Bioorganic & Medicinal Chemistry Letters 19 (2009) 4223–4227

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A stabilized demethoxyviridin derivative inhibits PI3 kinase

Hushan Yuan [a], Monica T. Pupo [c], Joe Blois [a], Adam Smith [a], Ralph Weissleder [a,d], Jon Clardy [b], Lee Josephson [a,*]

[a] Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School, 149 13th Street, Charlestown, MA 02129, United States
[b] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115, United States
[c] Universidade de Sao Paulo, Ribeirao Preto, SP, Brazil
[d] Center for Systems Biology, Massachusetts General Hospital and Department of Systems Biology, Harvard Medical School, 185 Cambridge Street, Boston, MA 02115, United States

ARTICLE INFO

Article history:
Received 17 February 2009
Revised 26 May 2009
Accepted 27 May 2009
Available online 30 May 2009

Keywords:
Viridins
P13 kinase
Natural products
Inhibitors

ABSTRACT

The viridins like demethoxyviridin (Dmv) and wortmannin (Wm) are nanomolar inhibitors of the PI3 kinases, a family of enzymes that play key roles in a host of regulatory processes. Central to the use of these compounds to investigate the role of PI3 kinase in biological systems, or as scaffolds for drug development, are the interrelated issues of stability, chemical reactivity, and bioactivity as inhibitors of PI3 kinase. We found that Dmv was an even more potent inhibitor of PI3 kinase than Wm. However, Dmv was notably less stable than Wm in PBS, with a half-life of 26 min versus Wm's half-life of 3470 min. Dmv, like Wm, disappeared in culture media with a half-life of less than 1 min. To overcome Dmv's instability, it was esterified at the C1 position, and then reacted with glycine at the C20 position. The resulting Dmv derivative, termed SA–DmvC20-Gly had a half-life of 218 min in PBS and 64 min in culture media. SA–DmvC20-Gly underwent an exchange reaction at the C20 position with *N*-acetyl lysine in a manner similar to a WmC20 derivative, WmC20-Proline. SA–DmvC20-Gly inhibited PI3 kinase with an IC_{50} of 44 nM, compared to Wm's IC_{50} of 12 nM. These results indicate that the stability of Dmv can be manipulated by reactions at the C1 and C20 positions, while substantially maintaining its ability to inhibit PI3 kinase. Our results indicate it may be possible to obtain stabilized Dmv derivatives for use as PI3 kinase inhibitors in biological systems.

The viridins, a class of fungally derived compounds that includes demethoxyviridin (Dmv) and wortmannin (Wm), are high affinity inhibitors of PI3 kinases.[1,2] As shown in Figure 1, Dmv and Wm are steroid-like structures featuring a common furan ring but which differ in other respects: (i) Dmv lacks the lactone found in the A ring of Wm, (ii) Dmv has a hydroxyl group at C1, while Wm has an acetoxy group at C11, (iii) Dmv features an unsaturated C ring and consequently a more extensive system of unsaturated double bonds.

Recently we showed that Wm derivatives synthesized by reaction of the C20 carbon with secondary amines were about 20 times less potent than Wm as inhibitors of PI3 kinase in 30 min assay but were about 10 times more potent than Wm in a 48 h anti-proliferative assay.[3] Thus, with WmC20 derivatives optimal bioactivity was obtained with a slowly Wm generating compound, rather than with a compound which had an optimal affinity for a presumed molecular target, PI3 kinase. A polymeric, slowly self-activating viridin ('SAV') derived from these studies had a potent anti-inflammatory effect in the ovalbumin model of lung inflammation, while a non-self-activating viridin ('NAV') lacked activity.[4] SAV also had powerful effects in an animal model of arthritis and in an A549 xenograft tumor animal model.[5,6]

Based on the hypothesis that virdin stability might be more important than target affinity in determining bioactivity, we initially hypothesized that Dmv would be superior to Wm as biological systems. We noted that Dmv lacked the lactone ring of Wm, and therefore lacked lactone ring opening as a potential

