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Slow Self-Activation Enhances The Potency of Viridin Prodrugs

Joseph Blois^{1,*}, Hushan Yuan^{1,*}, Adam Smith¹, Michael Pacold^{1,2}, Ralph Weissleder^{1,3,4}, Lewis C. Cantley^{4,5}, and Lee Josephson^{1,**}

¹Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School, 149 13th Street, Charlestown, MA 02129

²Center for Cancer Research, Massachusetts Institute of Technology, 40 Ames St., Cambridge, MA, 02142

³Center for Systems Biology, Massachusetts General Hospital, Boston, MA 02114

⁴Department of Systems Biology, Harvard Medical School, 200 Longwood Ave., Boston MA 02115

⁵Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, 77 Avenue, Louis Pasteur, Boston MA 02115

Abstract

When the viridin wortmannin (Wm) is modified at the C20 position, it exhibits a superior antiproliferative activity even though a covalent reaction between the C20 of Wm and a lysine in the active site of PI3 kinase is essential to Wm's mechanism of action. The improved potency results from a self-activation mechanism that slowly converts WmC20 derivatives from inactive prodrugs to the active species Wm (the parent compound with a reactive C20) over the 48 h incubation used to measure antiproliferative activity. Our results provide a guide for selecting Wm-like compounds to maximize kinase inhibition with the variety of protocols used to assess the role of PI3 kinase in biological systems, or for achieving optimal therapeutic effects *in vivo*. In addition, the slow self-activation of WmC20 derivatives provides a mechanism that can be exploited to obtain kinase inhibitors endowed with physical and pharmacokinetic properties far different from man-made kinase inhibitors, since they do not bind to kinase active sites.

Keywords

self-activation; wortmannin; PI3 kinase; potency

Introduction

Viridins, a class of compounds from fungi of which wortmannin (Wm) is a member, have long intrigued pharmacologists because of their potent antiproliferative and immune suppressive properties¹. Viridins are steroid-like molecules which have a "extra" furan ring as a common, key structural feature¹. Wm, the most commonly used viridin, has been widely employed to define the role of PI3 kinase in biological systems, with over 5000 Pubmed citations obtained when the combination "PI3 kinase" and "wortmannin" were searched. Wm is a nanomolar PI3 kinase inhibitor^{2, 3}, but at higher concentrations inhibits polo-like kinase⁴, mTOR⁵, DNA-dependent protein kinase⁶ and ATM⁷. Unlike man-made PI3 kinase inhibitors⁸⁻¹¹, a specific carbon on Wm, C20, reacts with a lysine in the ATP site of PI3 kinase^{1, 12, 13}. Wm's antiproliferative and immune suppressive activities have intrigued pharmacologists since at

*Contributed equally to this work.

**To whom correspondence should be addressed. E-mail: josephso@helix.mgh.harvard.edu, Tel: (617) 726-6478. Fax: (617) 726-5708.

least 1974¹⁴⁻²⁰, and it continues to be used as a scaffold for drug development^{21, 22}. Although virdins like Wm have frequently been used, both to define the role of PI3 kinase and as scaffolds for drug development, relationships between virdin chemistry (structure, stability and chemical reactivity) on the one hand, and their bioactivity on the other hand, are sometimes not easily reconciled.

A seeming inconsistency involves the bioactivity of Wm derivatives made by reaction of the electrophilic C20 carbon with nucleophiles: since a covalent reaction between the C20 of Wm and PI3 kinase is an essential part of its mechanism of kinase inhibition, can a reaction between C20 and nucleophiles yield WmC20 derivatives that are more potent than Wm? The inactivity of Wm derivatives made by modification of the electrophilic C20 carbon (see Figure 1 for Wm numbering) has long indicated the essential role a covalent reaction with PI3 kinase plays in the inhibition of PI3 kinase and in the compound's biological effects^{14, 23-25}, though others have often reported the bioactivity of Wm's modified at C20 are markedly superior to the starting Wm^{22, 26}.

We hypothesized that this inconsistency might be related to the rate at which inactive WmC20 derivatives form Wm²⁷, with slow release forms exhibiting higher activity in cell-based bioassays using long incubation times, a condition which may be more indicative of the behavior of compounds *in vivo*. To investigate this possibility, we modified a recently developed fluorescent Wm²⁷ with various constituent groups at C20, to obtain a panel of fluorescent WmC20 derivatives that formed the original fluorescent Wm at widely different rates. We then examined the activity of the panel with a short duration PI3 kinase assay and a long duration antiproliferative assay, and employed these compounds' fluorescence to determine their fates in cultured cells. We show that Wm modified at C20, WmC20 derivatives, are poor PI3 kinase inhibitors yet exhibit enhanced potency due to a self-activation mechanism that yields the active Wm over the course of a 48 hour antiproliferative assay. As the chemistry of Wm and its derivatives becomes fully understood, these unique natural products can be better exploited to define the role of the PI3 kinase in biological systems or to obtain compounds with acceptable efficacy and toxicity for use *in vivo*.

Materials and Methods

Compound synthesis

The synthetic strategy and compounds synthesized are shown in Figure 1. Compounds **3**, **2a**, **2b**, **2d** and **2e** were prepared as described^{27, 28}.

