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Covalent Reactions of Wortmannin under Physiological Conditions

Hushan Yuan,[1] Katie R. Barnes,[1] Ralph Weissleder,[1] Lewis Cantley,[2] and Lee Josephson[1,*]
[1] Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA
[2] Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, 77 Avenue Louis Pasteur, Boston, MA 02115, USA
*Correspondence: ljosephson@partners.org
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SUMMARY

Wortmannin (Wm), a steroid-like molecule of 428.4 Da, appears to be unstable in biological fluids (apparent chemical instability), yet it exhibits an antiproliferative activity in assays employing a 48 hr incubation period (prolonged bioactivity), a situation we refer to as the "wortmannin paradox." Under physiological conditions, Wm covalently reacts with nucleophiles such as the side chains of cysteine, N-methyl hexanoic acid, lysine, or proline at the C20 position on the furan ring. Like Wm, WmC20 amino acid derivatives had significant antiproliferative activities. Three Wm derivatives, WmC20-proline, WmC20-cysteine, and a WmC20-N-methyl hexanoic acid, generated Wm that then reacted with lysine in an exchange-type reaction. This unusual, reversible, covalent reaction of Wm with nucleophiles under physiological conditions provides an explanation for the wortmannin paradox.

INTRODUCTION

The central role played by PI3 kinases in a variety of metabolic processes suggests that the inhibition of this activity may be therapeutic in diverse diseases including cancer [1], inflammatory conditions [2], or coagulation disorders [3]. Wortmannin (Wm), a PI3 kinase inhibitor, is a frequent tool in chemical genomics and is used to help define the PI3 kinase dependence of biological processes such as histamine release and insulin action [4–6]. Wm inhibits PI3 kinases in a nonisotype-specific manner by covalently reacting with a lysine in the catalytic subunit of the enzyme [7, 8]. Current efforts to develop PI3 kinase inhibitors as drugs include both Wm-based pharmaceuticals [9, 10] and man-made, isotype-specific inhibitors [11, 12].

However, the comparison between isotype-selective PI3 kinase inhibitors and Wm-based pharmaceuticals as well as the use of Wm as a tool in chemical genomics are both complicated by the fact that Wm is a chemically reactive molecule and has been described as unstable (see [13–15]). We recently observed that Wm's stability was highly dependent on the presence of nucleophiles; in nucleophile-free, aqueous systems, Wm was stable (half-life = 57.8 hr in PBS), and the addition of nucleophiles like amino acids hastened its disappearance (half-life = 22 min in PBS plus 10 mM proline) [16]. If, however, Wm reacts with common nucleophiles, its ability to pass through nucleophile-rich intracellular or extracellular fluids, and to modify a molecular target like PI3 kinase, would seem to be unexplained. Also unclear is how the reactive Wm molecule might exert antiproliferative effects when it is added to nucleophile-rich culture media and cells are allowed to proliferate, typically for 48 hr. An improved understanding of the interrelated issues of stability and chemical reactivity would seem to be required if Wm is to be used to define the PI3 kinase dependence of biological processes or if Wm-based compounds are to be used as sources of potential pharmaceuticals.

Here, we present an explanation for what we term "the wortmannin paradox," or the ability of a chemically unstable molecule to exert biological effects that seem to require its persistence for hours to days. We show that under physiological conditions, the conjugated 2,4-diacylfuran system of Wm covalently and reversibly reacts at the C20 position with the side chains of certain amino acids, yielding WmC20 derivatives, which, like Wm, exhibit an antiproliferative activity. The 2,4-diacetylfuran system of the Wm molecule is a unique, to our knowledge, conjugated system that can both form and break covalent bonds at significant rates under physiological conditions.

RESULTS

Our investigation into the reaction of Wm with amino acids was initiated by the use of Wm in assays that measure the ability of a compound to antagonize cell proliferation over a 48–72 hr incubation period [17–19], when Wm's half-life in a variety of tissue culture media was reported to be about 10 min [13]. We first confirmed the instability of Wm in RPMI culture media by HPLC, as shown in Figure 1A, in which chromatograms of Wm in media for less than 1, 2, and 5 min are shown. The area of the Wm peak was fit to a first-order decay process (Figure 1B) to yield a rate constant of 0.70 min^{-1}, which corresponds to a half-life of 0.99 min. We therefore concluded that