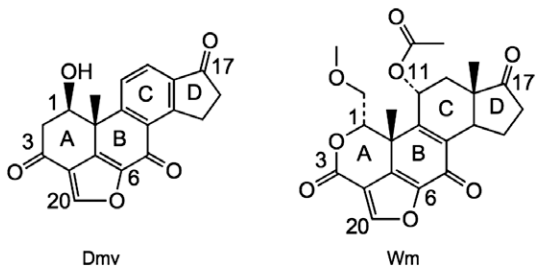

Figure 1. Structures of demethoxyviridin (Dmv) and wortmannin (Wm). Both compounds are steroid-like with an extra furan ring. Dmv lacks the lactone in ring A, the acetyl group at C11 and has an unsaturated ring C.

* Corresponding author. Fax: +1 617 726 5708.
E-mail addresses: mtpupo@fcfrp.usp.br (M.T. Pupo), ljosephson@mgh.harvard.edu (L. Josephson).

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doi:10.1016/j.bmcl.2009.05.105

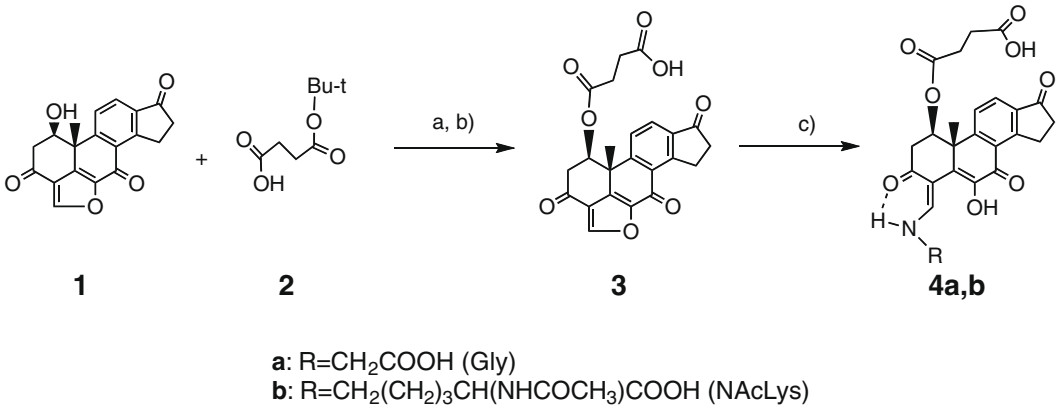


Figure 2. Synthesis of Dmv derivatives. C1 was esterified, yielding SA–Dmv, (**3**). C20 of SA–Dmv was reacted with primary amines to yield the open furan ring forms of SA–Dmv, compounds **4a** and **4b**. Reagents and conditions: (a) EDC, DMAP, CH_2Cl_2; (b) TFA/CH_2Cl_2; (c) NH_2R, PBS buffer, pH 7.3.

mechanism of instability. The opening of the lactone ring has been considered to be an important source of Wm instability by others.[1,7] We have argued an alternative view, that the lactone ring is highly stable at physiological pH's and that Wm's disappearance in culture media results from a reaction of its C20 carbon with nucleophiles.[8,9]

We undertook the current study with three goals. First, we sought add to our understanding of the stability, chemical reactivity and bioactivity of the viridins by comparing Dmv and Wm and their derivatives in different media. Second, given interrelationships between stability, chemical reactivity and bioactivity of Wm,[3] we sought to demonstrate that a stable Dmv derivative could be obtained that maintained an affinity for the presumed molecular target of the viridins, PI3 kinase. A Dmv derivative modified at C1 by the attachment of a succinic acid (SA) and at C20 by the attachment of glycine (we have termed this compound SA–DmvC20-Gly) achieved these goals. Finally, we sought to demonstrate that SA–DmvC20-Gly, like some Wm's modified at C20,[8] can undergo an unusual covalent exchange reaction at C20 under physiological conditions. This suggests that Dmv derivatives could be designed to serve as inactive, but self-activating prodrugs as has been done with the Wm scaffold.[3,4,9] Thus development of SA–DmvC20-Gly may serve to stimulate future interest in Dmv as the basis for drug development.