Wortmannin (Wm) was a gift of the natural products branch of the National Cancer Institute. Amino-dextran was a 70 kDa species from InVitrogen, Carlsbad, CA. All reagents and solvents were of standard quality. NMR was performed on a Varian 400 MHz and Varian Unity Inova 500MHz instruments with CDCl₃. High resolution mass spectra were obtained on a Micromass LCT instrument by using the time-of-flight ESI technique. Low resolution mass spectra were collected on a Waters Micromass ZQ MAA288 instrument. Compounds were purified by HPLC (Varian Prostar 210 with a variable wavelength PDA 330 detector) which employed reverse phase C18 columns (VYDAC, Cat. #: 218TP1022; 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 (for 2c and 4c): buffer A:buffer B (70:30) linear gradient to buffer A:buffer B (0:100) 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} : 410 nm and 495 nm. System 2 (for 2c and 4c): buffer A:buffer B (70:30) linear gradient to buffer A:buffer B (0:100) over 20 min, isocratic for 5 min, then gradient back to 70:30 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 4.9 ml/min, λ_{max} : 410 nm and 495 nm. System 3 (for 4a): buffer A:buffer B (80:20) isocratic for 5 min, linear gradient to buffer

A:buffer B (20:80) 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} : 410 nm. System 4 (for 4b): buffer A:buffer B (80:20) isocratic for 5 min, linear gradient to buffer A:buffer B (20:80) over 30 min, then isocratic for 5 min, gradient back to 80:20 (buffer A:buffer B) for 5 min and isocratic for 5 min, flow: 6.0 ml/min, λ_{max} : 408 nm. System 5 (for 4a): buffer A:buffer B (70:30) linear gradient to buffer A:buffer B (0:100) over 25 min, then isocratic for 5 min and gradient back to 70:30 (buffer A:buffer B) in 5 min and isocratic for 5 min, flow: 1.0 ml/min, λ_{max} : 280 nm and 490 nm. System 6 (for 4b): buffer A:buffer B (50:50) linear gradient to buffer A:buffer B (23:77) over 20 min, then isocratic for 5 min and gradient back to 50:50 (buffer A:buffer B) in 5 min and isocratic for 5 min, flow: 1.0 ml/min. λ_{max} : 280 nm and 490 nm.

Synthesis of 2c

A mixture of Wm **1** (12 mg, 0.028 mmol) and 4-(N-methylamino) pyridine (60 mg, 0.55 mmol) in anhydrous CH_2Cl_2 (1 mL) was stirred at room temperature for 15 hr. The mixture was purified by preparative silica gel TLC developed by CH_2Cl_2 :MeOH (10:1). A dark yellow solid was obtained. Crude yield: 40% with purity 96% (system 1). A further HPLC purification (system 2) was needed for both the PI3 kinase enzyme and anti-proliferative cell based assays. A bright yellow powder (**2c**) would be obtained after lyophilization. HRMS: $\text{C}_{29}\text{H}_{32}\text{N}_2\text{O}_8$, expd. 537.2237 ($\text{M} + \text{H}^+$), obsd. 537.2220. LRMS: 537.0. ^1H NMR (CDCl_3 , ppm, 500MHz): 0.86 (3H, s, C13-CH_3), 1.24 (1H, br, OH), 1.64 (3H, s, C10-CH_3), 1.79-1.84 (1H, q, $J_1 = 5.2$ Hz, $J_2 = 14.3$ Hz, H-12), 2.04-2.12 (4H, m, H-15, OCCH_3), 2.24-2.29 (1H, m, H-16), 2.39-2.44 (1H, q, $J_1 = 7.4$ Hz, $J_2 = 14.3$ Hz, H-12), 2.53-2.62 (1H, m, H-16), 2.89-3.02 (2H, m, H-14, OCH_2), 3.11-3.17 (4H, m, H-15, N- CH_3), 3.25 (3H, s, OCH_3), 3.27-3.32 (1H, q, $J_1 = 2.19$ Hz, $J_2 = 11.0$ Hz, OCH_2), 4.61-4.63 (1H, q, $J_1 = 2.5$ Hz, $J_2 = 6.6$ Hz, H-1), 6.07-6.10 (1H, m, H-11), 7.00 (2H, d, $J = 6.4$ Hz, Py-H), 8.39 (1H, s, H-20), 8.35 (2H, d, $J = 6.4$ Hz, Py-H).

Synthesis of 4a

NBD-Wm **3** (43.8mg, 0.065 mmol), 6-(N-methylamino)-hexanoic acid hydrogen chloride (53 mg, 0.3 mmol), and triethylamine (40 μL) were mixed in anhydrous DMSO (2 mL). The mixture was stirred at room temperature and completed immediately. After dilution with 50% acetonitrile in water (1:1) before the injection, the mixture was purified by HPLC (system 3) and gave a red powder after lyophilization. Analysis of **4a** by HPLC (system 5) showed less than 3% of NBDWm: 38.1 mg, 67.6%. HRMS: $\text{C}_{41}\text{H}_{51}\text{N}_5\text{O}_{13}$, calcd. 822.3561 ($\text{M} + \text{H}^+$), found 822.3594. LRMS: 822.4; ^1H NMR (CDCl_3 , ppm, 400MHz): 0.86 (3H, s, C13-CH_3), 1.42-1.48 (4H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 1.56 (3H, s, C10-CH_3), 1.61-1.78 (9H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$, H-12), 2.05-2.16 (1H, m, H-16), 2.22-2.47 (4H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 2.54-2.64 (1H, m, H-15), 2.73-2.87 (3H, H-12, H-14, H-16), 2.92-2.99 (1H, m, OCH_2), 3.09-3.17 (1H, m, H-15), 3.22 (3H, s, OCH_3), 3.30-3.60 (11H, br, NCH_3 , OCH_2 , NCH_2 , OH), 4.08 (2H, br, ArNCH_2), 4.48-4.49 (1H, d, $J = 6.25\text{Hz}$, H-1), 6.07-6.13 (2H, m, H-11, ArH), 8.26 (1H, s, H-20), 8.46 (1H, d, $J = 9.0\text{Hz}$, ArH).