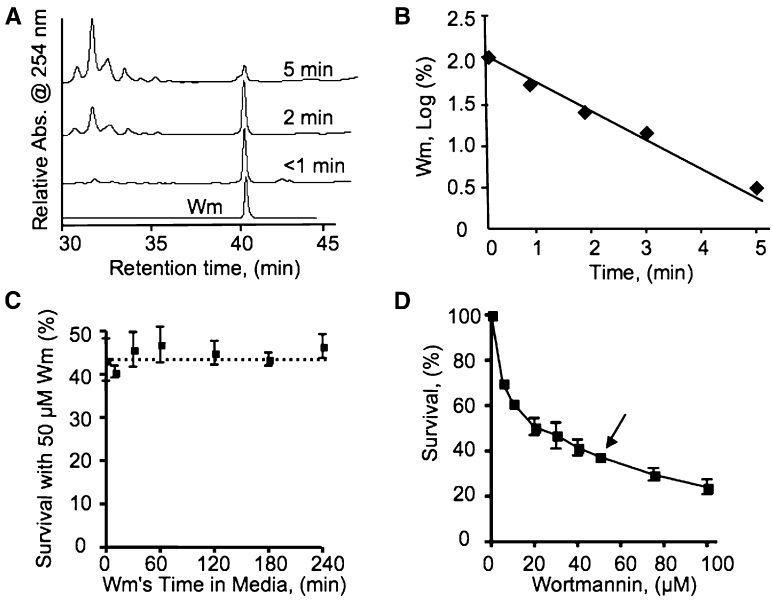

Figure 1. Fate of Wortmannin and Its Antiproliferative Activity in Tissue Culture Media

(A) Wortmannin (Wm) was incubated in culture media for the indicated times and subjected to HPLC. The rapid disappearance of Wm was associated with the appearance of a large number of peaks.

(B) First-order decay plot for the disappearance of Wm. The first-order decay constant was 0.70 min^{-1}, which corresponded to a half-life of 0.99 min.

(C) Wm was aged in tissue culture media for varying times and was added at 50 μM to cells that were then allowed to proliferate for an additional 48 hr. Survival was reduced to 40%–45% of cells not treated with Wm, regardless of Wm's incubation in culture media. Error bars indicate standard deviation.

(D) Inhibition of cell proliferation as a function of Wm concentration when Wm was added directly to media plus cells. The arrow indicates inhibition achieved by the 50 μM Wm concentration used in (C). The IC_{50} is given in Table 1. Wm rapidly disappears in culture media but exerts an antiproliferative activity after its disappearance. Error bars indicate standard deviation of four replicates.

Wm was at least as unstable in culture as reported [13]. We next examined the antiproliferative activity of Wm in tissue culture media by incubating Wm in the same RPMI media as that used in Figure 1A for varying time periods, up to 240 min. We then added the media with aged Wm to A549 cells whose proliferation was examined over an additional period of 48 hr, as shown in Figure 1C. Though the half-life of Wm was 0.99 min in media, Wm incubated in media for up to 4 hr inhibited cell proliferation in a manner similar to the same concentration of Wm (50 μM) added to media plus cells ($p > 0.5$ in all cases). The inhibition of cell proliferation as a function of Wm concentration is shown in Figure 1D (IC_{50} = 11.5 μM; also see Table 1).

We therefore hypothesized that when Wm was added to culture media, it was converted into a form or forms that had an antiproliferative activity. We had previously observed that Wm was stable in PBS, but that its disappearance was hastened by the addition of amino acids or protein (Table 1 of [16]), which suggested that Wm might be reacting with amino acids. We therefore hypothesized that the addition of Wm to culture media resulted in the formation of WmC20 amino acid derivatives that maintained an antiproliferative activity due to their ability to regenerate Wm through non-enzyme-catalyzed, reversible, covalent reactions at the C20 position, as shown in Figure 2. The model is supported by observations outlined below: (1) Wm reacts with amino acids under physiological conditions to form WmC20 derivatives; (2) WmC20 derivatives exhibit an antiproliferative activity and are inhibitors of PI3 kinase; (3) WmC20 derivatives generate Wm; (4)

Table 1. Reaction of Wortmannin with Amino Acid Derivatives or Proline in PBS or DMSO Plus TEA

Wm and Amino Acids in PBS	Wm	Wm and Proline	Wm and N-Acetyl-Lysine	Wm and N-Acetyl-Cysteine	Wm and NMeHA
Retention time (Figure 3)	40.80[a], 13.23[b]	34.09[a]	37.20[a]	35.33[a]	11.31[b]
Wm and amino acids in DMSO (Figure 4)		**1**	**2**	**3**	**4**
Yield		99.5%	78.4%	81.8%	68.9%
Retention time		34.12	37.10	35.20	11.34[b]
Molecular weight obtained		544.2179 ($M+H^+$)	617.2739 ($M+H^+$)	592.1856 ($M+H^+$)	574.2652 ($M+H^+$)
Molecular weight predicted		544.2182	617.2710	592.1852	574.2648
IC_{50}, (μM) Antiproliferation	11.5 ± 0.5	1.79 ± 0.18	29.8 ± 0.9	8.6 ± 0.3	2.10 ± 0.20

aa, amino acid; TEA, triethylamine; DMSO, dimethylsulfoxide.
[a] HPLC system 4.
[b] HPLC system 5.

