Published in final edited form as:

Bioorg Med Chem Lett. 2007 June 15; 17(12): 3281–3286. doi:10.1016/j.bmcl.2007.04.018.

Rational Design of Inhibitors of VirA-VirG Two-Component Signal Transduction

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Abstract

VirA-VirG two-component system regulates the *vir* (virulence) operon in response specific host factors (xenonogens) in the plant pathogen *A. tumefaciens*. Using whole cell assays, stable inhibitors inspired by the labile natural benzoxazinone inhibitor HDMBOA are developed. It is found that aromatic aldehydes represent a minimal structural unit for activity. In particular, 3-hydroxy-4,6-dimethoxy-3H-isobenzofuran-1-one (HDI) was found to have the highest activity, making it the most potent developed inhibitor of virulence gene expression in *Agrobacterium*.

Agrobacterium tumefaciens mediates the lateral transfer of genes to eukaryotic genomes *en route* to engineering the tumorous growths of crown gall disease. Switching wild-type transferred genes with other elements such as antibiotic resistance genes, biosynthetic pathway enzymes, and proteinaceous insecticides allows the bacterium to be used as a vector for plant genetic engineering.¹ Most cereal crops, however, are resistant to transformation by *A. tumefaciens*, limiting its biotechnological utility.¹⁻⁴ Efforts to better understand this limitation identified HDMBOA (**2**) exuded from maize roots as a potent inhibitor of host recognition (xenogenesis) mediated by the VirA-VirG two-component system (TCS).⁵ TCS environmental perception elements are widely used by both gram-negative and gram-positive pathogenic bacteria for xenogenesis.⁶ This natural strategy of blocking host perception suggests that similar prophylaxis anti-virulence agents could be found that avoid the selection pressure inherent to bactericidal agents, and/or in combination with antibiotics, extend therapeutic effectiveness.

Development of such novel agents from the natural product HDMBOA is complicated by the compounds unique reactivity. Recent mechanistic analyses have suggested that HDMBOA itself is not the active inhibitor, but instead functions as a pro-drug to deliver the active iminoquinone decomposition intermediate **3** intracellularly (Figure 1A). Intermediate **3** is also unstable and further decomposes to the inactive product MBOA (**4**).^{7, 8} This lability may be critical within the biological matrix, but we sought to capture structural features of **3** necessary for inhibition while retarding hydrolytic breakdown. Here we describe a successful approach that accomplishes both goals.

HDMBOA (**2**) and the related *vir* inhibitor DIMBOA (**5**) are members of the benzoxazinone family of secondary metabolites.^{9, 10} Their decomposition is dependent on both the redox activity of the hydroquinone-like ring system and the reactivity of the aldehyde (red and blue respectively in Figure 1).⁷ Likewise, the biological activity of **2**, **3**, and **5** may also depend on

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both of these elements. Analogs **6** – **11** (Table 1A) were prepared to evaluate this requirement by eliminating redox activity (and formation of species like **3**). To assay *vir* inhibition, whole cell assays were performed in the presence of 100 μ M acetosyringone (a potent *vir* inducer perceived by the VirA/VirG TCS) as described in Supplementary Information. Analog **6** retained the hydroxamic acid/aldehyde groups found in DIMBOA and showed comparable inhibitory activity to that of that natural product. Unfortunately, poor aqueous solubility and elevated toxicity prevented accurate IC₅₀ determination. Analogs **8** – **11** lacked the hydroxamic acid and all were acceptably soluble, less toxic, and more stable under aqueous conditions.⁷ The similar biological activities of **8** – **11** suggested that stable aldehyde inhibitors could be designed and neither the hydroxamate nor the quinone functional groups were essential.

The complex instability of the benzoxazinone nucleus can be attributed largely to the *ortho* heteroatoms on the aryl ring.⁷ As a template for stable inhibitors, these heteroatoms were removed and the electrophilic carbonyls (blue in Figure 1) retained, leading to the simple 3*H*-isobenzofuran-1-one skeleton of **12**. A series of compounds based on this skeleton (**12** – **25**) were readily prepared through two separate approaches. Compounds containing an acid were prepared by electrophilic aromatic substitution of an acyl chloride or formyl equivalent on the corresponding ester. Since this approach was incompatible with the synthesis of many of the keto/aldehyde and keto/keto structures, a unique rearrangement of hydrazone phenols by [(diacetoxy)iodo]benzene was employed.¹¹ Although yields by this latter method were poor, the generality of this procedure permits facile access to a potentially diverse library of *o*-carbonyl compounds. Attempts to synthesize aldehyde/aldehyde analogs were unsuccessful.