Synthesis of Dmv derivatives (*procedures* see Supplementary data): The reactions used to modify Dmv **(1)** are shown in Figure 2. The C1 hydroxy group was esterified by reaction with mono-*tert*-butyl succinate by using *1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride* as a coupling agent in the presence of DMAP (*4-dimethylaminopyridine*). SA–Dmv (**3**) was obtained by

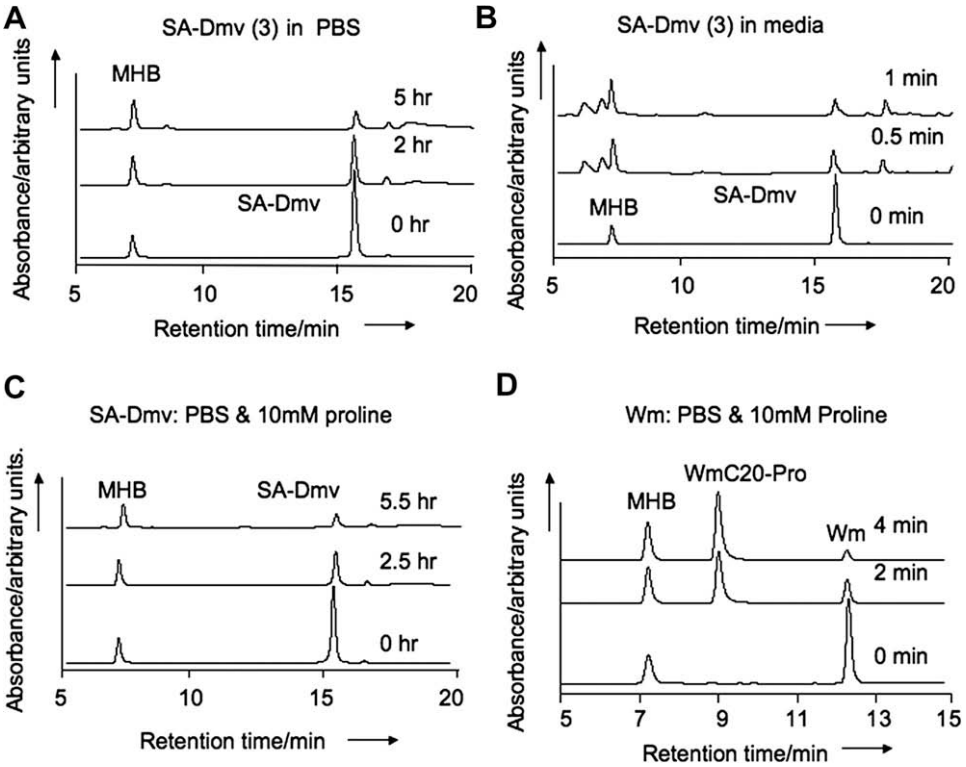


Figure 3. Illustrative HPLC chromatograms showing the fate of SA–Dmv (**3**). SA–Dmv was incubated in PBS (A), media (B) or PBS plus proline (C). Absorbance was monitored at 258 nm. Half-times for the loss in peak area from these and additional time points are given in Table 1. Unlike Wm, the stability of SA–Dmv in PBS was unaffected by the addition of proline, compare (A) and (C). In contrast, Wm reacted with proline to form a single compound, WmC20-Pro.