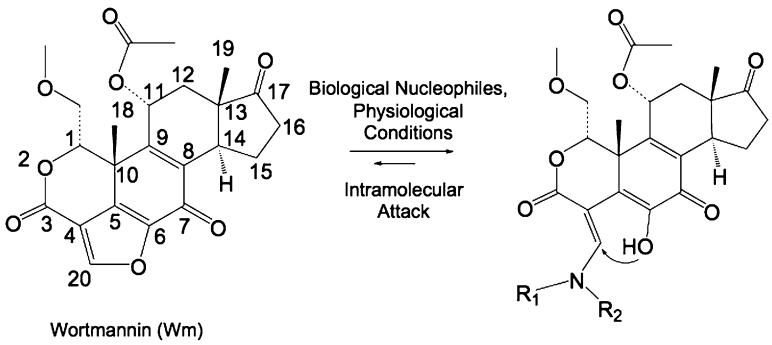

Figure 2. Proposed Mechanism of Wortmannin Disappearance in Culture Media
The structure and number systems of wortmannin (Wm) are given with the 2,4-diacetylfuran ring denoted. The model proposes that Wm reacts with biological nucleophiles at the C20 position. Due to an intramolecular attack by the hydroxyl group at C6, WmC20 derivatives can form Wm, but the position of the equilibrium is to the right. Wm numbering is shown.

when a WmC20 derivative generates Wm in the presence of a large excess of a second reactive amino acid, the newly formed Wm should react with the second amino acid; and (5) the WmC20 derivatives that generate Wm inhibit PI3 kinase.

To understand the reaction of Wm with amino acids under physiological conditions, we incubated 1.35 μM Wm with amino acid derivatives or proline (1.35 mM, PBS, pH 6.8, 37°C) for 22 hr and subjected the resulting mixtures to HPLC. No evidence of reaction was seen with N-acetyl-tryptophan, N-acetyl-tyrosine, N-benzoyl-arginine, or N-benzoyl-histidine. However, when Wm was incubated with proline (Figure 3A), N-acetyl-lysine (Figure 3B), N-acetyl-cysteine (Figure 3C), or N-methyl hexanoic acid (NMeHA) (Figure 3D), new peaks were readily apparent. To investigate the Wm derivatives obtained in PBS, we then prepared four WmC20 amino acid derivatives in DMSO and TEA, as shown in Figure 4: **1**, or WmC20-pro; **2**, or WmC20-lys; **3**, or WmC20-cys; and **4**, or WmC20-NMeHA. Each of the four new peaks formed when Wm was incubated with proline, NMeHA, or amino acid derivatives (Figures 3A–3D) had retention times corresponding to those of the expected WmC20 derivative prepared in DMSO and are therefore identified as such in the figure. The retention times of the new peaks obtained in PBS are compared with the retention times of WmC20 derivatives prepared in DMSO in Table 1. Table 1 also gives the mass of the Wm derivatives prepared in DMSO compared to those predicted for these compounds. Reaction at the C20 position of the Wm molecule was indicated by an analysis of NMR peaks. We conclude that Wm reacts with the epsilon amino group of lysine, the sulfhydryl group of cysteine, and the secondary amine of proline under physiological conditions to yield WmC20 derivatives.

To examine the second prediction of the model shown in Figure 2, we determined the IC_{50}s of Wm, **1**, **2**, **3**, and **4** by using A549 cells in an antiproliferative assay in which these compounds were added to media plus cells once, at the start of the 48 hr assay period. IC_{50}s are given in Table 1. The IC_{50}s of compounds **1**, **2**, **3**, and **4** were moderately higher or lower than Wm, but all had significant antiproliferative effects.

The hypothesis that WmC20 derivatives might undergo exchange reactions is shown in Figure 5A, in which the exchange reactions between **1**, **3**, and **4**, and N-acetyl-lysine are shown. As shown in Figure 5B, when WmC20-pro or **1** (0.3 mM) was incubated with N-acetyl-lysine (3 mM), Wm

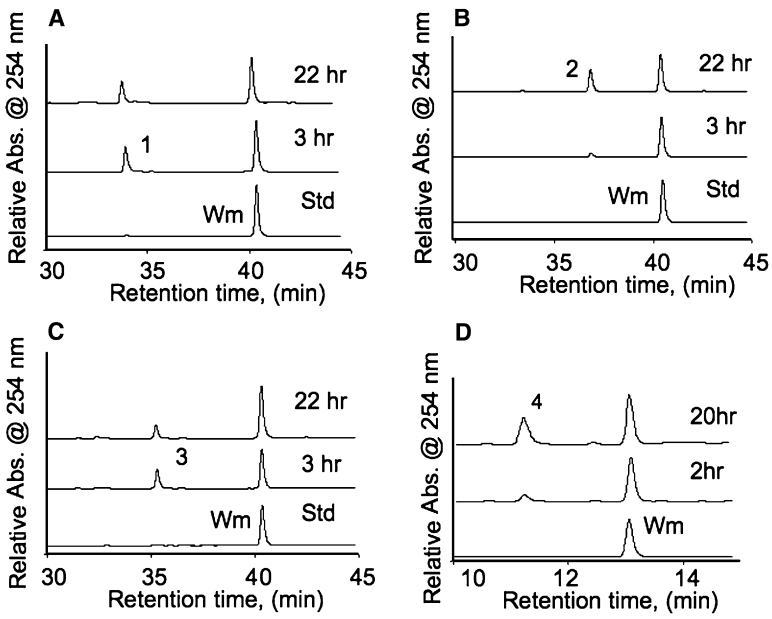

Figure 3. Nonenzymatic Reaction of Wortmannin with Amino Acid Side Chains under Physiological Conditions
(A–D) (A) Wortmannin (Wm) (1.5 μM) was incubated with 1.5 mM proline (PBS, pH 6.8, 37°C) for 3 and 22 hr and was analyzed by HPLC. The compound formed cochromatographed with compound **1** made in DMSO. (B–D) Wm was incubated with (B) N-acetyl-lysine, (C) N-acetyl-cysteine, or (D) NMeHA. Again, Wm derivatives formed in PBS cochromatographed with compounds made in DMSO. Retention times are given in Table 1. HPLC system 4 was used for (A)–(C), while system 5 was used for (D).