Synthesis of 4b

NBD-Wm **3** (13mg, 0.02 mmol) and 6-amino-hexanoic acid (13 mg, 0.1 mmol) were mixed in anhydrous DMSO (1 mL). The mixture was stirred at room temperature for 1.5 hr. After dilution with 50% acetonitrile in water (1:1) before the injection, the mixture was purified by HPLC (system 4) and gave a red powder after lyophilization. HPLC analysis (System 6) showed a single peak to prove its high purity. 13.5 mg, 83.9%. HRMS: $\text{C}_{40}\text{H}_{49}\text{N}_5\text{O}_{13}$, calcd. 808.3405 ($\text{M} + \text{H}^+$), found 808.3423. LRMS: 808.4 ($\text{M} + \text{H}^+$), 825.4 ($\text{M} + \text{NH}_4^+$), 830.4 ($\text{M} + \text{Na}^+$). ^1H NMR (CDCl_3 , ppm, 400MHz): 0.82 (3H, s, C13-CH_3), 1.42-1.50 (4H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$), 1.52 (3H, s, C10-CH_3), 1.67-1.82 (9H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$, H-12), 2.22-2.42 (7H, m, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CO}$, H-12,

H-15, H-16), 2.53-2.61 (1H, m, H-16), 2.73-2.95 (2H, H-14, OCH₂), 3.14-3.18 (1H, m, H-15), 3.23 (3H, s, OCH₃), 3.39-3.68 (8H, m, br, NCH₃, NCH₂, OCH₂, OH), 4.08 (2H, br, ArNCH₂), 4.29 (1H, d, J=7.6Hz, H-1), 5.99-6.02 (1H, m, H-11), 6.11 (1H, d, J=9.0Hz, ArH), 8.46 (1H, d, J=9.0Hz, ArH), 8.53 (1H, d, J=13.9Hz, H-20), 9.82-9.88 (1H, m, NH); ¹³C NMR (ppm): 16.65, 22.62, 24.28, 26.02, 26.26, 26.53, 30.42, 33.51, 33.80, 36.62, 38.63, 42.43, 42.50, 43.88, 49.97, 49.98, 55.93, 59.43, 67.51, 73.31, 77.43, 81.31, 88.41, 101.31, 129.05, 135.70, 137.09, 137.56, 145.10, 145.57, 150.83, 159.61, 166.15, 172.62, 177.22, 178.65, 218.10.

Synthesis of 4c

A mixture of NBD-Wm **3** (21 mg, 0.031 mmol) and 4-(N-methylamino) pyridine (0.268 g, 0.6 mmol) in anhydrous CH₂Cl₂ (2 mL) was stirred at room temperature for 2 days. The mixture was purified by preparative silica gel TLC developed by CH₂Cl₂:MeOH (10:1). A dark red waxy solid was obtained. Crude yield: 16.7% with purity 95% (system 1). A further HPLC purification (system 2) was needed for both the PI3 kinase enzyme and anti-proliferative cell based assays. A bright red powder (**4c**) would be obtained after lyophilization. HRMS: C₄₀H₄₄N₆O₁₁, expd. 785.3141 (M + H⁺), obsd. 785.3145. LRMS: 807.4 [M+Na⁺]; ¹H NMR (CDCl₃, ppm, 400MHz): 0.90 (3H, s, C13-CH₃), 1.44-1.49 (2H, m, J = 7.3 Hz, NCH₂CH₂CH₂CH₂CH₂CO), 1.62 (3H, s, C10-CH₃), 1.71-1.83 (5H, m, NCH₂CH₂CH₂CH₂CH₂CO, H-12), 2.02-2.12 (1H, m, H-15), 2.24-2.34 (1H, m, H-16), 2.37 (2H, t, J = 7.32, NCH₂CH₂CH₂CH₂CH₂CO), 2.45-2.49 (1H, q, J₁ = 7.32 Hz, J₂ = 13.7 Hz, H-12), 2.57-2.63 (1H, q, J₁ = 8.8 Hz, J₂ = 19.5Hz, H-16), 2.66 (1H, s, OH), 2.92-2.98 (2H, m, H-14, OCH₂), 3.17-3.19 (1H, m, H-15), 3.20 (3H, s, OCH₃), 3.21 (3H, s, N-CH₃), 3.34-3.37 (1H, q, J₁ = 2.9 Hz, J₂ = 10.7 Hz, OCH₂), 3.46 (3H, br, NCH₃), 4.12 (2H, br, NCH₂), 4.73-4.74 (1H, q, J₁ = 2.9 Hz, J₂ = 5.9 Hz, H-1), 6.10-6.13 (2H, m, H-11, NBD-H), 7.22 (2H, d, J = 6.8 Hz, Py-H), 8.26 (1H, s, H-20), 8.46 (1H, d, J = 8.8 Hz, NBD-H), 8.63 (2H, d, J = 6.8 Hz, Py-H).

Synthesis of 5a

Amino dextran (300mg, 0.00429mmol) in 4 mL PBS, pH 7 was added to the NHS ester of **2a** in 4 mL DMSO (28.7mg, 0.0429 mmol) and the mixture incubated for 1.5 hr at 37 °C. Low molecular weight impurities were removed with Sephadex G-50 chromatography in 1mM phosphate buffer at pH 7.0. A yellow powder was obtained after lyophilization. The Wm attached was determined from Wm absorbance by using UV standard curves at 418 nm. The ratio of **2a** to dextran was 5.9.

Synthesis of 5b

Amino dextran (202mg, 0.00429mmol) in 3 mL PBS, pH 7 was added to the NHS ester of **2b** in 5 mL DMSO (56 mg, 0.0858 mmol) and the mixture incubated for 3 hr at 37 °C. Low molecular weight impurities were removed as above and a yellow powder was obtained after lyophilization. The Wm attached was determined from Wm absorbance by using UV standard curves at 408 nm. The ratio of **2b** to dextran was 7.6.

Synthesis of 6a

The NHS ester of Cy5 (Amersham Biosciences) was attached to a 70 kDa amino dextran- (Invitrogen) following the Amersham protocol and the molar ratio of Cy5:dextran (0.58) determined spectrophotometrically. The remainder of the amino groups on the amino dextran (Cy5) were then reacted with the NHS ester of **4a**. To amino dextran(Cy5) (35mg, 0.0005mmol) in 1 mL PBS, pH 7 was added to the NHS ester of **4a** in 4 mL DMSO (6.6mg, 0.00718mmol) and the mixture incubated for 2 hr at 37 °C. Low molecular weight impurities were removed with lyophilization as above. The NBD-Wm **3** attached was determined from NBD absorbance by using UV standard curves at 490 nm. The ratio of **4a** to dextran was 3.7.

