 4°C , and then centrifuging the 29,500g supernatant at 105,000g for 60 min and 4°C . The resulting supernatant was designated as the cytosolic fraction. The sedimented pellet was designated as the microsomal fraction and was resuspended in 0.1 M potassium phosphate buffer (pH 7.4). All hepatic fractions were stored at -70°C until used.

Expression and Purification of Human Carbonyl Reductase. A human carbonyl reductase (secondary alcohol:NADPH⁺ oxidoreductase, EC 1.1.1.184) cDNA sequence (GenBank accession number 504056) isolated from the MCF-7 breast cancer cell line (Forrest et al., 1990) was inserted between the restriction sites, NdeI and BamHI, of the expression vector pET11a (Novagen, Madison, WI). Expression of recombinant carbonyl reductase with this clone was achieved in *Escherichia coli* BL21(DE3) (Novagen). A single bacterial colony was inoculated into 50 ml of Luria-Bertani broth containing 100 $\mu\text{g}/\text{ml}$ ampicillin and was incubated overnight at 37°C . The overnight culture was expanded further in 2 liters of Luria-Bertani broth with ampicillin until the optical density at 600 nm was between 0.4 and 0.6. Protein expression was induced by the addition of 0.5 mM isopropyl β -D-thiogalactopyranoside and incubation for an additional 3 h at 37°C . The induced bacteria were harvested by centrifugation and resuspended in 100 ml of a buffer containing 10 mM Tris-HCl (pH 8), 0.1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 5 $\mu\text{g}/\text{ml}$ lysozyme. The mixture was rocked gently at 4°C for 30 min, after which bacteria were lysed on ice by sonication with a Branson Sonifier (Branson Ultrasonics Corp., Danbury, CT). After the addition of streptomycin sulfate to a final concentration of 3 mg/ml and stirring for 10 min at 4°C , the homogenate was centrifuged at 20,000g for 15 min. Carbonyl reductase was purified from the resulting supernatant using glutathione-agarose (Sigma-Aldrich) and a procedure described by Toft et al. (1994). The purity of the isolated protein was verified by electrophoresis on 5% SDS-

polyacrylamide gels (Bio-Rad, Hercules, CA) and staining with Coomassie Blue (Bio-Rad). Final carbonyl reductase preparations were divided into 200- μl aliquots and stored at -70°C until use, which was less than 30 days. During that period there was no statistically significant loss of enzymatic activity.

Measurement of Protein Concentrations. Protein concentrations of tissue homogenates, hepatic fractions, and carbonyl reductase preparations were determined using the Coomassie Plus protein assay reagent kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

Incubation of Tissue Preparations or Carbonyl Reductase with Wortmannin. All incubations were performed in 12×75 mm borosilicate glass tubes, under aerobic conditions, and in a Dubnoff shaking water bath set at 37°C and 33 to 36 oscillations/min. Incubation mixtures were of 200- μl volume and contained the following enzyme sources: 20 μl of mouse liver, lung, kidney, or brain 2500g supernatant; 20 μl of red blood cell homogenate; or 10 μl of mouse hepatic S9 fraction, microsomes, or cytosol. Reaction mixtures also contained 10 to 20 μl of designated combinations of 30 mM NADPH and 30 mM NADH and 10 μl of 200 $\mu\text{g}/\text{ml}$ (0.46 mM) wortmannin in ethanol/water (1:4, v/v). In experiments evaluating the necessity of NADPH or NADH for wortmannin metabolism, these cofactors were omitted from the incubation mixture. Buffering was accomplished with 100 μl of 0.2 M potassium-phosphate buffer, pH 6.0, or phosphate-buffered saline when red blood cell homogenate was the enzyme source in the incubation mixture. Distilled water was used to complete the 200- μl incubation volume. Reactions were initiated by addition of coenzymes. After 5 min of incubation, 1 ml of ethyl acetate was added to each tube, the tubes were vortexed briefly, and 5 μl of 2 $\mu\text{g}/\text{ml}$ internal standard in water were added to each tube. A 5-min incubation was chosen because preliminary experiments had documented that time to be in the linear portion of the time course of in vitro metabolism under the conditions used in these studies. The samples were then transferred into 1.5-ml microcentrifuge tubes, which were vortexed for 5 min on a Vortex Genie-2 (Scientific Products, Bohemia, NY) and then centrifuged at room temperature for 5 min at 16,000g. The resulting upper, organic layers were transferred into clean 12×75 mm glass tubes and dried under a stream of nitrogen. The dried residues were resuspended in 135 μl of methanol/water (35:65, v/v) and sonicated for 5 min. The solutions were transferred to autosampler vials and 50 μl were injected, by autosampler, into the LC/MS system described below.