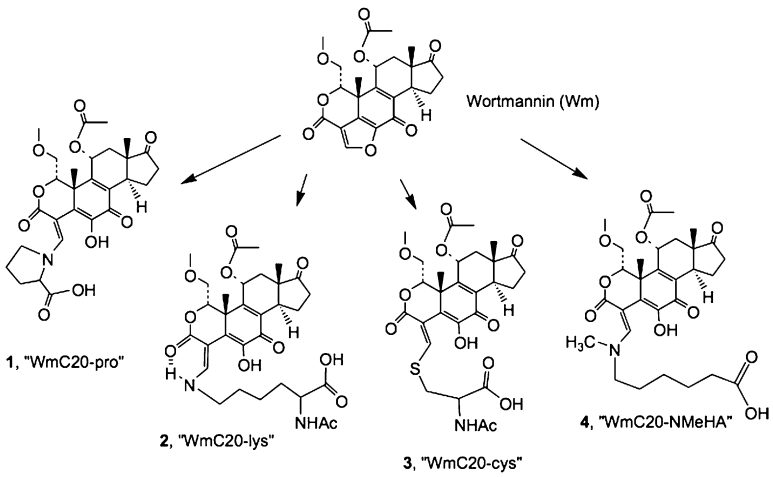

Figure 4. Synthesis of Wortmannin C20 Amino Acid Derivatives
Wortmannin C20 amino acid derivatives were prepared in DMSO and TEA.

formed in transient fashion during incubations of 1–5 hr. However, by 20 hr, both Wm and **1** had disappeared, and a peak corresponding to pure WmC20-lys, **2**, made in an organic solvent was the only peak obtained. When the same experiment was performed with WmC20-cys, **3**, the transient formation of Wm was again seen, with the final formation of **2** (Figure 5C). Identical results were obtained when **4**, WmC20-NMeHA, was incubated with N-acetyl-lysine (data not shown). However, when **2** was incubated with proline or N-acetyl-cysteine, no evidence of a reaction was seen (Figures 5D and 5E). We conclude that the incubation of **1**, **3**, or **4** with N-acetyl-lysine produced Wm as a stable intermediate, which then reacted with the epsilon amino group N-acetyl-lysine to yield **2**. The net effect is a replacement of N-acetyl-cysteine, proline, or NMeHA with N-acetyl-lysine at the C20 position of Wm.

Finally, we assessed the ability of the four WmC20 derivatives to inhibit PI3 kinase, which employs a 30 min incubation, as shown in Table 2. Each derivative was also evaluated for its ability to produce Wm. WmC20 derivatives that inhibited PI3 kinase produced Wm under conditions of the enzyme assay, a correlation discussed further below.

DISCUSSION

We propose that an equilibrium exists between Wm and WmC20-modified derivatives under physiological conditions (pH 6.8, 37°C, presence of nucleophiles), as shown in Figure 2. Evidence supporting this view is derived from four observations: (1) Wm reacts at the C20 position with amino acids under physiological conditions; (2) WmC20 amino acid derivatives are active in antiproliferative assays; (3) WmC20 amino acids produce Wm, which then reacts with other amino acids in exchange reactions; and (4) the strength of WmC20 derivatives as inhibitors of PI3 kinase, IC_{50}, parallels their ability to generate Wm.

Reaction of Wm at C20 with Amino Acid Side Chains

The C20 position of Wm reacts with amino acid side chains of lysine and cysteine or proline (PBS, pH 6.8, 37°C), not with the side chains of tryptophan, histidine, tyrosine, or arginine. The nucleophilicites of the nitrogens or oxygens of histidine, tryptophan, tyrosine, and arginine are reduced because of the partial electron delocalization via a p-π conjugation when they are deprotonated. On the other hand, the nitrogens of lysine, proline, and NMeHA and the sulfur of cysteine feature more localized electrons and are more strongly nucleophilic. When incubated with Wm, these nucleophiles react with the C20 of Wm, which is electrophilic due to (1) the electron-withdrawing nature of the two carbonyl groups of the 2,4-diacylfuran ring and (2) the strain on the furan ring system conferred by the rest of the Wm molecule. Earlier studies on WmC20 derivatives used organic solvents and base for their preparation [20–22]. Here, we show that the reactions between Wm and amino acids at the C20 position occur under physiological conditions, and that the products made are the same as those made under organic conditions.

Antiproliferative Activity of WmC20 Derivatives

The WmC20 derivatives that we prepared exhibited IC_{50}s in an antiproliferative assay that were modestly lower (**1**, or WmC20-pro; **3**, or WmC20-cys; and **4**, or WmC20-NMeHA) or higher (**2**, or WmC20-lys) than Wm. The antiproliferative activity of WmC20 derivatives has been reported [9, 21, 22].