Synthesis of 6b

Amino-dextran (36 mg, 0.0005mmol, 70 kDa) was reacted with the NHS ester of **4b** (5.5 mg, 0.005mmole) and purified as above. The ratio of **4b** to dextran was 6.3.

Because dextran interfered with the PI3 kinase assay (data not shown), the ability of **5a**, **5b**, **6a** and **6b** to inhibit PI3 kinase was obtained with compounds where an amino glucose carrier replaced the amino dextran. Compound **2a** (or **2b**, **4a**, **4b**) (0.04 mmol) was mixed with 2-amino glucose hydrogen chloride (0.4 mmol) in anhydrous DMSO (1ml). After the addition of triethylamine (60 μ l), the mixtures were incubated under 37°C for 2.5 hr. The solution was diluted with water (1 ml) and applied to HPLC using system 5: buffer A:buffer B (90:10) linear gradient to buffer A:buffer B (40:60) over 25 min, then isocratic for 5 min and gradient back to 90:10 (buffer A:buffer B) in 5 min and isocratic for 5 min, flow: 4.9 ml/min. λ_{max} : 280 nm and 410 nm. Yield: 65-80%. HRMS: **5a**: C₃₆H₅₀N₂O₁₄: exp. 735.3335 [M+H⁺], obs. 735.3325, LRMS: 757.4 [M+Na⁺]; **5b**: C₃₅H₄₈N₂O₁₄: exp. 721.3178 [M+H⁺], obs. 721.3176, LRMS: 743.4 [M+Na⁺]; **10a**: C₄₇H₆₂N₆O₁₇: exp. 983.4244 [M+H⁺], obs. 983.4256, LRMS: 1005.4 [M+Na⁺]; **10b**: C₄₆H₆₀N₆O₁₇: exp. 991.3907 [M+Na⁺], obs. 991.3914, LRMS: 991.7 [M+Na⁺].

Generation of Wm or NBD-Wm 3

The half-lives for Wm formation were determined by incubating WmC20 compounds at 1.5 mM Wm equivalents in PBS, pH 6.8 and 37°C. The mixture was analyzed by HPLC using MHB as an internal standard (1 μ g of methyl-4-hydroxybenzoate). With **4a**, **4b** and **4c**, 10% acetonitrile and 5% DMSO were added to insure solubility of the NBD-Wm that was formed. System 1 (for **2c** and **4c**), system 5 (for **4a**) and system 6 (for **4b**) were used for HPLC analysis. With the WmC20 dextran conjugates Wm and NBD-Wm insolubility was utilized as a separation mechanism. A solution of **5a** or **6a** (1.5 mM Wm equivalents) in 300 μ L in PBS (pH 7.0) was incubated at 37 °C. Released Wm or NBD-Wm was removed by centrifugation (5 minutes, 13,000 rpm, Eppendorf benchtop microfuge). The absorbance of the supernatant at 322 nm or 480 nm for **5a** and **6a**, respectively, were used to obtain Wm released. Data were fit to a first order decay process (log Wm concentration versus time), with coefficients of correlation of greater than 0.95 in all cases. First order decay constants were converted to half-lives.

Activity based bioassays

The antiproliferative assay employed the A549 cell line, which was used in all facets of this study, and was performed as described²⁹. The PI3 kinase assay employed the gamma catalytic subunit of PI3 kinase and was the fluorescence energy transfer assay³⁰.

Cellular pharmacokinetic bioassays

The formation of NBDylated (wortmannylated) proteins was monitored as described²⁹. Briefly, cells were treated with 10 μ M of NBD-Wm **3** or an NBD-WmC20 derivative for the indicated time and whole cell lysates generated. Cell protein was normalized between samples and wortmannylated (NBDylated) protein was visualized using an anti-NBD rabbit polyclonal antibody (Biogenesis, Munich, Germany) and quantified using densitometry. Labeled cell protein was taken as the sum of the areas of three prominent bands (35, 55 and 75 kDa). Values of labeled cell protein as a function of time (L(t)) were fit to a two step sequential model where k_1 was the rate of increase of labeled protein, k_2 was the rate of decrease of labeled protein, and L_0 was the maximum using GraphPad Prism Software (Irvine, CA): $L(t) = L_0 * k_1 / (k_2 - k_1) * \exp((-k_1 * t) - \exp(-k_2 * t))$. To obtain the uptake of compounds, cells were incubated with 10 μ M of **3** or an NBD-WmC20 derivative for the indicated time. Cells were detached with trypsin and fluorescence determined by FACS²⁹. Cell fluorescence as a function of time (F(t)) was

measured as the relative cellular fluorescence (RCF), which is the geometric mean of cells exposed to an NBD compound divided by geometric mean of unexposed cells^{29, 31, 32}. All data for the first hour were fit to a single exponential curve ($F(t) = F_0 \cdot (1 - \exp(-k \cdot t))$). Calculated standard errors were less than 10% and have not been provided.

The X-ray crystal structure of Wortmannin bound to PI3K gamma (1E7U,12) was loaded into PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002), DeLano Scientific, Palo Alto, CA, USA) and the 6-carbon linker and NBD were attached using the PyMOL Build function.

Results

Our strategy for the synthesis of Wm based viridin derivatives is shown in Figure 1. Low molecular weight WmC20 derivatives were prepared by reacting the C20 of NBD-Wm **3** or Wm **1**, which behave similarly as PI3 kinase inhibitors and in the antiproliferative assay²⁹, with nucleophiles to yield **2a-e** and **4a-c**. Modification of Wm at C11 for NBD attachment and modification at C20 occur independently. Low molecular weight WmC20 derivatives featuring a carboxylic acid (**2a**, **4a**, **2b** and **4b**) were then reacted with the amino group of a 70 kDa amino-dextran, which increases their molecular weight and increases their hydrophilicity through an association with many hydroxyls on the polyglucose-like dextran polymer.