The keto/aldehyde structures **13** – **16** were active inhibitors, independent of the aldehyde position on the skeleton (Table 1B). While the aldehyde was necessary (**17** – **19**), it was not sufficient (**20** – **21**) for activity. The reason for the loss of inhibitory activity resulting from moving the carbonyl center one atom from the aromatic ring in **22** and **7** remains unclear. Nonetheless, the acid/aldehyde **12** seemed the best candidate with an IC₅₀ of 8 μ M.

To evaluate the importance of the aryl methoxy substituents in **12**, an additional series of 3*H*-isobenzofuran-1-one analogs was prepared. The diethoxy analog **26** was easily prepared by electrophilic aromatic formylation of the benzoic acid methyl ester, but mono-methoxy benzoic acid methyl esters were inactive to formylation. Analogs **27** – **30** were therefore prepared by activation of the aromatic nucleus via *ortho*-lithiation of the corresponding methoxy benzamide followed by addition of DMF as the electrophile.¹²⁻¹⁴ Hydrolysis of the amide afforded the 2-formyl monomethoxy benzoic acids in good yield. ¹H-NMR indicated that the 3*H*-isobenzofuran-1-one tautomer was dominant for **26**, **27**, **28** and **30** in D₂O and DMSO-*d*₆, however **29** existed equally in both ring and chain tautomers in DMSO-*d*₆ (see Supporting Information).¹⁵

The simplest acid/aldehyde 3*H*-isobenzofuran-1-one skeleton represented by **23** was completely inactive, as was the diethoxy analog **26**, suggesting some structural role for the methoxy groups of **12**. The 6-methoxy analog **29**, structurally most analogous to **3**, also showed no *vir* inhibitory activity. Only **27** with its methoxy substituent *ortho* to the aldehyde had significant inhibitory activity. The 4,6-dimethoxy compound, HDI (**12**) remained the most potent stable *vir* induction inhibitor yet discovered.

To evaluate the biological specificity of the natural products HDMBOA (**2**), DIMBOA (**5**), and their synthetic analog HDI (**12**), two protocols were employed. Both protocols were developed and used to establish that α -bromo-acetosyringone (ASBr), an analog of the inducer acetosyringone (AS), is an irreversible inhibitor of VirA/VirG mediated induction.¹⁹ First, the octopine-inducible *occ* regulon with the fusion reporter construct *occ::lacZ* was developed as a control.²⁰ Neither of the benzoxazinones **2** and **5**, nor the synthetic analog **12**, significantly

induced or inhibited *occ* gene expression (Figure 2). The simple vinylogous acid/aldehyde **35** was non-specific by this assay and was included as a positive control. These results establish that the compounds are not affecting reporter lacZ expression through the general transcription and translation machinery, nor are they compromising cell viability.

Second, a simple washing assay was employed to evaluate reversibility. After 2 hours of co-incubation with inducing AS, **2**, **5**, and **12** inhibited *vir* expression equivalently. Significant recovery of gene expression was observed following removal of these from the media (**35** and ASBr were included as positive and negative controls for reversibility respectively, Figure 3), and no significant effects on cell growth rates were observed, even for HDMBOA concentrations 100-fold higher than the IC₅₀ for *vir* gene inhibition. Thus, all three compounds appear to specifically inhibit the *vir* gene signal transduction pathway and to inhibit AS induction through reversible interaction with signal perception elements rather than through irreversible inactivation. By analogy to other benzoxazinones,²¹ the open-chain form of **2** may reversibly react with protein nucleophiles; forming adducts with reactive amines,²² cysteines,²³ or serine residues.²⁴