Exchange Reaction of WmC20 Derivatives Due to Wm Formation

The ability of Wm to react with amino acids under physiological conditions (forward reactions in Figure 2 and Figure 5A), together with our earlier observation that WmC20 derivatives can produce Wm [16] (reverse reaction in Figure 2), suggested that an exchange reaction involving WmC20 amino acid derivatives and amino acids might be possible. When incubated with N-acetyl-lysine, WmC20 derivatives **1** and **3** produced Wm and eventually formed **2** (Figures 5B and 5C). (Compound **4** was also incubated with N-acetyl-lysine with similar results [data not shown].) The accumulation of **2** or WmC20-lys with exchange reactions requires that this Wm derivative, one

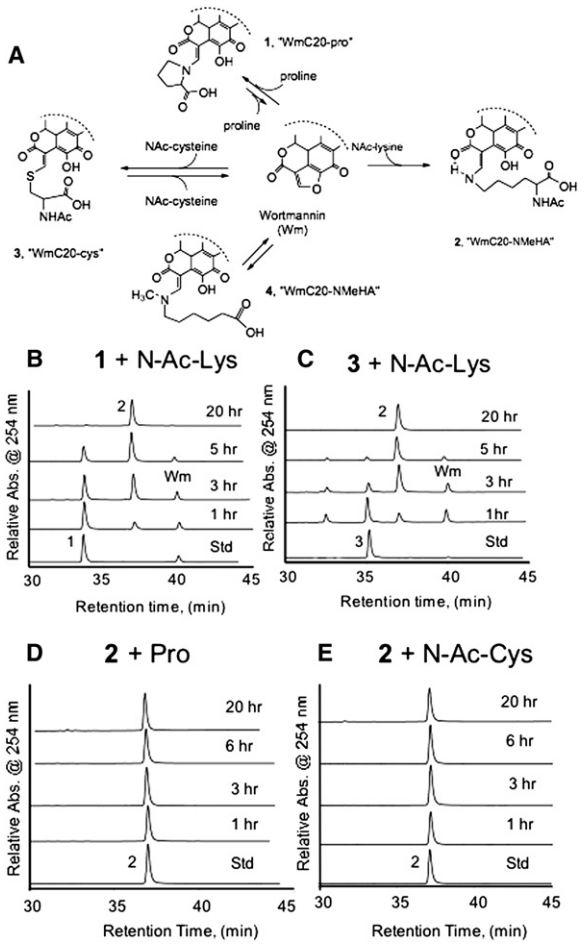

Figure 5. Exchange Reactions Involving Wortmannin C20 Amino Acid Derivatives

(A) Wortmannin C20 derivatives lacking hydrogen-bond stabilization, **1**, **3**, and **4**, generate Wm, which reacts with the N-acetyl-lysine present to form **2**. Since **2** is the low-free energy compound due to hydrogen-bond stabilization, a reaction of N-acetyl-cysteine or proline with **2** does not yield **1** or **3**. The exchange reactions proposed in (A) were monitored by HPLC in (B)–(E).

(B) Incubation of **1** with N-acetyl-lysine (PBS, pH 6.8, 37°C). With time, **1** disappears with the formation of Wm (1–5 hr), which then disappears with the formation of **2** by 20 hr.

(C) Incubation of **3** with N-acetyl-lysine. With time, **3** disappears with the formation of Wm (1–5 hr); **2** eventually forms at 20 hr.

(D and E) No exchange occurs between **2** and (D) proline or (E) N-acetyl-cysteine.

with a secondary nitrogen at C20, be more stable than other derivatives. The increased stability of **2** relative to **3**, **4**, or **1** results from the presence of a hydrogen-bond stabilization of **2**, as shown in Figure 5, a structure first proposed by Norman et al. [20] and confirmed by Yuan et al. [16]. The stability of **2** was also apparent from the inability of its N-acetyl-lysine to exchange with proline or N-acetyl-cysteine (Figures 5D and 5E). It should be noted that for the exchange reaction to occur, Wm must be stable in the aqueous media employed, consistent with our observation of its stability [16].

Table 2. Inhibition of PI3 Kinase and Wortmannin Generation by WmC20 Derivatives

Compound	IC_{50} PI3 K (nM)	Relative IC_{50}[a]	Wm Generation[b] (30 min, 25°C, PBS)
Wm	12	1.00	1.00
WmC20-lys, **2**	>1000	0	<0.001
WmC20-pro, **1**	68	0.18	0.051
WmC20-cys, **3**	1144	0.010	0.006
WmC20-NMeHA, **4**	108	0.11	0.036

[a] IC_{50} for Wm divided by IC_{50} of compound.
[b] Wm generation (30 min, 25°C) was measured by HPLC. Wm is expressed as fraction of the initial Wm present. Incubation for PI3 kinase assay was also performed at 30 min at 25°C.