The half-lives of Wm formation of the twelve WmC20 compounds shown in Figure 1 were then determined. Compounds featuring a N(Me)pyridine group at C20 (**2c** and **4c**), had half-lives for Wm formation of 2.30 and 1.89 h, respectively. These compounds are termed “fast Wm forming” WmC20 derivatives and are shown in italics in Table 1. Five WmC20 derivatives featured an alkyl based tertiary amine at C20 (**2a**, **2d**, **4a**, **5a** and **6a**) and formed Wm with half-lives between 8.0 and 9.9 hours. These compounds are termed “slow Wm forming” WmC20 compounds and are shown in bold lettering in Table 1. Five WmC20 compounds featuring a secondary amine at C20 (**2b**, **2e**, **4b**, **5b** and **6b**) were termed “non Wm forming” WmC20 derivatives. With these compounds the formation of Wm could not be detected (less than 2% of the WmC20 compound) after 10 hours in PBS, our standard assay, or when the incubation period was extended to 100 hours. Based on their behavior under these conditions, they are termed “non-Wm formers,” though Wm formation might occur below our detection limits or in biological systems. They are listed as having half-lives of greater than 100 h in Table 1. The designation of WmC20 derivatives as “fast,” “slow” or “non” Wm reflected their rate of formation, which in turn was determined by the nature and type of atoms near C20 (leaving group), and which was independent of the group present at C11 (NBD adduct vs. acetyl group) or the attachment of dextran attached to the carboxyl group of **2a** and **2b**, or **4a** and **4b**.

We next measured the behavior of fast, slow and non-Wm forming WmC20 compounds using Wm's which lacked modifications at C20, that is Wm **1** and NBD-Wm **3** as controls, in our four bioassays. Data obtained is provided in Table 1.

Activity based antiproliferative and PI3 kinase assays

With the antiproliferative assay, fast Wm releasing compounds had IC₅₀'s similar to Wm, slow Wm releasing compounds had IC₅₀'s 3 to 12 times lower than the Wm IC₅₀ (better than Wm), and non Wm releasers had IC₅₀'s at least 2.6 times higher than that of Wm (worse than Wm). With the *in vitro* PI3 kinase assay on the other hand, all WmC20 compounds tested were notably less effective inhibitors than Wm. Fast or slow Wm releasing compounds had IC₅₀'s 5 to 30 times higher than Wm. Non-Wm formers did not inhibit PI3 kinase even at the highest concentration employed (10000 nM). All WmC20 derivatives tested were weaker PI3 kinase inhibitors than Wm, reflecting the incomplete generation of Wm during this 0.5 h assay, with

the rate of Wm formation governed by the nature of the leaving group at C20. To ascertain whether the variable rates of Wm formation achieved with our panel of WmC20 derivatives in PBS determined the pharmacokinetics with which compounds interacted with cells, we exposed intact cells to fluorescent WmC20 compounds and determined their fate.

Labeled cell protein assay

The time course for the formation of wortmannylated cell protein provided a measure of a cell's exposure to the reactive species (NBD-Wm **3**), when an NBD-WmC20 compound was applied to cells. Cells were exposed to 10 μ M of the non-C20 protected NBD-Wm or **3**, the fast Wm releasing **4c**, the slow releasing **4a** and **6a**, and the non releasing **4b** and **6b** for various times, then lysed and the wortmannylated proteins determined via NBD immunoreactivity as shown in Figure 2. With all compounds, the 55 kDa band was detected before other bands and was the only band seen with the non Wm releasing **4b**. The ability of **4b** to label the 55 kDa protein indicates some NBD-Wm formation was occurring under these conditions (48 h incubation with cells), though **4b** was designated as a "non" Wm forming compound based on its behavior in PBS. This small difference may reflect slightly different activation mechanisms in cells versus PBS, or reflect a higher sensitivity of the enzyme amplified Western blot method for detecting wortmannylated protein in Figure 2.

To more quantitatively determine the time course of cell protein labeling, films of Western blots were scanned and areas of the 35 kDa, 55 kDa and 75 kDa bands were summed and plotted versus time as shown in Figure 3A, with the relative initial rates of cell protein labeling given in Table 1. The formation of wortmannylated cell protein reflects a reaction between the C20 of Wm, either added as NBD-Wm **3** or released from a NBD-WmC20 derivative (**4a-c**, **6a**, **6b**), and the epsilon amino groups of protein lysines. Reactions between Wm and lysine (an N acetylated lysine), including exchange reactions between WmC20 protected derivatives and lysine, occur under physiological conditions *in vitro*²⁸. The decrease of wortmannylated protein with long incubation times likely reflects protein degradation and synthesis. All WmC20 protected compounds labeled protein more slowly than the non-C20 protected NBD-Wm. The low molecular weight **4c** labeled protein similarly to **4a**, which in turn was faster than **4b** (**4c** ~ **4a** > **4b**). The difference between **4a** and **4b** (**4a** > **4b**) was maintained when they were attached to dextran (**6a** > **6b**), though attachment to dextran resulted in a decreased rate of protein labeling (**4a** > **6a** and **4b** > **6b**).

Cell fluorescence assay

To obtain another measure of the pharmacokinetics of NBD-WmC20 compounds, cells were incubated with these compounds and the resulting NBD-derived cellular fluorescence as a function of time determined as shown in Figure 3B. Relative initial rates of fluorescence increase, which reflect compound uptake, are provided in Table 1. Here **4c** was taken up faster than **4a**, which was taken up faster than **4b** (**4c** > **4a** > **4b**). Again this pattern (**4a** > **4b**) maintained when **4a** or **4b** were attached to dextran (**6a** > **6b**), though as with the labeled cell protein assay, dextran attachment resulted in a decreased rate of compound uptake (**4a** > **6a** and **4b** > **6b**).