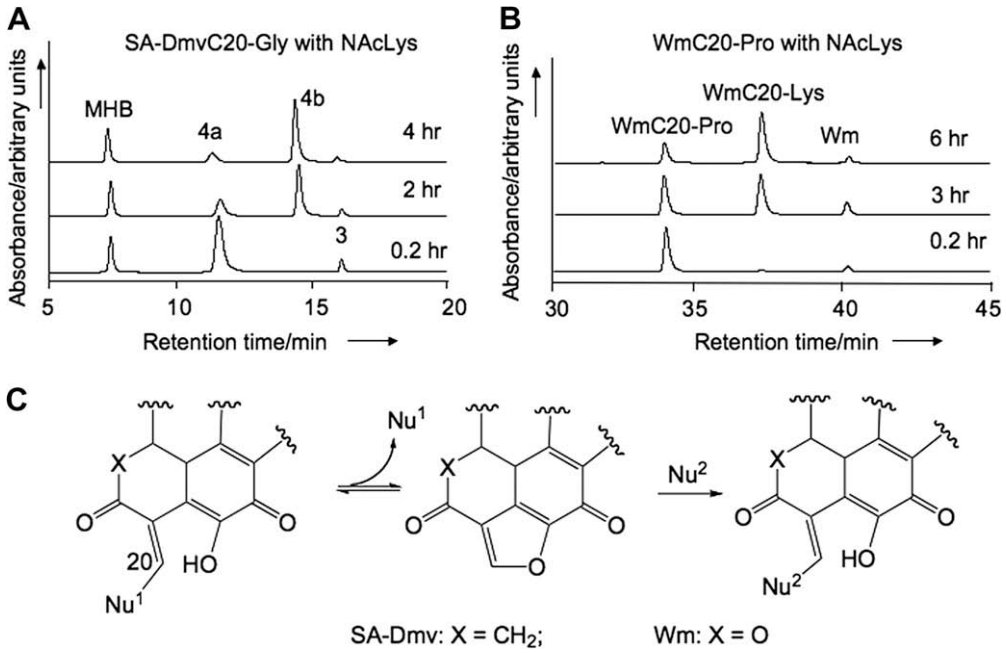


Figure 4. Exchange reactions at the C20 position with SA–DmvC20-Gly or WmC20-pro. (A) Chromatograms obtained when SA–DmvC20-Gly (**4a**) was incubated with 10 mM NAc-Lys. **4a** disappeared and a new peak 14.5 min formed. The 14.5 min peak was identified as **4b**, SA–DmvC20-NAcLys, by retention time and molecular weight by mass spec. (B) Chromatograms obtained when WmC20-Pro was incubated with 10 mM NAc-Lys. WmC20-Pro disappeared with the formation of WmC20-NAcLys which had a retention time of 37 min. (C) Open furan ring derivatives of SA–Dmv or Wm undergo exchange reactions with nucleophiles in the media.

cleaving the *tert*-butyl group with trifluoroacetic acid in dichloromethane. Because of the improved bioactivity of some Wm's modified at C20,[3,10] the C20 of SA–Dmv **(3)** was reacted with glycine (for **4a**) or *N*-acetyl lysine (for **4b**). Below we compare the stability and activity of Dmv, SA–Dmv and SA–DmvC20-Gly, with **4b** serving a reference compound when the exchange reactions of SA–DmvC20-Gly was examined in NAc-Lys, see Figure 4.

Stability and reactivity of Dmv derivatives (*procedures* see Supplementary data)*:* Dmv (**1**), and three Dmv derivatives (**3, 4a, 4b**) were incubated in PBS, PBS plus amino acids or media and the decrease in peak area monitored by HPLC. The decrease in HPLC peak area versus incubation time was then fit to a first order decay model.[9] An excellent fit ($r^2 > 0.9$ in all cases was obtained), permitting the calculation of first order decay constants and half-lives expressed in minutes in Table 1.

Dmv was moderately stable in PBS, exhibiting a half-life of 26 min but had a half-life of less than a minute in culture media. The reaction of the C1 hydroxyl of Dmv, to yield SA–Dmv (**3**), resulted in a modest stabilization with the half-life in PBS increasing from 26 min for Dmv to 120 min for SA–Dmv. Exemplary HPLC chromatograms for the stability of SA–Dmv are shown in Figure 3. SA–Dmv decayed slowly in PBS (Fig. 3A), but rapidly in media (Fig. 3B). As was the case with Dmv, SA–Dmv disappearance was associated with the formation of multiple products. Thus, while addition of a SA to Dmv resulted in an improvement in stability in PBS, both Dmv and SA–Dmv still had half-lives of less than 1 min in media (Table 1).

