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Hinnuliquinone, a C_2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease

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Abstract

HIV-1 protease is one of several key enzymes required for the replication and maturation of HIV-1 virus. An almost two-decade research effort by academic and pharmaceutical institutions resulted in the successful commercialization of seven drugs that are potent inhibitors of HIV-1 protease activity and which, if used correctly, are highly effective in managing viral load. However, identification of clinical viral isolates that are resistant to these drugs indicates that this is a significant problem and that new classes of inhibitors are continually needed. Screening of microbial extracts followed by bioassay-guided isolation led to the discovery of a natural hinnuliquinone, a C_2-symmetric bis-indolyl quinone natural product that inhibited the wild-type and a clinically resistant (A44) strain of HIV-1 protease with K_i values of 0.97 and 1.25 μM, respectively. Crystallographic analysis of the inhibitor-bound HIV-1 protease helped explain the importance of the C_2-symmetry of hinnuliquinone for activity. Details of the isolation, biological activity, and crystallographic analysis of the inhibitor-bound protease are herein described.

Keywords: Hinnuliquinone; Fungal metabolite; HIV-1 protease inhibitor; Inhibitor-bound X-ray structure of protease

HIV-1 encodes for an aspartic protease that is essential for maturation of infectious virus. This is one of two key biological targets (HIV-1 reverse transcriptase is the other) to which therapeutic drugs have been developed and approved by the FDA. Currently, at least seven HIV-1 protease inhibitors are commercially available and include saquinavir, nelfinavir, ritonavir, indinavir, amprenavir, lopinavir, and atazanavir [1]. The clinical use of HIV-1 protease inhibitors as monotherapy, in the absence of other anti-viral agents, is contraindicated due to the rapid emergence of drug-resistant strains of HIV. The current drug treatment for HIV-1 usually consists of a cocktail of anti-viral and other drugs including anti-fungal and/or anti-bacterial drugs. The identification and approval of novel HIV-1 protease inhibitors would add significantly to the management of the disease in this and other countries.

Currently approved protease inhibitors are peptidomimetics and are one of the first examples of structure-based drug design that utilized structural information of inhibitor binding to the active site of HIV-1 protease as well as inhibitors of other aspartic proteases (e.g., renin) [1]. This approach successfully identified chemical leads for this target and accelerated the clinical availability of therapeutically useful drugs. The unresolved issue of viral resistance to current therapy is a daunting challenge and continues to hamper efforts to halt the progression and

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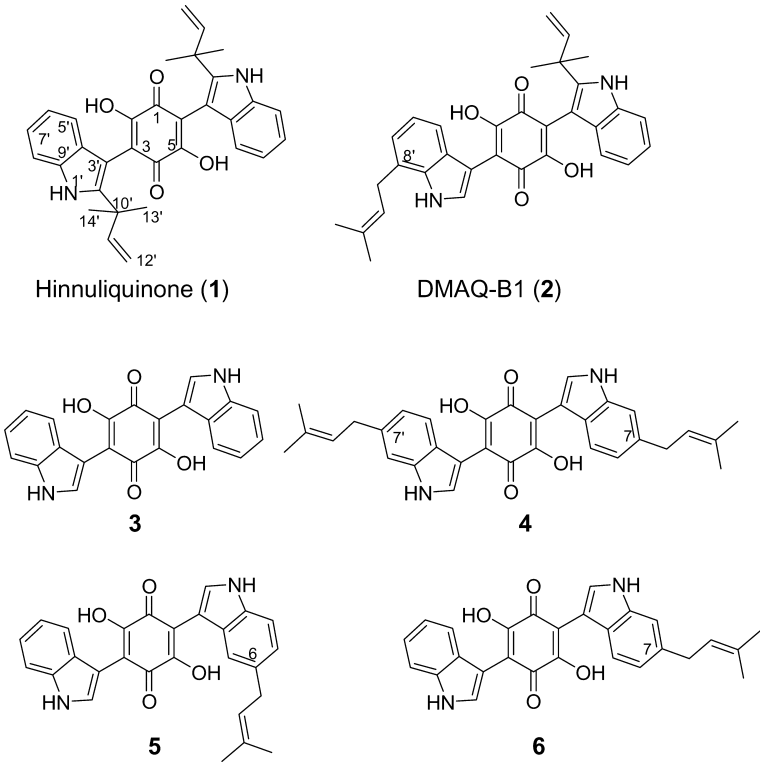

Scheme 1. Chemical structures of hinnuliquinone (**1**), DMAQ-B1 (**2**), and compounds **3**–**6**.

spread of the disease. The identification of structurally novel classes of inhibitors that are less susceptible to the development of resistance could potentially alleviate the continued spread of the disease and decrease the mortality rate that usually accompanies infection.