Discussion

WmC20 compounds have variable rates of Wm formation

WmC20 compounds formed Wm at highly variable rates that depended on the nature of the leaving group at C20. With an N-methyl pyridine at C20, the delocalization of electrons of the pyridine ring together with a tertiary amine at C20, resulted in fast Wm formation. Half-lives for Wm formation were 1.89 and 2.3 h for **2c** and **4c**, respectively (Table 1). With alkyl group based tertiary amines at C20, a slow Wm formation was obtained: half-times were between 8.0 and 9.9 h. Slow Wm forming compounds included N(Me) hexanoic acid based WmC20

derivatives (**2a**, **4a**, **5a**, **6a**) or **2d**, which featured a proline leaving group. With secondary amines at C20, Wm formation was not observed: the half-time for Wm formation was greater than 100 h. Compounds in this class included N(H) hexanoic acid based compounds (**2b**, **4b**, **5b**, **6b**) or **2e** which featured a lysine leaving group. The rates of Wm release were independent of the presence of the group at C11 (acetyl or NBD-adduct) and the attachment of the low molecular carboxylic acid WmC20 derivatives (**2a**, **2b**, **4a**, **4b**) to a dextran carrier (**5a**, **5b**, **6a**, **6b**). The attachment of low molecular weight WmC20 derivatives to dextran increased the size, and water solubility of the Wm (data not shown) without altering Wm release kinetics. For example, the half-life for Wm formation with **2a** was 8.7 h, while that for **2a** with dextran attached, (**5a**), was 9.1 h.

WmC20 compounds as inhibitors of PI3 kinase

All WmC20 compounds were substantially weaker PI3 kinase inhibitors than Wm, though they fell into two classes. Compounds which did not form Wm (or NBD-Wm), that is **2b**, **4b**, **5b**, **6b** or **2e**, featured a secondary amine at C20 and failed to inhibit PI3 kinase at the highest concentration employed (10000 nM). Fast Wm forming (**2c**, **4c**) or slow Wm forming compounds (**2a**, **4a**, **5a**, **6a**, **2d**) inhibited the enzyme but with higher IC₅₀'s than Wm, due to the incomplete production of Wm in the 0.5 h time of the assay. These results were consistent with our earlier work on the inhibition of PI3 kinase by **2a** and **2b** which used a radioactive lipid phosphorylation assay²⁷, while the current study employed a fluorescence energy transfer method³⁰.

WmC20 compounds as inhibitors cell proliferation

The conclusion that the kinetics of Wm formation controlled the antiproliferative activity of WmC20 compounds was supported by the correlation between the rates of Wm formation and the antiproliferative activities of the WmC20 compounds in culture (Table 1). Fast Wm releasers with half-times for Wm formation of 1.87 to 2.30 h, had IC₅₀'s similar to Wm, which were short compared to the 48h time for the antiproliferative assay and reflects the negligible effect of the Wm formation reaction under these conditions. Slow Wm releasers (half-time for Wm formation about 9 hours) had lower IC₅₀'s than Wm (more potent than Wm), a behavior we related to their slow generation of Wm over the 48 h time of the assay. On the other hand, non-Wm formers had IC₅₀'s higher than Wm. Since their half-times for Wm formation were greater than 100h, about twice the 48 h assay period, these compounds would be expected to largely remain as inactive C20 modified Wm's for the duration of the assay. Although the rate of Wm formation with WmC20 compounds appeared to explain the activity of these compounds in PI3 kinase and antiproliferative assays, we sought to confirm that Wm release controlled the pharmacokinetic behavior of these compounds in more complex situations involving cells.

With assays for the cellular uptake of NBD-Wm (cell fluorescence and the formation of wortmannylated cell protein), the widely varying rates of NBD-Wm formation *in vitro* correlated with very different behavior when NBD-WmC20 derivatives were exposed to cells (Table 1). Thus the fast Wm forming **4c** entered cells faster than the slowly Wm forming **4a**, which in turn was faster in these respects than the non-Wm forming **4b** (**4c** > **4a** > **4b**). The non-Wm forming compounds **4b** and **6b** exhibited extremely slow pharmacokinetics in cell protein labeling, and were poorly internalized by cells. Results with the labeled cell protein assay generally paralleled those with the cell fluorescence assay, the exception being that the rate of cell protein labeling for **4c** was slightly slower than **4a**, though on the basis of Wm formation rates **4c** should be faster.

The view that the energy for the high affinity between PI3 kinase and Wm is largely derived from the reaction between the electrophilic C20 and a lysine in the ATP site of the catalytic

subunit of PI3 kinase stems from observations that Wm when modified at C20 inhibits PI3 kinase either very poorly or not at all^{14, 23}, though the opposite result, that WmC20 derivatives are better PI3 kinase inhibitors than Wm have also been described^{22, 26}. In light of the different reports on the inhibition of PI3 kinase by WmC20 compounds, it is of interest to consider the crystal structure of Wm in the ATP site of PI3 kinase, and what it implies for the self-activating mechanism of action proposed here.

A model of NBD-Wm in the ATP site of PI3 kinase p110 gamma subunit based on earlier crystallographic studies is shown in Figure 4¹² provides important support for two key features of the postulate that NBD-WmC20 derivatives are in effect “fluorescent Wm prodrugs.” First, when NBD-Wm is formed, it can bind in the ATP site of the kinase similarly to Wm. As shown in Figure 4A, the rings of NBD-Wm **3** (orange) bind deeply in the ATP pocket, while NBD (cyan) protrudes far out into the solvent, consistent with similar IC₅₀'s for PI3 kinase that result when NBD is attached to Wm (Table 1). Second, WmC20 derivatives should not be able to bind in the ATP site of the kinase, a possibility examined in Figure 4B. An NBD-Wm modified at C20 cannot bind to PI3 kinase because of the proximity of surface of the enzyme (blue). Other aspects of the Wm binding to PI3 kinase not shown in Figure 4 indicate a deeply buried C20 is essential for formation of important stabilizing hydrogen bonds between (i) the carbonyl oxygen at the C3 of Wm and hydroxyl group of S806 and, (ii) between the hydroxyl group at C6 and an oxygen of D964¹². As shown in Figure 1, C3 and C6 are positioned close to the reactive C20.