HDMBOA therefore appears to protect maize from *A. tumefaciens* infection by inhibiting virulence activation mediated by the VirA-VirG TCS. TCSs play a central role in the response of prokaryotes, and some eukaryotes, to environmental changes and biotic stimuli including xenognosis (host recognition).⁶ The defining proteins of two-component systems – the histidine kinase sensor and response regulator – are potential targets for antibiotic development since they are specific and often unique to a particular pathogen.⁶ Although it would be tempting to speculate that the pressure to develop pathogen-resistance has generally driven the evolution of antagonists to two-component virulence proteins, HDMBOA is currently the only known example of a host-derived inhibitor that targets two-component signal perception. The unique and varied reactivity of this successful natural inhibitor may underlie the apparent rarity of this strategy. Benzoxazinones are typically protected from hydrolysis by storage as stable D-glucosides that are released by endogenous plant glucosidases when needed (**1** in Figure 1). The instability of exuded active species **2** creates a steady-state concentration around the root that is below the level of cyto-toxicity but sufficient for inhibition of xenognosis in *A. tumefaciens*.⁵ Therefore, HDMBOA does not accumulate to the point where selection pressure could create resistance; but merely disables the sensory system of those pathogens very close to the host. As researchers overcome the difficulties inherent in studying unstable natural products, the generality of this apparent resistance strategy may be revealed.

The emergence of antibiotic resistance in pathogenic bacteria has inspired research and development of two-component system inhibitors. The results of these efforts have been reviewed,^{6, 25} and unfortunately all compounds identified by several different approaches were either too hydrophobic or detergent-like for further development as therapeutic agents. As a natural two-component system inhibitor, HDMBOA may hold the key to novel strategies for development of anti-virulence two-component system inhibitors with improved pharmacokinetics. Accordingly, this study examined the specific features of HDMBOA required for inhibition of the host recognition elements VirA-VirG.

Simple *in vivo* assays suggested that the inhibitory activity of **2** and the weaker inhibitor **5** can be removed by media exchange, implying a reversible inhibition mechanism (Figure 3). Yet most of the possible chemical reactivities ascribed to benzoxazinones are irreversible in nature and associated with the unstable iminoquinone nucleus.²⁶⁻²⁸ We therefore focused inhibitor design on the reversible aldehyde electrophile. Indeed, the aldehyde was found to be essential to the activity of analogs **12** – **16** in Table 1B, with HDI (**12**) being the most potent of these analogs. Replacement of the aldehyde with a ketone abolished activity (compounds **17** – **19**). Synthesis of additional HDI analogs revealed that structural effects of the methoxy substituents,

possibly for active-site recognition, are apparently more critical than their electronic effects as the 4,6-diethoxy analog **26** was completely inactive. The 4-methoxy group is required for activity (Table 1C). Moreover, many ketone substitutions at R₁ were tolerated. Therefore, derivatization of this position will permit convenient access to labeled compounds and screening for more potent analogs.

Despite the simplicity of the assumptions in its design, HDI is in fact a reversible inhibitor with higher potency for *vir* inhibition than DIMBOA and all other synthetic *vir* inhibitors. The design of HDI assumed that HDMBOA was competitive with phenol induction through reversible covalent binding to VirA via an electrophilic aldehyde. Although a site of phenol binding on VirA has not been precisely identified, the structural requirements and limits have been identified. Methoxy groups proximal to the phenol hydroxy contribute positively to phenol sensitivity. This dependence is observed in the trend of increasing inducer potency for 4-hydroxy-acetophenone $\ll$ 4-hydroxy-3-methoxy-acetophenone < 4-hydroxy-3,5-dimethoxy-acetophenone (AS).²⁹ By simple analogy, the 3*H*-isobenzofuran-1-one derivative HDI (**12**), may be described as a stable structure combining the critical features of HDMBOA with known structural features of phenol inducers.

Given the instability of HDMBOA and the generality of these assumptions, HDI may in fact function as an analog of decomposition intermediate **3**, which has been shown to possess *in vivo* inhibitory activity. In a previous report, an analog of imidoquinone **3** which lacked the aldehyde functional group was an active *vir* inhibitor.⁷ In these experiments, we have now uncoupled the aldehyde from the imidoquinone and demonstrated that stable aromatic aldehydes are also *vir* inhibitors.

The high potency of HDMBOA is likely to depend on both functional elements, though they may act on different steps of signal transduction. *In vivo* and *in vitro* assays are being developed to assess the specificity of HDI, HDMBOA, and DIMBOA for a particular step in two-component system signal perception and transmission, and for specificity to other two-component systems. While HDI is an apparently specific inhibitor of VirA/VirG signal recognition, these results raise the possibility that the unique reversible electrophilic reactivity of 3*H*-isobenzofuran-1-ones may find wider application as an initial scaffold for library construction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Andrew G Palmer for his assistance with this manuscript.