Experiments evaluating the potential role of carbonyl reductase in wortmannin metabolism used 10 μl of purified, recombinant human carbonyl reductase instead of mouse tissue homogenates or hepatic fractions. Carbonyl reductase incubations also included 10 μl of 0.5 mM FAD and 10 μl of 2% bovine serum albumin.

Experiments investigating the ability of quercetin to inhibit wortmannin metabolism included designated quercetin concentrations between 0 and 460 μM . Due to the limited solubility of quercetin in water, quercetin was dissolved in ethanol. Therefore, each experiment also included control incubations that contained the appropriate volume of ethanol.

Samples used for LC-MS/MS identification of which carbonyl functionality on wortmannin was reduced by carbonyl reductase differed from the standard incubation conditions described above in that 80 μl of mouse cytosol was used and the incubation time was 60 min.

Liquid Chromatography/Mass Spectrometry. The LC/MS system consisted of an Agilent 1100 autosampler with a 100- μl sample loop (Agilent Technologies, Palo Alto, CA) and an Agilent 1100 quaternary pump fitted with a Brownlee NewGuard RP-18 (7- μm particle size, 3.2 mm \times 15 mm) and a Waters μ Bondapak C18 column (10- μm particle size, 3.9 mm \times 300 mm) (Waters, Milford, MA). The mobile phase was a 40-min gradient that contained 0.1% formic acid in all solvents and was pumped at 1 ml/min. Initial conditions were methanol/water (35:65, v/v) and were maintained for 5 min. The methanol content of the mobile phase was then increased, in a linear manner, over 25 min to methanol/water (45:55, v/v). Over the next minute, the mobile phase was returned to initial conditions, which were maintained for 9 min before the next sample was injected. Column eluate was split by a P-470 micro-splitter valve (Upchurch Scientific, a division of Scivex, Oak Harbor, WA), so that 10% of the flow entered the mass spectrometer.

Column eluate was analyzed with a Thermo Finnigan aQa Mass Spectrometer (Thermo Finnigan, San Jose, CA) operating in electrospray, positive single-ion mode to monitor $429.3 \pm 0.5 m/z$ for wortmannin, $431.3 \pm 0.5 m/z$

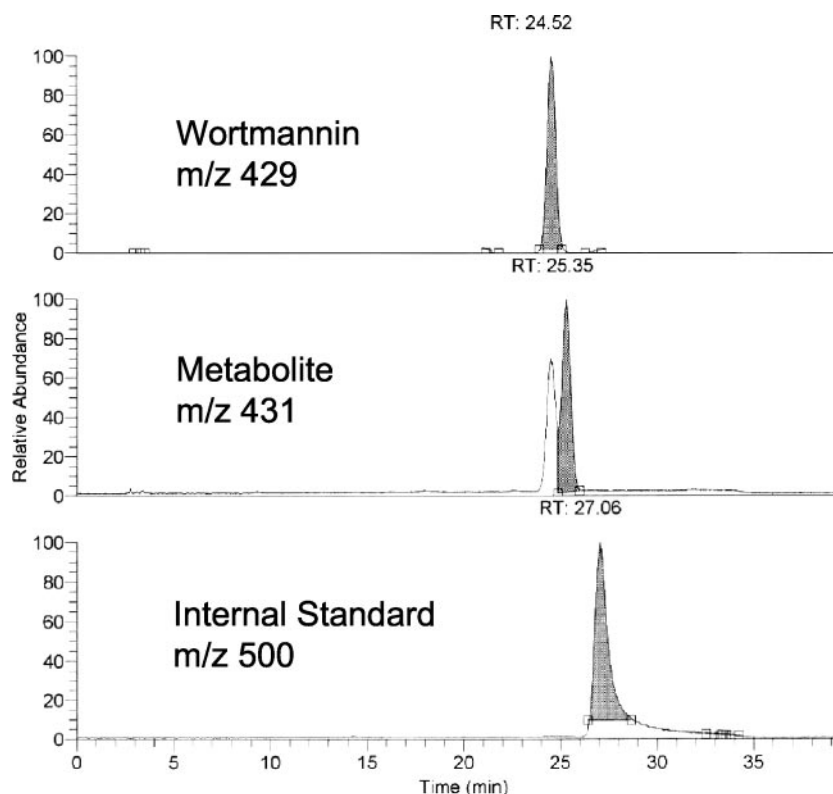


FIG. 1. LC/MS chromatograph of wortmannin, its proposed alcohol metabolite, and internal standard detected in a reaction mixture containing mouse liver homogenate, wortmannin, NADPH, and NADH, as indicated under Materials and Methods.

for the reduced metabolite of wortmannin, and 500 ± 0.5 m/z for the internal standard. The insert probe temperature was set at 300°C with 4000 V applied as the ion spray voltage and 10 V as the orifice voltage. Nitrogen gas flow was fixed by the tank head unit set at 75 psi (520 kPa). The system was operated with Thermo Finnigan Excalibur Software.