As summarized in Figure 5 the behavior NBD-Wm C20 derivatives in the PI3 kinase assays (5A) and anti-proliferative assay (5B) depends on a combination of assay duration and the rate of release of NBD-Wm. In the 0.5 h kinase assay, the fast activating (**4c**) and slow activating (**4a**, **6a**) NBD-WmC20 derivatives partially generate NBD-Wm **3** and have higher IC₅₀'s (are weaker) than the non C20 protected NBD-Wm. NBD-Wm does not have to self-activate and therefore has a lower IC₅₀. Non-Wm forming compounds (**4b**, **6b**) failed to inhibit PI3 kinase. If NBD-Wm is added to culture media (Figure 5B), reactions occur at the non-protected C20 and with nucleophiles like amino acids as previously described²⁸ and as shown in 5B. An uncontrolled mixture of NBD-WmC20 compounds is formed, components of which slowly generate Wm, endowing the culture media with a continuing antiproliferative activity though NBD-Wm has disappeared. When a slowly NBD-Wm forming compound (**4a** or **6a**) is added, NBD-Wm slowly forms over the assay period, which is about 5 half-lives (48/9). Cell fluorescence increases slowly (Figure 3B), and wortmannylated proteins form slowly (Figure 2), indicating the rate limiting step in culture media with these compounds is the slow release of NBD-Wm. WmC20 derivatives that form NBD-Wm slowly behave as self-activating, slow release prodrugs, though they are relatively poor PI3 kinase inhibitors.

Results with the slow Wm forming, dextran conjugate **6a** are of particular interest for two general reasons. First, with **6a** the formation of NBD-Wm **3** is associated with a drastic change in physical properties. When present as the **6a** prodrug, NBD-Wm has a molecular weight of 72 kDa, and is hydrophilic and water soluble, properties conferred by its attachment to the 70 kDa dextran carrier. Released from **6a**, NBD-Wm (MW=677 daltons) is poorly soluble in water. Therefore with **6a** predominant release of NBD-Wm is extracellular, and the released NBD-Wm undergoes a rapid cellular uptake. Supporting this view is the fact that when NBD-Wm is attached to dextran with a non-releasing linkage (**6b**), it exhibited no antiproliferative activity and failed to enter cells, both by the cell fluorescence and modified protein assays. A second reason for focusing on **6a** is that when used *in vivo*, the dextran can be an inert macromolecular carrier, extending blood half-life, slowing hepatic uptake, and providing enhanced tumor targeting due to the enhanced permeability and retention effect^{33, 34}. The results of **6a**, termed the SAV prodrug (self-activating viridin prodrug), in animal models will be presented shortly.

Our slow release mechanism shown in Figures 5A and 5B provides a basis for two recommendations for selecting Wm or Wm derivatives to analyze signal transduction pathways. For short-duration assays (minutes to about 1 h), Wm should be used, since WmC20 derivatives are inactive and will fail to form Wm during these time periods, as evident from results with our PI3 kinase assay. In addition, Wm's lack of charge and hydrophobicity allows it to enter cells rapidly, as was the case for the minimally charged NBD-Wm (Figure 3B). For assays where long incubations are employed, greater than about 6 hours, a slowly Wm forming, C20 protected Wm should be used. For animal studies or pharmaceutical applications, WmC20 derivatives are also recommended. As indicated by Wm half-life of less than 10 minutes in culture media³⁵, the use of unprotected, C20 reactive Wm will result in the formation of an uncontrolled mixture of WmC20 compounds, which can give rise to variable results depending not on the design of the compound but on the nucleophiles present in the media it encounters.

The improved potency as antiproliferative agents of compounds like **6a** is related to their mechanism of slow self-activation and generation of active NBD-Wm **3**, and neither implies nor requires that PI3 kinase be the molecular target of the released NBD-Wm. Our use of the PI3 kinase assay and antiproliferative assay to assess the bioactivity was based on the incubation times employed by these assays, and by their use in many laboratories, rather than a mechanistic relationship between them. In fact determining the molecular target responsible for Wm's inhibition of cell proliferation is complicated by the micromolar concentrations necessary to achieve this effect, see Table 1 and^{15, 16, 22}. These concentrations are considerably above the IC₅₀'s of Wm not only for PI3 kinase (IC₅₀ = 1-5 nM,^{2, 3, 8}, but also for polo-like kinase (IC₅₀= 24 nM,⁴), mTOR (IC₅₀=200 nM,⁵), DNA-dependent protein kinase (IC₅₀= 200 nM,⁶ and ATM (IC₅₀= 200 nM,⁷). A second observation for the specificity of Wm for PI3 kinase *in vivo* was its selective modification of the p110 subunit of PI3 kinase from neutrophils^{13, 36}. However, more recent studies with a variety of Wm's and cancer cell lines have shown Wm can label multiple cell proteins^{4, 29, 37}. Our assay for wortmannylated cell protein (Figure 3A) does not assume that the labeled bands visualized (bands at 35, 55 and 75 kDa) are involved in generating the biological effects of Wm. Instead, this assay assumes that the degree of modification of these proteins provides a quantitative indication of the exposure of the cell to the chemically reactive C20 of NBD-Wm, in much the same way that the level of glucose modified proteins like hemoglobin A1c can be used as an indication of the exposure of cells to glucose³⁸.

Wm's unique chemistry, the ability to attach the NBD fluorochrome to the C11 carbon and then further modify Wm at C20, permits the design of fluorescent WmC20 derivatives which self-activate to produce the active NBD-Wm **3** and whose disposition in biological systems can be readily ascertained. The chemistry outlined here can be expanded to obtain more chemically diverse NBD-Wm releasing libraries of compounds whose physical properties or interaction with molecular targets could be determined by replacing the amino-dextran of **6a** with polysaccharides, peptides, antibodies, etc. The ability to rapidly determine the fate of such materials *in vivo* could then be based on NBD's fluorescence or immunoreactivity, providing a preliminary assessment of a compound's biodistribution and pharmacokinetic properties. Since man-made kinase inhibitors achieve selectivity by binding to the active sites of target kinases with high affinity, the delivery-based design of self-activating Wm natural product prodrugs can be a completely distinct and therefore valuable approach to obtaining kinase inhibitors.