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Figure 1.

The critical elements of HDMBOA *vir*-inhibition activity. Endogenous plant glucosidases activate the activity of **1** to generate a multi-centered electrophile **2**. In water, **2** decomposes to an *ortho*-imidoquinone (**3**) *en route* to the inactive product MBOA (**4**). The *ortho*-(hydro)quinone nucleus (red) and electrophilic carbonyls (blue) of **2** (and **5**) are potentially required for activity. Structure **12** retains the essential structural features of the active natural products (shown in box) and aldehyde reactivity.

Figure 2.

Specificity of HDMBOA (2), DIMBOA (5), HDI (12), and HF (35). Inhibition of *occ::lacZ* activity was evaluated in A348 pSM102 induced by 100 μ M octopine. For HDMBOA and DIMBOA, inhibition of *vir* activity was monitored in A358mx (*virE::lacZ*).²⁰ For HDI and HF, inhibition of *vir* activity was monitored in A348/pSW209 (*virB::lacZ*). All *vir* assays were induced with 100 μ M AS in 1% sucrose induction media.

Figure 3.

Reversibility of inhibition. *A. tumefaciens* strain A348/pSW209 was incubated with 100 μ M of *vir* inducer AS and inhibitors at the indicated concentration in parentheses for two hours at which time samples were washed, split, and incubated for an additional six hours with (white bars) or without (gray bars) inhibitor. The final *virB::lacZ* activity is presented as percent of AS-only ("Control" gray bar). The "Control" white bar was incubated without AS for the final six hours to represent a minimum activity.

Table 1

Structures and *vir* inhibition data of (A) aldehyde-based benzoxazinone analogs, (B) various 1,2-diacyl-3,5-dimethoxy-benzene derivatives, and (C) HDI analogs

A	Compound	IC ₅₀ (μM)
2 R=CH ₃		<1
5 R=H		30 ± 4
6		25 ± 2
7 ^a		>400
8 ^b		25 ± 5

A	Compound	IC ₅₀ (
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9

40 ± 6

A	Compound	IC ₅₀ (
10		34 ± 6
11		35 ± 3

B

	R ¹	R ²	R ³	IC ₅₀ (μM)
HDI 12	COOH	CHO	OMe	8 ± 2
13	CHO	COMe	OMe	25 ± 2
14	COMe	CHO	OMe	25 ± 2
15	CHO	CO- <i>n</i> -Bu	OMe	10 ± 2
16	CO- <i>n</i> -Bu	CHO	OMe	10 ± 2
17	COOH	COMe	OMe	>200
18	COOH	CO- <i>n</i> -Bu	OMe	>200
19	COMe	COMe	OMe	>200
20	H	CHO	OMe	>200 ^c
21	CHO	H	OMe	>200 ^c
22	CH ₂ COOH	CHO	OMe	>200
23	COOH	CHO	H	>200
24	COMe	CHO	H	25 ± 2
25	CO- <i>n</i> -Bu	CHO	H	20 ± 2

	X	IC ₅₀ (μM)
HDI 12	4,6-OMe	8 ± 2
26	4,6-OEt	>200
27	4-OMe	41 ± 8
28	5-OMe	>200
29	6-OMe	>200
30	7-OMe	>200
23	H	>200
31	4,6-OMe,7-NO ₂	>200
32	4,6-OMe,7-NH ₂	>200
33	4,6-OMe,7-NHAc	>200
34	4,6-OMe,7-OH	>100 ^d
35		25 ± 4

Activity was monitored in *A. tumefaciens* strain A348/pSW209 by *virB::lacZ* expression in the presence of 100 μM AS inducer at 28 °C. An IC₅₀ assignment of '>200' indicates that no inhibitory activity was observed up to 200 μM.

^aNMR assignment in CHCl₃ was most consistent with a cyclic hemiacetal which may interfere with the activity of the aldehyde.

^bSynthesized as the methyl hemiacetal which was rapidly labile in aqueous solution.

^cInhibition <50% was observed near 200 μM, but toxicity at and above 200 μM prevented meaningful evaluation of IC₅₀.

^dCompound was insoluble above 100 μM in induction media supplemented with 2% DMSO.