The IS ratio was calculated for each standard by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves of wortmannin, containing concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 $\mu\text{g}/\text{ml}$, were constructed by plotting the IS ratio versus the known concentration of wortmannin in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. With this assay procedure, the extraction efficiency for wortmannin was 86%. The lower limit of quantitation of the assay was 0.01 $\mu\text{g}/\text{ml}$, and the assay was linear over the concentration range of 0.01 to 100 $\mu\text{g}/\text{ml}$.

LC-MS/MS analyses used an Agilent model 1100 autosampler and pump that provided linear gradients and a constant flow rate of 200 $\mu\text{l}/\text{min}$. All chromatography was performed on a Phenomenex Luna C18 (2) column (3- μm particle size, 2 mm \times 150 mm) (Phenomenex, Torrance, CA). A mobile phase consisting of acetonitrile/water (65:35, v/v), buffered with 0.2% formic acid, was used for all separations. Under these conditions, wortmannin eluted at 13 min, and the reduced metabolite eluted at 7.9 min.

TABLE 1

Metabolism of wortmannin by unfractionated mouse tissue homogenates		
Tissue	Rate of Metabolite Production ^a	Rate Relative to Liver
	<i>nmol/min/mg protein</i>	
Liver	0.18 ± 0.01^b	1.00
Kidney	0.06 ± 0.01	0.35
Lung	0.03 ± 0	0.16
Brain	N.D. ^c	
Red blood cells	0.01 ± 0.00	0.03

^a Both NADPH and NADH were included as cofactors.

^b Mean \pm S.D.

^c N.D., no detectable metabolite in reaction mixture.

Mass spectrometry was performed on a Micromass Quattro micro triple quadrupole mass spectrometer (Waters), using electrospray ionization. Collision-induced MS/MS spectra were acquired from positive ion parents at a rate of 1 scan/s, over a mass range of 20 to 450 Da. MS/MS mass spectra were acquired with a collision cell pressure of approximately 1 mTorr, and a collision energy of 30 V.

Enzyme Kinetic Parameter Estimations. Protein concentrations used in incubation reactions were chosen so that reaction rates were linear for at least 20 min. Because authentic standards of reduced wortmannin metabolite were not available, ratios of metabolite area to that of internal standard were related to the wortmannin standard curve and expressed as molar equivalents of wortmannin. The concentrations of wortmannin used as substrate were limited to a maximum of 300 μM by the solubility of wortmannin. The apparent kinetic parameters, K_M and V_{max} , were determined by nonlinear regression analysis of substrate concentration versus rate data. Specifically, the Michaelis-Menten equation was used.

TABLE 2

Metabolism of wortmannin by mouse hepatic fractions and purified, human carbonyl reductase

Fraction	Cofactor	Rate of Metabolite Production	Rate Relative to S9 and NADPH
		<i>nmol/min/mg protein</i>	
S9	NADPH	1.89 ± 0.22^a	1.00
S9	NADH	0.53 ± 0.07	0.28
S9	None	0	0
Cytosol	NADPH	1.88 ± 0.47	0.99
Cytosol	NADH	0.13 ± 0.19	0.07
Cytosol	None	0	0
Microsomes	NADPH	0.18 ± 0.02	0.10
Microsomes	NADH	N.D. ^b	
Carbonyl reductase	NADPH	8.85 ± 0.10	4.68
Carbonyl reductase	None	0	0

^a Mean \pm S.D.

^b N.D., no detectable metabolite in reaction mixture.

TABLE 3

Quercetin inhibition of wortmannin metabolism by mouse hepatic fractions and purified, human carbonyl reductase

Quercetin/ Wortmannin	S9		Cytosol		Carbonyl Reductase	
	Rate of Metabolite Production	Percentage Inhibition	Rate of Metabolite Production	Percentage Inhibition	Rate of Metabolite Production	Percentage Inhibition
	<i>nmol/min/mg protein</i>		<i>nmol/min/mg protein</i>		<i>nmol/min/mg protein</i>	
Ethanol control	0.26 ± 0.03 ^{a,b,c}	0	0.31 ± 0.02	0	3.42 ± 1.16	0
5:1	0.16 ± 0.02	37	0.22 ± 0.01	28	0 ± 0	100
10:1	0.15 ± 0.02	44	0.19 ± 0.01	37	0 ± 0	100
20:1	0.09 ± 0.01	64	0.15 ± 0.02	51	0 ± 0	100

^a Mean ± S.D.^b NADPH was the cofactor.^c Quercetin was dissolved in ethanol and added to reaction mixtures in 9.2 μl to give inhibitor/substrate ratios at 5:1, 10:1, and 20:1. Ethanol alone (2.5 μl) was added to control reactions.

lis-Menten equation was fit to data from individual experiments using the computer program ADAPT II (D'Argenio and Schumitzky, 1979).