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Figure 1. Synthesis of fluorescent WmC20 derivatives

Wm **1** is modified at C11 by the attachment of NBD and a 6 carbon linker to obtain NBD-Wm, **3**. When **3** is modified at C20, it becomes a self-activating fluorescent derivative by virtue of an intramolecular attack of the hydroxyl at C6 which forms Wm as shown in greater detail in Figure 1 of (29). The physical properties of low molecular weight fluorescent derivatives (**4a**, **4b**) can be further altered by reaction with an amine “carrier,” as illustrated by their reaction with amino dextran to form **6a** and **6b**. Oxygens at C3 and C6 flanking C20 form hydrogen bonds when Wm (or NBD-Wm) has reacted with a lysine in the ATP binding site of PI3 kinase gamma, see discussion regarding Figure 4.

Figure 2. Time course for the formation of wortmannylated cell protein when cells were incubated with NBD-Wm or NBD-WmC20 compounds

Multiple wortmannylated (NBDylated) proteins were seen with the principal bands at 35 kDa (possibly a doublet), 55 kDa and 75 kDa. With all compounds the 55 kDa band appears first. Time course of cell protein labeling (total area of 35, 55 and 75 kDa bands) was further analyzed as shown in Figure 3A.

Figure 3. Time courses for the behavior of fluorescent WmC20 derivatives in cells

(A) Time course for the formation of wortmannylated cell protein with the C20 reactive NBD-Wm **3** or NBD-WmC20 compounds. (B) Time course for the change in cell fluorescence, reflecting uptake of NBD. The relative cell fluorescence (RCF) is shown on a short time scale (0-1 h) and long time scale (0-24 h). The y-axis is the same for both. Lines in (A) or (B) (0-1 h) were obtained by fitting data to the equations given in Methods, with the relative initial rates given in Table 1.

Figure 4. Binding of NBD-Wm 3 into the active site of PI3 kinase gamma

Model is based on the published structure of Wm in the ATP site of PI3 kinase gamma (PDB: 1E7U) with NBD adduct added. (A) Desacyl Wm (Wm lacking the acetyl group at C11) is orange. The NBD adduct (NBD and six carbon linker) is colored cyan, with nitrogen in blue and oxygen in red. The NBD and the six-carbon linker protrude out of the ATP-binding pocket into the solvent while Wm is buried deeply in the ATP-binding pocket. (B). Another view of NBD-Wm showing the relationship between the surface of the enzyme and NBD-Wm. Lysine 833 covalently reacted with the C20 of Wm, is in magenta and extends through a blue, positively, charged surface. The proximity of the surface prevents Wm's modified at C20 from binding in the ATP-binding pocket.

(A) PI3 Kinase Assay: 0.5 h

(B) Antiproliferative Assay: 48 h

Figure 5. Fate of NBD-WmC20 derivatives in biological systems

(A) PI3 kinase assay: With a 0.5 h incubation, fast and slow activating WmC20 derivatives partially activate by generating NBD-Wm 3 and have higher IC₅₀'s than non C20 protected forms of Wm like 3. 3 is stable and active under these conditions and has a low IC₅₀. (B) Antiproliferative assay: NBD-Wm in culture media reacts with nucleophiles like amino acids, yielding a mixture of NBD-WmC20, components of which slowly regenerate NBD-Wm, endowing the culture media with a continuing antiproliferative activity though NBD-Wm has disappeared. When a slowly NBD-Wm forming prodrug (4a or 6a) is used, the viridin NBD-Wm forms over the assay period. The slow formation of 4a or 6a an increased potency relative

to **3**, though the slow formation of NBD-Wm makes these compounds poor PI3 kinase inhibitors. NBD-Wm rapidly enters cells after release.

Table 1

Wm Formation and Activities of WmC20 Compounds

Compound	Group @ C11	Group @ C20	Carrier	Wm Formation $t_{1/2}$ (h)	Antiprolif. IC50, (μ M)	PI3K IC50, (nM)	Cell Prot Label. Initial Rate	Cell Fluor. Initial Rate
1	Acetyl	none	None	Not Appl.	11.4 $\pm$ 0.5	17.6 $\pm$ 1.7		
3	NBD	none	None	Not Appl.	12.2 $\pm$ 0.8	24.2 $\pm$ 1.6	1.00	1.00
2c	Acetyl	N(Me)-Pyridine	None	2.30 (2.13-2.42)	9.86 $\pm$ 2.52	119 $\pm$ 11		
4c	NBD	N(Me)-Pyridine	None	1.87 (1.69-2.73)	9.99 $\pm$ 1.25	262 $\pm$ 20	0.206	1.199
2a	Acetyl	N(Me)-alkyl	None	8.7 (7.83-9.75)	0.96 $\pm$ 0.33	128 $\pm$ 16		
4a	NBD	N(Me)-alkyl	None	8.0 (6.96-9.53)	3.85 $\pm$ 0.36	174 $\pm$ 12	0.246	0.187
2d	Acetyl	Pro	None	9.9 (7.29-15.53)	1.79 $\pm$ 0.18	105 $\pm$ 11		
5a	Acetyl	N(Me)-alkyl	Dextran	9.1 (7.42-11.65)	1.05 $\pm$ 0.42	458 $\pm$ 21		
6a	NBD	N(Me)-alkyl	Dextran	9.2 (8.54-10.08)	1.04 $\pm$ 0.10	453 $\pm$ 17	0.0656	0.0661
2b	Acetyl	N(H)-alkyl	None	>100	27.9 $\pm$ 3.3	>10000		
4b	NBD	N(H)-alkyl	None	>100	48.7 $\pm$ 13.3	>10000	0.00613	0.0271
5b	Acetyl	N(H)-alkyl	Dextran	>100	Not detect	>10000		
6b	NBD	N(H)-alkyl	Dextran	>100	Not detectable	>10000	None	0.0064
2e	Acetyl	NAc---lys	None	>100	29.8 $\pm$ 0.9	>10000		