Inhibition of PI3 Kinase

To determine whether the inhibition of PI3 kinase by WmC20 derivatives was consistent with the model shown in Figure 2, in which Wm is the predominant active compound, we measured the ability of WmC20 derivatives to inhibit PI3 kinase and compared that with the generation of Wm by HPLC. As shown in Table 2, pure WmC20-lys did not inhibit PI3 kinase and did not generate detectable Wm in the time of the assay (30 min). Three other WmC20 derivatives (WmC20-pro, WmC20-cys, and WmC20-NMeHA) inhibited PI3 kinase with higher IC_{50}s than Wm; the higher IC_{50} results from the partial conversion to Wm during the 30 min incubation time of the PI3 kinase assay. For the four WmC20 derivatives evaluated, the amount of Wm generated was roughly proportional to the potency of WmC20 derivatives as PI3 kinase inhibitors. The IC_{50}s for the inhibition of PI3 kinase were far lower than their IC_{50}s for the inhibition of cell proliferation (Table 2), as noted by others [9, 19].

The model of reversible Wm formation (Figure 2) provides a general fate of Wm in nucleophile-rich biological systems, proposing that the bulk of the Wm exists as WmC20 derivatives that have a bioactivity due to the reversible formation of Wm. Evidence for the formation of WmC20 derivatives under physiological conditions (forward reaction shown in Figure 2) is provided by the disappearance of Wm in culture media (Figure 1A) and the reaction of Wm with amino acid side chains (Figure 3). Evidence for simultaneous forward and reverse reactions (an equilibrium) is provided by the exchange reactions demonstrated in Figures 5B and 5C. Evidence for the formation of Wm from pure WmC20 derivatives is provided by the ability of WmC20 derivatives to inhibit PI3 kinase in proportion to the amount of Wm generated (Table 2). Thus, the model provides an explanation for the observation that some WmC20 derivatives inhibit PI3 kinase (see Table 2 and [20]), in spite of the fact that the C20 of the furan ring reacts with a lysine in the ATP site of PI3 kinase [23]; WmC20 derivatives that inhibit PI3 kinase are those that generate Wm over the 30 min assay period (reverse reaction of Figure 2). A feature of the model is the slow, transient generation of Wm that occurs (Figures 5B and 5C),

which provides an explanation for how WmC20 derivatives can be antiproliferative during a 48 hr assay (Table 1).

The model shown in Figure 2 requires a specific relationship between two potential sources of Wm instability in biological systems—a slow hydrolytic opening of the lactone ring and fast reaction of nucleophiles at C20. The stability of Wm required to obtain the simple chromatograms shown in Figure 5 is consistent with a long half-life for Wm in PBS (without nucleophiles) and demonstrates the slow opening of the lactone ring. The stability of Wm in PBS was previously determined to be 57.8 hr [16]. The major source of "instability" of Wm is the reaction of nucleophiles at C20, which causes Wm to rapidly disappear in culture media (half-life of 0.99 min in culture media, Figure 1A), or 22 min in PBS plus proline [16]. Consistent with the view that Wm instability is due to a reaction at the C20 of the 2,4-diacetylfuran ring system with nucleophiles, and not to lactone hydrolysis, is the stability of lactone-containing natural products. The stability of compounds like digoxin, warfarin, etoposide, and irinotecan is sufficient for their use as pharmaceuticals.

Wm-related compounds have long been investigated as antiinflammatory [24] and anticancer agents [9, 18, 19, 21], and they have been rejected for pharmaceutical development due to toxicity and stability considerations [14, 15]. The complex behavior of Wm in biological systems suggests that an understanding of the fate of Wm-derived compounds may have to accompany all investigations into their efficacy and toxicity. For example, the relative activity of panels of Wm derivatives may reflect the conversion of Wm-based compounds to active and inactive molecules, rather than structure activity relationships predicated on the binding of compounds in the form synthesized to a molecular target(s). However, determining the fate of Wm in biological systems is complicated by the large number of molecules with which it can react (primary amines, secondary amines, thiols), yielding products with widely varying properties. Not only can the nonpolar Wm molecule form low-molecular weight, charged, or uncharged compounds by reaction with low-molecular weight nucleophiles, but it can likely react with high-molecular weight nucleophiles as well. On gels, a number of wortmannylated proteins were observed, when a fluorescent Wm was reacted with cells or cell lysates [25], and may reflect the formation of WmC20-modified proteins. Multiple methodologies will likely be needed for following Wm, a steroid-like molecule of 428.4 Da that can react with a variety of nucleophilic species. The model shown in Figure 2, proposing the interconversion between Wm and WmC20 derivatives, provides a useful hypothesis for further investigations into the complex behavior of Wm in biological systems.