Results

Because wortmannin was known to be unstable at alkaline pH, preliminary studies were performed to characterize the stability of wortmannin at pH 7.4. These studies showed that 10 μg/ml solutions of wortmannin in phosphate-buffered saline, pH 7.4, decomposed when incubated at 4°C so that by 15 min, only 81 ± 6% of the original wortmannin remained. Subsequent studies demonstrated that wortmannin was stable at pH 6.2, and therefore, all subsequent metabolism studies were done at this pH.

Incubation of wortmannin with liver homogenate, NADPH, and NADH resulted in production of a new material with a molecular weight that was 2 amu greater than that of wortmannin (Fig. 1). Under the chromatographic conditions used, the retention times of wortmannin, its presumed metabolite, and internal standard were approximately 24.5, 25.4, and 27.1 min, respectively. Of the tissues examined, liver had the highest rate of conversion of wortmannin to its presumed reduced metabolite, although some metabolism of wortmannin occurred when it was incubated with kidney or lung homogenate, NADPH, and NADH (Table 1). There was very little metabolism of wortmannin by brain or red blood cell homogenates (Table 1).

When liver homogenate was fractionated by centrifugation, the S9 fraction retained the ability to metabolize wortmannin, and NADPH was preferred as a cofactor over NADH (Table 2). Subsequent studies showed that cytosol metabolized wortmannin in the presence of NADPH and, to a much lesser extent, in the presence of NADH (Table 2). Microsomal metabolism of wortmannin was minimal (Table 2), even though microsomal metabolic activity at pH 6.2 was confirmed by their ability to metabolize 17-allylaminogeldanamycin to its metabolite 17-aminogeldanamycin (Egorin et al., 1998). Metabolism of wortmannin occurred after incubation with purified, recombinant human carbonyl reductase and at a rate greater than that associated with the S9 fraction or cytosol (Table 2). There was no metabolism of wortmannin by liver homogenate, S9 fraction, cytosol, or purified carbonyl reductase in the absence of NADPH or NADH (Table 2).

Quercetin, a carbonyl reductase inhibitor (Forrest and Gonzalez, 2000), greatly reduced wortmannin metabolism by the S9 fraction, cytosol, and purified, recombinant human carbonyl reductase (Table 3). This inhibition increased with increasing ratios of quercetin to wortmannin. Although ethanol controls indicated that ethanol alone reduced the metabolism of wortmannin by approximately 33%, the inhibition by quercetin was dose-dependent and always greater than that observed with ethanol alone (Table 3).

With the strong evidence that carbonyl reductase was the enzyme responsible for reduction of wortmannin, kinetic studies were under-

taken with purified, recombinant human carbonyl reductase. These studies demonstrated the K_M for wortmannin reduction by carbonyl reductase to be 119 ± 9 μM and the V_{max} for this process to be 58 ± 9 nmol/min/mg of protein (Fig. 2, A and B).

LC-MS/MS spectra of wortmannin and the metabolite produced by carbonyl reductase indicated that the carbonyl group reduced by carbonyl reductase was located at position 17 (Fig. 3). This was