Natural products have a rich and diverse history of providing useful and novel structural leads for various biological targets and have been particularly productive with respect to anti-infective targets. Previous efforts from our group resulted in the identification and isolation of a series of novel cytochalasins [2,3]. Continued screening led to the isolation of hinnuliquinone (**1**), a C_2-symmetric dimeric isoprenyl-indolyl-dihydroxyquinone (Scheme 1) from a fungal species, which inhibited wild-type HIV-1 protease activity with an IC_{50} of 2.5 μM. When tested against a highly resistant mutant enzyme A44 [4] **1** exhibited an IC_{50} value of 1.8 μM, suggesting the possibility that this compound may be useful against drug-resistant strains of HIV-1. The details of the fermentation, isolation, HIV-1 protease inhibitory activity of hinnuliquinone, and the co-crystallization of hinnuliquinone with HIV-1 protease and other related natural products are described in this report.

Materials and methods

General procedure and reagents. All reagents were purchased from Fisher Scientific or Sigma–Aldrich. Demethylasterriquinone-B1 (DMAQ-B1) was provided by Dr. Gino Salituro of Merck [5].

Source and fermentation of the producing organism. The producing fungus was isolated from leaves of *Quercus coccifera* collected in Ontígola, Aranjuez (Madrid, Spain), using a surface sterilization technique [6]. Lack of sporulation of the culture prevented full characterization of the sterile mycelium.

For the production of the compound, seed flasks were prepared from potato dextrose agar (PDA, Difco) using fresh slants of the isolate as described by Peláez et al. [7]. Two-milliliter portions of the resulting cultures were used to inoculate 250 mL unbaffled Erlenmeyer flasks containing 50 mL MV8 medium [8], which were incubated at 25 °C in a rotatory shaker at 220 rpm for 21 days. The MV8 medium contained (in g/L): maltose (75), V8 juice (200 mL), soy flour (1), L-proline (3), and Mes (16.2, pH 6.5). The compound was also produced by growing the fungus in a rice-based BRFT solid medium [8] in 250 mL Erlenmeyer flasks inoculated with 2-mL portions of the seed culture. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for 28 days.

Isolation of hinnuliquinone. A 6 L fermentation broth was grown for 28 days and was extracted with 7.2 L of methyl ethyl ketone (MEK) by shaking for 2 h and subsequently filtered through celite. The MEK layer was separated and concentrated on a rotovapor under reduced pressure to a volume of 500 mL and extracted twice with methylene chloride (250 mL each). The combined methylene chloride extract was concentrated to dryness under reduced pressure, dissolved in methanol (550 mL), and washed twice with 550 and 300 mL hexane each. The methanol layer was concentrated to dryness under reduced pressure to give an oily residue which was washed again with 100 mL hexane leading to yield 7.73 g of residue as a black powder. A 160 mg aliquot of the residue was dissolved in 0.5 mL methanol and chromatographed on a Waters symmetry reversed phase C-18 column (19 × 300 mm) eluting with a 30 min 60–90% gradient of aqueous methanol at a flow rate of 8 mL/min. The fractions eluting from 24 to 25 min were concentrated under reduced pressure and lyophilized to give hinnuliquinone (40 mg, 305 mg/L) as a black powder. RP-HPLC retention time was 9.48 min (Waters symmetry C-18 4.6 × 250 mm, 50% aqueous

acetonitrile +0.1% trifluoroacetic acid, 1 mL/min). HREIMS (*m/z*) found a mass 506.2193 (calculated for $C_{32}H_{30}N_2O_4$, 506.2206).