A feature of Wm stability is the ability of amino acids like proline or NAc-Lys to hasten Wm's disappearance in PBS, due to the formation of WmC20 derivatives.[8] However, proline had little effect on the decay of SA–Dmv (compare Fig. 3A and C), while the addition of proline to Wm decreased its half-life from 3470 to 1.5 min (Table 1), due to the formation of a single peak with a retention time of 8.9 min (Fig. 3D). The peak was identified as a non-furan ring C20 proline derivate of Wm termed WmC20-Pro which has been previously synthesized and characterized.[8]

We hypothesized that the instability of SA–Dmv in culture media (half-life 0.43 min) might be remedied by reaction of its C20 with an amino acid. We therefore reacted the C20 of SA–Dmv with glycine, to obtain SA–DmvC20-Gly (**4a**). As indicated in Table 1, **4a** had a half-life of 64 min in media, indicating that SA–DmvC20-Gly was a Dmv derivative with greatly improved stability compared to the starting point of this study, Dmv, which had a half-life of 0.47 min in media.

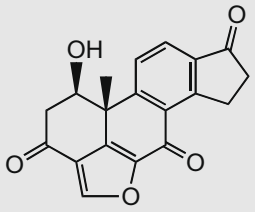
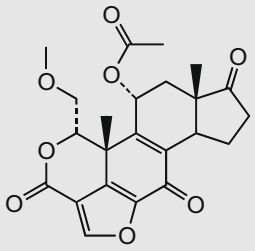
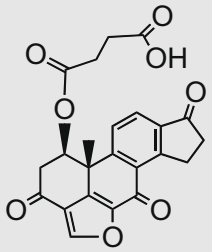
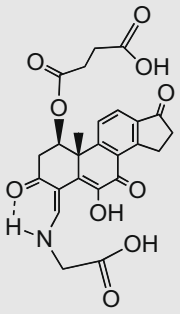
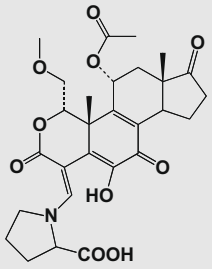
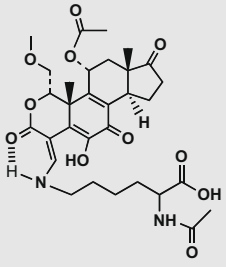
A feature of some open furan ring forms of Wm is their ability to generate Wm that then reacts with other nucleophiles present, exchanging amino acids covalently bound at the C20 position,[3,8] as shown in Figure 4C. To examine whether SA–DmvC20-Gly (**4a**) would undergo such exchange reactions, it was incubated with NAc-Lys and the formation of SA–DmvC20-Lys (**4b**) determined as shown in Figure 4A. A small amount of SA–Dmv (**3**) formed transiently, with the loss of **4a** and production of a single compound with a retention time of 14 min. This compound was identified as **4b** by a common retention time and molecular weight. Figure 4B shows the results of incubating WmC20-Pro with NAc-Lys. Again a small amount of Wm formed briefly, with the loss of WmC20-Pro and accumulation of a single compound that was identified as WmC20-Lys. Thus it appears that certain open ring derivatives of SA–Dmv or Wm can participate in exchange reactions as shown in Figure 4C.