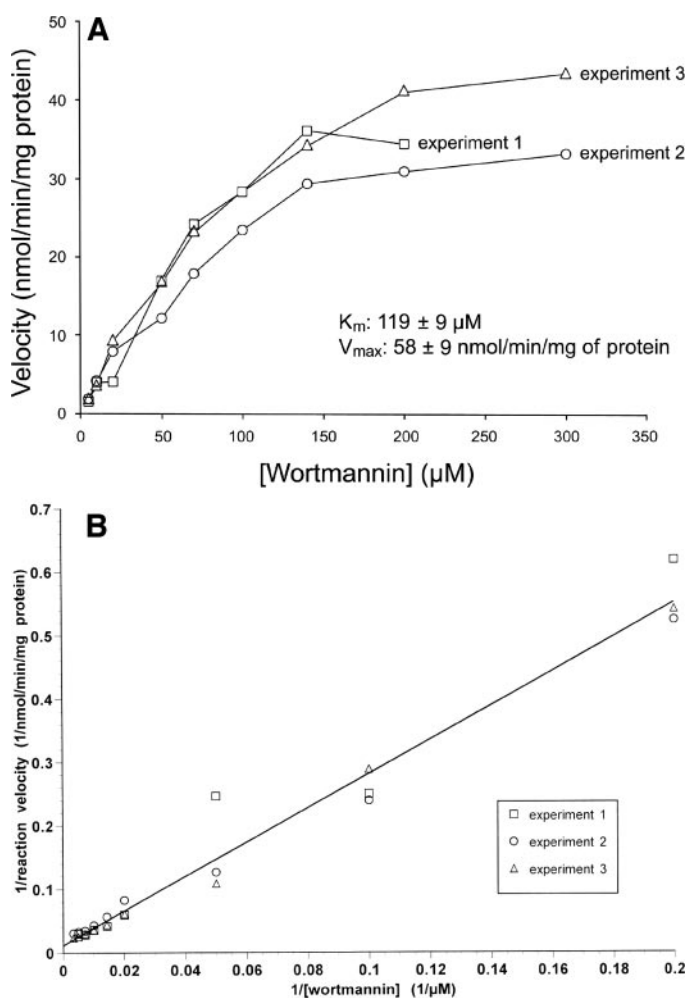
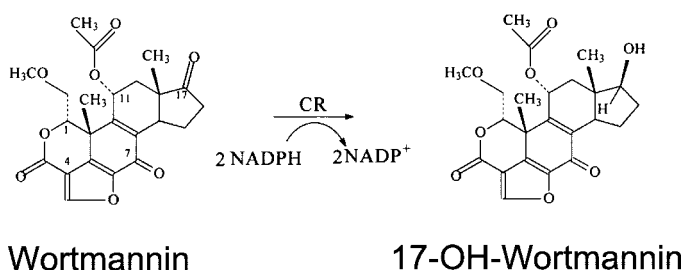


FIG. 2. Plot of wortmannin concentration versus rate of metabolism by purified recombinant human carbonyl reductase (A) and double-reciprocal plot of the data displayed in A (B).

Wortmannin was incubated under aerobic conditions and at pH 6.2 with carbonyl reductase and NADPH. Symbols represent the means of three separate experiments, each performed in triplicate. The line in B represents the best fit to the combined data from experiments 1, 2, and 3.



M.W. 428

M.W. 430

FIG. 3. Chemical structure of wortmannin and scheme by which it is metabolized by carbonyl reductase to produce 17-OH-wortmannin.

demonstrated by performing sequential MS/MS experiments on fragments of both compounds until a common ion with the same mass was identified. Those experiments showed similar fragmentation patterns, with a persistence of a 2-amu difference in fragments from wortmannin and its metabolite. This sequence was observed until loss of the portion of the molecules containing the 17 position resulted in a common ion with m/z 225 (Figs. 4, A and B, and 5)

Discussion

PI3-kinase, a heterodimeric protein consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (Stephens et al., 1993; Fry, 1994), has been implicated as playing an essential role in signal-

ing cascades related to a number of important cellular processes (Nakanishi et al., 1995; Carpenter and Cantley, 1996a,b; Ward et al., 1996; Turchi and Ho, 2000; Wymann et al., 2000; Balla, 2001; Vanhaesebroeck et al., 2001). Wortmannin, a natural product initially isolated from the fungus *Penicillium wortmanni* (Brian et al., 1957) and now prepared from various *Fusarium* species (Abbas and Mirocha, 1988; Abbas et al., 1989), has been utilized extensively as a tool for studying PI3-kinase signaling cascades and pathways (Ui et al., 1995). As a result of the importance of PI3-kinase in cellular growth control and the specificity of inhibition of PI3-kinase by wortmannin, wortmannin has been considered for development as a potential antineoplastic agent.

One obstacle to evaluating the in vivo pharmacology of wortmannin has been the lack of an assay suitable for quantitation of wortmannin in biological matrices such as plasma, normal tissues, and tumors. To date, the only published assay for quantitation of wortmannin involves assessment of potential contamination of agricultural products with wortmannin and utilizes 40 g of corn kernels as the sample to be studied (Xu and Mirocha, 1994). The LC/MS assay described in the current article addresses the need for a method suitable for pharmacology studies. As demonstrated, the assay is applicable to small volumes of proteinaceous matrices and has a sensitivity that should be suitable for animal and clinical pharmacokinetic studies, should they be performed. Furthermore, the use of mass spectrometric detection rather than absorbance detection allows specificity of analyte determination.