SIGNIFICANCE

A model for the fate of Wm in biological fluids, in which the active species, Wm, exists in equilibrium with inactive WmC20 derivatives, and which accounts for Wm's apparent instability and prolonged bioactivity, is proposed. Wm covalently reacts with a lysine in the ATP site of PI3 kinase to inhibit the enzyme; however, Wm's target specificity is limited, and non-target-related covalent reactions, such as the reaction with certain amino acids, occur. Other natural products that modify biological targets and react more generally include the penicillins, which modify peptidases involved with bacterial cell wall synthesis [26] and also react with the epsilon amino groups of lysines [27]. Similarly, aspirin acetylates its target cyclooxygenase and a variety of other proteins [26]. Thus, similar to what is seen with other reactive natural products, Wm reacts both with a biological target and other nucleophiles. However, the reactions of Wm are unusual for the following reasons. First, Wm's reaction with amino acids results in only moderate decreases of its antiproliferative activity, as is the case with lysine, or results in increased antiproliferative activity, as is the case with proline, cysteine, and NMeHA. Second, Wm's reaction with amino acids is reversible, generating Wm. Understanding the unusual and complex fates of chemically reactive natural products like Wm in biological systems is essential if we are to properly use such materials as selective inhibitors of biological processes or design pharmaceuticals based on them.

EXPERIMENTAL PROCEDURES

General

Wortmannin (Wm) was a gift of the natural products branch of the National Cancer Institute. All reagents and solvents were of standard quality. NMR was performed on a Varian 400 MHz instrument with $CDCl_3$. Mass spectra were obtained on a Micromass LCT instrument by using the time-of-flight ESI technique. HPLC (Varian Prostar 210 with a variable wavelength PDA 330 detector) employed reverse-phase C18 columns (VYDAC, Cat. #: 218TP1022 for synthesis; Varian, Cat. #: R0086200C5 for analysis) with water (Millipore, containing 0.1% trifluoroacetic acid) (buffer A) and acetonitrile (containing 20% buffer A) (buffer B) as the elution buffers. System 1 (used for purification of **1**): buffer A:buffer B (80:20) isocratic for 5 min, linear gradient to buffer A:buffer B (20:80) over 20 min, then gradient back to 80:20 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 4.9 ml/min, λ_{max}: 410 nm). System 2 (used for purification of **2**): buffer A:buffer B (80:20) isocratic for 5 min, linear gradient to buffer A:buffer B (0:100) over 30 min, then gradient back to 80:20 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 6.0 ml/min, λ_{max}: 255 nm. System 3 (used for purification of **3**): buffer A:buffer B (80:20) isocratic for 5 min, linear gradient to buffer A:buffer B (20:80) over 45 min, then gradient back to 80:20 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 4.9 ml/min, λ_{max}: 410 nm. System 4: buffer A:buffer B (99:1) linear gradient to buffer A:buffer B (40:60) over 46 min, then gradient back to 90:1 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 1.0 ml/min, λ_{max}: 254 nm. System 5: buffer A:buffer B (70:30) linear gradient to buffer A:buffer B (20:80) over 15 min, then gradient back to 70:30 (buffer A:buffer B) for 3 min and isocratic for 5 min, flow: 1.0 ml/min. λ_{max}: 258 nm.

Syntheses of Wm C20 Derivatives 1, 2, and 3

To synthesize **1**, Wm (10.7 mg, 0.025 mmol) and proline (5.75 mg, 0.05 mmol) were mixed in anhydrous DMSO (0.5 ml). The mixture was stirred at room temperature for 1 hr. After dilution with 50% acetonitrile in water (1:1), the mixture was purified by HPLC (system 1) and gave a yellow powder after lyophilization. Analysis of **1** by HPLC (system

4) showed 89.5% purity. Yield: 13.5 mg, 99.5%. MS: $C_{28}H_{33}NO_{10}$, cal. 544.2182, found 544.2179 ($M+H^+$); ^1H NMR, ($CDCl_3$): 0.86 (3H, s, 13-CH_3), 1.31–1.35 (1H, t, J = 8 Hz), 1.57 (3H, s, 10-CH_3), 1.70–2.00 (5H, b), 2.15 (3H, s, CH_3COO), 2.20–2.35 (2H, m), 2.35–2.45 (1H, m), 2.55–2.64 (2H, m), 2.90–3.00 (2H, m), 3.10–3.20 (3H, m), 3.25 (3H, s), 3.40–3.50 (1H, b), 4.50–4.65 (2H, m), 6.00–6.10 (1H, b, H-11), 8.28 (1H, s, H-20).

To synthesize **2**, Wm (12.8 mg, 0.03 mmol), N-acetyl-lysine (28.2 mg, 0.15 mmol), and triethylamine (30 μl) were mixed in a mixture of DMSO (1 ml), methanol (1 ml), and water (100 μl). After 1 hr of stirring, the reaction mixture was purified by HPLC (system 2), and a yellow powder was obtained after lyophilization. The purity of compound 2 was confirmed by HPLC as 100% (system 4). Yield: 14.5 mg, 78.4%. MS: $C_{31}H_{40}N_2O_{11}$, cal. 617.2710, found 617.2739 ($M+H^+$); NMR ($CDCl_3$, ppm): 0.81 (3H, s, 13-CH_3), 1.38–1.60 (2H, m), 1.52 (3H, s, 10-CH_3), 1.64–1.76 (2H, m), 1.82–1.96 (3H, m), 2.04 (3H, s, $NHCOCH_3$), 2.06 (3H, s, CH_3COO), 2.22–2.40 (3H, m), 2.50–2.63 (1H, m), 2.80–2.99 (2H, m,), 3.16–3.24 (2H, m), 3.26 (3H, s, CH_3OCH_2), 3.36–3.52 (2H, m), 3.52–3.89 (2H, b), 4.30–4.35 (1H, m), 4.70–4.78 (1H, m, $CH_2CHCOOH$), 5.97–6.01 (1H, m, H-11), 6.94 (1H, d, J = 8 Hz, $NHCOCH_3$), 8.55 (1H, d, J = 12 Hz, H-20), 9.83–9.88 (1H, m, C20NH).