Based on the improved stability of SA–DmvC20-Gly, we next examined its ability to inhibit PI3 kinase, Table 2. Using the IC_{50} of 12 nM for Wm as reference point, Dmv's IC_{50} was 12 times lower, while the IC_{50}'s of SA–Dmv and SA–DmvC20-pro were 1.7 and 3.6 times higher than Wm. Thus SA–DmvC20-Gly was a stabilized Dmv derivative that maintained a substantial ability to inhibit PI3

Table 1
Stability of Dmv and Dmv derivatives first order decay half-lives ($t_{1/2}$) are in minutes

	PBS	PBS + Pro	PBS + NAc-Lys	PBS + Gly	Media
Wm	3470	1.5	18.4	4.1	0.99
Dmv	26	0.59	0.61	0.99	0.47
SA–Dmv (**3**)	120	108	23	13.5	0.43
SA–DmvC20-Gly (**4a**)	218	126	94	—	64

Table 2
Dmv and Wm derivatives as PI3 kinase inhibitors and participants in C20 exchange reactions

Compound		Exchange reaction	IC_{50} (nM)
Dmv		Not relevant	1.0
Wm		Not relevant	12
SA–Dmv (**3**)		Not relevant	20
SA-DmvC20-Gly (**4a**)		Yes	44
WmC20-Pro (Table 2, Ref. [8])		Yes	68
WmC20-Lys (Table 2, Ref. [8])		No	>1000

kinase. Our study of the stability, reactivity and activity Dmv and its derivatives permits comparisons to be made with the better-studied viridin, Wm.

Wm versus Dmv: We previously found that Wm was stable in PBS with a half-life of 57.8 h.[9] Here we report that Dmv was considerably less stable, with a half-life of 26 min. Second, both Dmv and Wm were highly unstable in culture media, with half-lives of less than 1 min. However, when Dmv was reacted with NAc-Lys, Pro or Gly in PBS, a complex mixture was obtained, while Wm produced single compounds due to reaction of its C20 position. Since Dmv lacked the lactone ring of Wm, and lactone ring opening is a potential mechanism of Wm instability,[1,7] the chemical basis of its instability cannot lie in an opening of the lactone ring. Instead the more extensive conjugated ring structure of Dmv may result in more numerous chemically reactive, electrophilic centers, an interpretation favored by the high multiplicity of products obtained when Dmv was incubated in PBS with a variety of amino acids (Yuan and Josephson, unpublished observations).

SA–Dmv versus Dmv: The reaction of the C1 OH of Dmv to form SA–Dmv produced a significant improvement in stability in PBS and in PBS with amino acids added (Table 1). Since the C1 of Dmv does not appear to participate in resonance stabilization as the compounds are conventionally drawn (Fig. 1), a more complete analysis of the resonance structure and potential electrophilic carbons of Dmv seems needed. The ability to modify Dmv at C1 with retention of PI3 kinase inhibitory activity has been noted.[11]

SA–DmvC20-Gly versus Dmv: SA–DmvC20-Gly was significantly more stable than Dmv achieving a modest half-life of 64 min in media compared to a half-life of less than 0.47 min for Dmv. Although modification at C1 and C20 increased its IC_{50} for PI3 kinase from 1.0 nM (Dmv) to 44 nM (SA–DmvC20-gly), SA-DmvC20-gly was only 3.6 times less potent than Wm (IC_{50} = 12 nM). Thus SA–DmvC20-Gly was a stabilized Dmv derivative that maintained a substantial ability to inhibit PI3 kinase.

SA–DmvC20-Gly versus WmC20-Pro: Both C20 modified viridins underwent exchange reactions under physiological conditions when incubated with NAc-Lys (Fig. 4). The ability to undergo this exchange reaction generates SA–Dmv, which features an intact furan ring and an IC_{50} of 20 nM for PI3 kinase (Table 2).

Although there have been many efforts to use Wm as a scaffold for drug development,[10,12,13] we know of no efforts to employ Dmv, perhaps because of its instability. Our results demonstrate for the first time that the stability of Dmv can be manipulated while substantially maintaining its ability to inhibit PI3 kinase. In addition, some Dmv derivatives, like some Wm derivatives,[3,8,9] can undergo exchange reactions at the C20 position. Our results indicate it may be possible to obtain stabilized Dmv derivatives for use as PI3 kinase inhibitors in biological systems.

Acknowledgment

This work was supported in part by P50-CA86355 (R.W.) and, CA24487 (J.C.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.105.

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