The results of the in vitro metabolism studies presented have

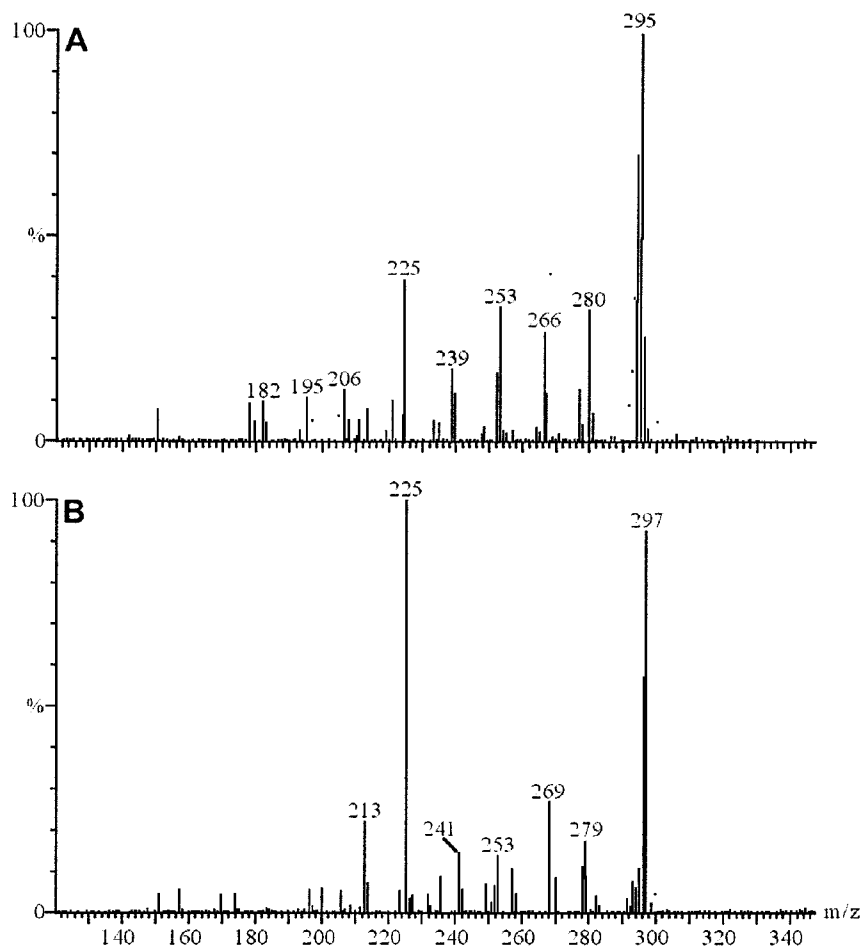


FIG. 4. MS/MS spectra of major ionization-induced fragment ions for wortmannin (m/z 295; A) and the reduced metabolite (m/z 297; B) of wortmannin produced by carbonyl reductase.

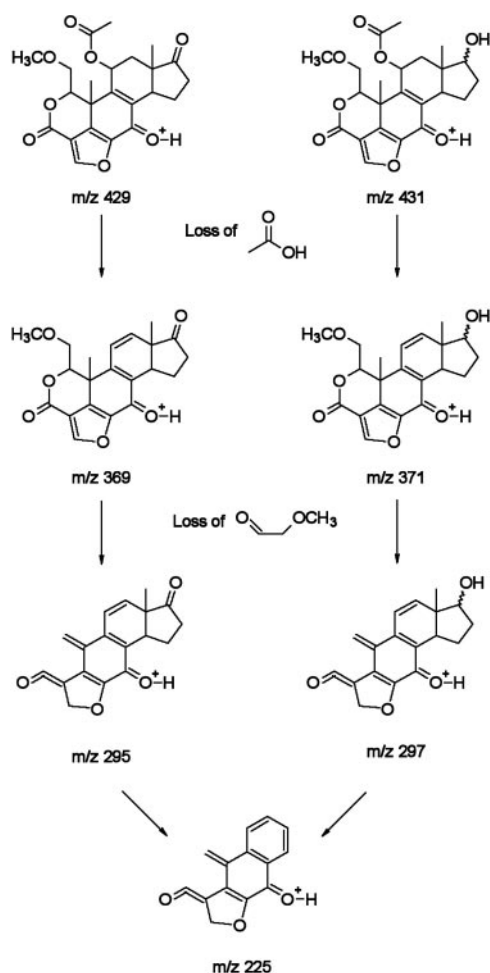


FIG. 5. Fragmentation patterns of wortmannin (left-hand series) and its reduced metabolite (right-hand series) demonstrating a persistent 2-amu difference in ions until a common ion with m/z 225 resulted from the loss of that portion of the molecule containing carbon 17.

several implications. It is clear that wortmannin is a substrate for carbonyl reductase (Bachur, 1976; Flynn and Green, 1993; Jez et al., 1997; Forrest and Gonzalez, 2000). This could be inferred from the sequential demonstration that 1) wortmannin was metabolized by mouse hepatic cytosol; 2) NADPH was the favored cofactor; and 3) the carbonyl reductase inhibitor, quercetin, decreased wortmannin metabolism in a dose-dependent fashion. These observations led to the conclusive demonstration that purified, recombinant human carbonyl reductase metabolized wortmannin and the calculation of the apparent K_M and V_{max} for that process.