To synthesize **3**, Wm (17.1 mg, 0.04 mmol), N-acetyl-cysteine (13 mg, 0.08 mmol), and triethylamine (0.4 ml) were mixed in CH_2Cl_2 (1 ml). After 1.5 hr of stirring, the reaction mixture was concentrated under reduced pressure. The residue was purified by HPLC (system 3), and a yellow powder was obtained after lyophilization. Subsequent purity analysis by HPLC (system 4) revealed the presence of a minor impurity, accounting for less than 20% of total. Yield: 19.4 mg, 81.8%. MS: $C_{28}H_{33}N_2O_{11}S$, cal. 592.1852, found 592.1856 ($M+H^+$); ^1H NMR, ($CDCl_3$): 0.82 (3H, s, 13-CH_3), 1.52 (3H, s, 10-CH_3), 1.70–2.01 (3H, b), 2.05 (3H, s, $NHCOCH_3$), 2.09 (3H, s, CH_3COO), 2.22–2.40 (3H, m), 2.54–2.64 (1H, m), 2.80–2.90 (1H, m), 2.94–3.00 (1H, m), 3.14–3.34 (2H, m, H-15), 3.26 (3H, s, CH_3OCH_2), 3.40–3.50 (2H, AB, J_1 = 4 Hz, J_2 = 16 Hz, SCH_2), 4.46 (1H, d, J = 4 Hz, H-1), 4.98–5.00 (1H, m, CHCOOH), 5.98–6.01 (1H, m, H-11), 6.59 (1H, d, J = 8 Hz, $NHCOCH_3$), 8.78 (1H, s, H-20).

To synthesize **4**, WmC20-NMeHA was synthesized as described [16].

Reaction of Wm with Amino Acid Side Chains or Proline

A total of 10 μl Wm (0.058 mM in DMSO) was diluted in 400 μl PBS buffer (pH 6.8). To this solution, 1000 equivalents of the appropriate N-protected amino acid in DMSO or PBS buffer were added. If necessary, 10–30 μl acetonitrile was added to completely solubilize the reaction mixture. Solutions were incubated at 37°C for the indicated time and were analyzed by applying 10 μl plus 1 μl internal standard (1 μg methyl-4-hyrdroxybenzoate [MHB]) to the HPLC column. System 4 and system 5 were used for HPLC.

Exchange Reactions

WmC20 derivatives **1**, **2**, or **3** at 0.3 mM in PBS (pH 6.8) were incubated with 3 mM N-acetyl-lysine, proline, or N-acetyl-cysteine at 37°C for 24 hr. The reaction was monitored by HPLC with system 4, by injecting the reaction mixture (10 μl) with the MHB internal standard (1 μl, 1 μg/μl).

Antiproliferative and PI3 Kinase Assays

A549 cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All cells were grown in a humidified atmosphere at 37°C and 5% CO_2. Growth inhibition was evaluated by the SRB assay [28]. Cells were seeded in 96-well plates at 5000 cells per well in 100 μl media and were incubated at 37°C for 24 hr. Cells were then treated with 0–100 μM compounds **1**–**3** at 37°C for 48 r. Cells were fixed with 50 μl cold 50% TCA, incubated at 4°C for 1 hr, and washed five times with tap water. The 96-well plates were allowed to air dry, and 100 μl 0.4% sulforhodamine B (SRB) in 1% acetic acid was added to each well. After 10 min, the unbound SRB was washed away with 1% acetic acid, and the plates were dried. Protein-bound dye was solubilized by the addition of 200 μl 10 mM Tris buffer and subsequent shaking at room temperature for 10 min. Absorbance was measured at 490 nm, by a microtiter plate reader. Percent cell survival was calculated for each concentration by the ratio of the measured absorbance to the absorbance of the untreated cells. The PI3 kinase enzyme assay was the fluorescent energy transfer method [29]. The assay has higher IC_{50}s for Wm (12 nM) than radioactive lipid phosphorylation assays (1–4 nM) because it uses a higher ATP concentration (100 μM). IC_{50}s for both assays were determined by curve fitting performed by using Prism 4.0a software.

Antiproliferative Activity of Wm Aged in Culture Media

Wm was incubated in RPMI 1640 media at 37°C for up to 240 min and then added to A549 cells. Cell survival was determined after 48 hr as described above.

Generation of Wm from WmC20 Derivatives

Solutions of **1, 2, 3**, and **4** (1.88 mM) were made by reconstituting powdered WmC20 derivatives with PBS (pH 7.0) and were incubated at 25°C for 30 min. They were analyzed by applying 10 μl plus 1 μl internal standard (1 μg MHB) to the HPLC. System 4 was used for compounds **1**–**3**, and system 5 was used for compound **4**.

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