Identification of carbonyl reductase as an enzyme responsible for metabolism of wortmannin implies that such metabolism involves reduction of a carbonyl functionality to an alcohol and is consistent with the known broad substrate specificity of carbonyl reductase and the fact that carbonyl reductase is known to have a pH optimum of 6.2 (Bachur, 1976; Felsted et al., 1977; Flynn and Green, 1993; Jez et al., 1997; Forrest and Gonzalez, 2000). Identification of 17-OH-wortmannin as the metabolite produced by carbonyl reductase has important mechanistic, and potentially toxicologic, implications because 17-OH-wortmannin is known to bind to PI3-kinase (Thelen et al., 1994). Moreover, 17-OH-wortmannin has been shown to be approximately 10-fold more potent than wortmannin in its ability to inhibit PI3-kinase (Norman et al., 1996). Furthermore, additional *in vivo* studies with chemically produced 17-OH-wortmannin have shown it to be

more toxic than wortmannin but inactive against the C3H mammary tumor model that is sensitive to wortmannin (Norman et al., 1996).

Although the exact structure of the wortmannin metabolite produced by carbonyl reductase has been identified and shown to be different from the compounds that result from pH-dependent chemical degradation of wortmannin (Holleran et al., 2003), the full extent of wortmannin metabolism after it is administered to an animal is unknown. Moreover, it should be recognized that although the studies described in this article used a pH that was known to be a pH optimum for carbonyl reductase and prevented wortmannin decomposition, pH 6.2 may not represent the intracellular pH where wortmannin metabolism would occur. It is unclear whether other enzymes might produce wortmannin metabolites other than the alcohol produced by carbonyl reductase. In addition, the fact that quercetin was so much more efficient in inhibiting the metabolism of wortmannin by carbonyl reductase than by hepatic S9 or cytosolic fractions may indicate the ability of other enzymes to convert wortmannin to 17-OH-wortmannin. Furthermore, whereas carbonyl reductase has been shown to reduce wortmannin, subsequent phase II metabolism, such as glucuronidation, of 17-OH-wortmannin, could occur *in vivo* but would not have been possible under the *in vitro* conditions used in the current studies. Finally, the relative contributions of chemical degradation versus metabolism in *in vivo* systems remains to be defined.

Although development of antitumor agents targeted against specific molecules and pathways related to cell growth and other critical cellular functions occupies a central role in antineoplastic pharmacology, a number of potential barriers must be recognized, and possibly overcome, before the success of this strategy becomes a common reality. Among the potential barriers are differences between studies done with isolated molecular targets in cell-free systems or cells in tissue culture and studies done *in vivo* in which drug metabolism to potentially inactive or toxic metabolites can occur. The data in the current article provide important insight into the behavior of wortmannin should further preclinical and possible clinical studies of that agent be pursued. Furthermore, the LC/MS assay developed for quantitation of wortmannin should be a valuable tool for use in those studies.

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References

- Abbas HK and Mirocha CJ (1988) Isolation and purification of a hemorrhagic factor (wortmannin) from *Fusarium oxysporum* (N17B). *Appl Environ Microbiol* **54**:1268–1274.
- Abbas HK, Mirocha CJ, and Gunther R (1989) Mycotoxins produced by toxic *Fusarium* isolates obtained from agricultural and nonagricultural areas (Arctic) of Norway. *Mycopathologia* **105**:143–151.
- Bachur NR (1976) Cytoplasmic aldo-keto reductases: a class of drug metabolizing enzymes. *Science (Wash DC)* **193**:595–597.
- Balla T (2001) Pharmacology of phosphoinositides, regulators of multiple cellular functions. *Curr Pharm Des* **7**:475–507.
- Berrie CP (2001) Phosphoinositide 3-kinase inhibition in cancer treatment. *Expert Opin Investig Drugs* **10**:1085–1098.
- Brian PW, Curtis PJ, Hemming HG, and Norris GLF (1957) Wortmannin, an antibiotic produced by *Penicillium wortmanni*. *Trans Br Mycol Soc* **40**:365–368.
- Cardenas ME, Sanfridson A, Cutler NS, and Heitman J (1998) Signal-transduction cascades as targets for therapeutic intervention by natural products. *Trends Biotechnol* **16**:427–433.
- Carpenter CL and Cantley LC (1996a) Phosphoinositide 3-kinase and the regulation of cell growth. *Biochim Biophys Acta* **1288**:M11–M16.
- Carpenter CL and Cantley LC (1996b) Phosphoinositide kinases. *Curr Opin Cell Biol* **8**:153–158.
- Cuenda A and Alessi DR (2000) Use of kinase inhibitors to dissect signaling pathways. *Methods Mol Biol* **99**:161–175.
- D'Argenio DZ and Schumitzky A (1979) A program package for simulation and parameter estimation in pharmacokinetic systems. *Comput Programs Biomed* **9**:115–134.

- Demetri GD (2001) Targeting c-kit mutations in solid tumors: scientific rationale and novel therapeutic options. *Semin Oncol* **28**:19–26.
- Dong Z, Huang C, and Ma WY (1999) PI-3 kinase in signal transduction, cell transformation and as a target for chemoprevention of cancer. *Anticancer Res* **19**:3743–3747.
- Egorin MJ, Rosen DM, Wolff JH, Callery PS, Musser SM, and Eiseman JL (1998) Metabolism of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) by murine and human hepatic preparations. *Cancer Res* **58**:2385–2396.
- Fabbro D, Ruetz S, Buchdunger E, Cowan-Jacob SW, Fendrich G, Liebetanz J, Mestan J, O'Reilly T, Traxler P, Chaudhuri B, et al. (2002) Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharmacol Ther* **93**:79–98.
- Felsted RL, Richter DR, and Bachur NR (1977) Rat liver aldehyde reductase. *Biochem Pharmacol* **26**:1117–1124.
- Flynn TG and Green NC (1993) The aldo-keto reductases: an overview. *Adv Exp Med Biol* **328**:251–257.
- Forrest GL, Akman S, Krutzik S, Paxton RJ, Sparkes RS, Doroshow J, Felsted RL, Glover CJ, Mohandas T, and Bachur NR (1990) Induction of a human carbonyl reductase gene located on chromosome 21. *Biochim Biophys Acta* **1048**:149–155.
- Forrest GL and Gonzalez B (2000) Carbonyl reductase. *Chem Biol Interact* **129**:21–40.
- Fry MJ (1994) Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* **1226**:237–268.
- Hazeki O, Hazeki K, Katada T, and Ui M (1996) Inhibitory effect of wortmannin on phosphatidylinositol 3-kinase-mediated cellular events. *J Lipid Mediat Cell Signal* **14**:259–261.
- Holleran JL, Egorin MJ, Zuhowski EG, Parise RA, Musser SM, and Pan S (2003) Use of high-performance liquid chromatography to characterize the rapid decomposition of wortmannin in tissue culture media. *Anal Biochem* **323**:19–25.
- Jez JM, Bennett MJ, Schlegel BP, Lewis M, and Penning TM (1997) Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* **326** (Pt 3):625–636.
- Murray RZ and Norbury C (2000) Proteasome inhibitors as anti-cancer agents. *Anticancer Drugs* **11**:407–417.
- Nakanishi S, Yano H, and Matsuda Y (1995) Novel functions of phosphatidylinositol 3-kinase in terminally differentiated cells. *Cell Signal* **7**:545–557.
- Noonberg SB and Benz CC (2000) Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs* **59**:753–767.
- Norman BH, Shih C, Toth JE, Ray JE, Dodge JA, Johnson DW, Rutherford PG, Schultz RM, Worzalla JF, and Vlahos CJ (1996) Studies on the mechanism of phosphatidylinositol 3-kinase inhibition by wortmannin and related analogs. *J Med Chem* **39**:1106–1111.
- Shoemaker RH, Scudiero DA, Melillo G, Currens MJ, Monks AP, Rabow AA, Covell DG, and Sausville EA (2002) Application of high-throughput, molecular-targeted screening to anticancer drug discovery. *Curr Top Med Chem* **2**:229–246.
- Stein RC (2001) Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocr Relat Cancer* **8**:237–248.
- Stephens LR, Jackson TR, and Hawkins PT (1993) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim Biophys Acta* **1179**:27–75.
- Thelen M, Wymann MP, and Langen H (1994) Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes. *Proc Natl Acad Sci USA* **91**:4960–4964.
- Toft E, Soderstrom M, Ahlberg MB, and DePierre JW (1994) A novel 34 kDa glutathione-binding protein in mature rat ovary. *Biochem Biophys Res Commun* **201**:149–154.
- Turchi JJ and Ho M (2000) Physiological functions of protein kinase inhibitors. *EXS (Basel)* **89**:109–121.
- Ui M, Okada T, Hazeki K, and Hazeki O (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* **20**:303–307.
- Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, and Waterfield MD (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**:535–602.
- Ward SG, June CH, and Olive D (1996) PI 3-kinase: a pivotal pathway in T-cell activation? *Immunol Today* **17**:187–197.
- Wymann MP, Sozzani S, Altruda F, Mantovani A, and Hirsch E (2000) Lipids on the move: phosphoinositide 3-kinases in leukocyte function. *Immunol Today* **21**:260–264.
- Xu Y and Mirocha CJ (1994) Analysis of wortmannin (H-1) by liquid chromatography and gas chromatography. *J AOAC Int* **77**:942–947.