Separation of atropisomers of hinnuliquinone. Hinnuliquinone (200 μg in 20 μL) was injected onto a chiral Phenomenex Chirex NGLY&DND HPLC column (4.6 × 250 mm) and eluted with 4:1 hexane: isopropanol +0.2% trifluoroacetic acid at a flow rate of 1 mL/min. Isomers 1 and 2 eluted at 14 and 19 min, respectively. The process was repeated 15 times and fractions were concentrated under reduced pressure to give 1 mg each of the two isomers. Isomer **1**: ^{1}H NMR (CD_2Cl_2) at 25 °C: 8.33 (2H, s, NH), 7.21 (2H, brd, $J = 7.6$ Hz, H-5′), 7.09 (2H, dt, $J = 1.2$, 7.6 Hz, H-6′), 7.20 (2H, dt, $J = 1.2$, 7.6 Hz, H-7′), 7.40 (2H, dd, $J = 8.0$, 0.8 Hz, H-8′), 6.16 (2H, dd, $J = 17.2$, 10.4 Hz, H-10′), 5.23 (2H, dd, $J = 17.2$, 0.8 Hz, H-11′), 5.20 (2H, dd, $J = 10.4$, 0.8 Hz, H-12′), 1.53 (12H, s, H_3-13′ and H_3-14′); ^{13}C NMR (CD_2Cl_2) at −55 °C: 182.5 (C-1), 154.3 (C-2), 111.2 (C-3), 142.4 (C-2′), 98.6 (C-3′), 127.6 (C-4′), 117.8 (C-5′), 119.7 (C-6′), 121.8 (C-7′), 110.7 (C-8′), 134.0 (C-9′), 38.9 (C-10′), 144.8 (C-11′), 112.0 (C-12′), 25.9 (C-13′), 26.7 (C-14′). Isomer **2**: ^{1}H NMR (CD_2Cl_2) at −50 °C: 8.31 (2H, s, NH), 7.28 (2H, brd, $J = 7.6$ Hz, H-5′), 7.10 (2H, ddd, $J = 1.2$, 7.2, 8.0 Hz, H-6′), 7.21 (2H, ddd, $J = 1.2$, 7.2, 7.6 Hz, H-7′), 7.39 (2H, dd, $J = 7.6$, 0.8 Hz, H-8′), 6.12 (2H, dd, $J = 17.6$, 10.8 Hz, H-10′), 5.20 (2H, dd, $J = 17.6$, 1.2 Hz, H-11′), 5.11 (2H, dd, $J = 10.8$, 0.8 Hz, H-12′), 1.51 (12H, s, H_3-13′ and H_3-14′); ^{13}C NMR (CD_2Cl_2) at −55 °C: 182.5 (C-1), 154.3 (C-2), 111.2 (C-3), 142.5 (C-2′), 98.6 (C-3′), 127.6 (C-4′), 118.1 (C-5′), 119.6 (C-6′), 121.8 (C-7′), 110.6 (C-8′), 134.0 (C-9′), 38.9 (C-10′), 144.8 (C-11′), 112.0 (C-12′), 25.9 (C-13′), 26.7 (C-14′).

HIV-1 protease natural product screening assay. Fermentation extracts were screened using a HIV-1 protease assay as described earlier [3].

HIV-1 protease peptide cleavage assay. For a detailed description of the assay see Olsen et al. [4].

Hinnuliquinone bound HIV-1 protease X-ray crystallography. Wild-type HIV-1 protease was purified as described previously [9]. Co-crystals of HIV-1 protease and hinnuliquinone complex were obtained by mixing the protease and hinnuliquinone in 1:3 molar ratio [9,10]. Diffraction data were recorded at ambient temperature on an R-AXIS II imaging-plate system using Cu-Kα radiation from a rotating anode X-ray source. Data were reduced and scaled using the HKL suite [11]. The structure of the complex was determined by direct refinement, using coordinates of the HIV-1 protease structure, previously determined in the same space group [10].

Results and discussion

Isolation and structure of hinnuliquinone

Hinnuliquinone was originally isolated from *Nodulisporium hinnuleum* [12,13]. The purification of hinnuliquinone reported here is based on bioassay guided separation using HIV-1 protease assay and is different from that of the previously reported one and the specifics of the purification have been provided above and are summarized here. Briefly, the broth was extracted with methyl ethyl ketone, partitioned into methylene chloride followed by trituration with hexane to provide semi-purified product which was subsequently chromatographed on reversed phase HPLC yielding 305 mg/L. Purified hinnuliquinone presents as a black power with a molecular weight of 506 and a molecular formula of $C_{32}H_{30}N_2O_4$.

Searching the Chapman and Hall database provided hits for bis-indolyl-quinone, hinnuliquinone or DMAQ-B1, as potential structural possibilities. While these compounds have the same molecular formula and are generally dimeric in nature only hinnuliquinone is a C_2-symmetric dimer and should show only half of the signals in the NMR spectra. The ^{13}C NMR spectrum in *deuterio*-methylene chloride at ambient temperature displayed only 14 of 32 possible signals, indicating that it could be a symmetrical dimer. Additionally, these signals were generally broad. The symmetrical dimeric nature of the compound was also suggestive from the analysis of the ^{1}H NMR spectrum. These data together with the comparison of the UV spectrum indicated that the isolated compound was hinnuliquinone [12,13]. However, the NMR spectral analysis indicated that hinnuliquinone displayed atropisomerism due to the restricted rotation around C3–C3′. The two atropisomers were purified by normal phase HPLC chromatography using a chiral column. The ^{1}H NMR spectrum of the two purified atropisomers exhibited distinct spectra for each of the isomers. The ^{13}C NMR spectrum at 25 °C still displayed only 14 carbons and lacked the signals for C-1 and C-2. These carbons were easily observed when the spectra were recorded at −55 °C similar to what was reported for semicochliodinols [14]. These data represent the first description of the complete ^{1}H and ^{13}C NMR spectral assignment of both atropisomers of hinnuliquinone and are presented in detail in the Materials and methods section. The atropisomers equilibrated slowly at room temperature and within a week were fully equilibrated to the starting ratio of ∼1:1. The rate of equilibration could be decreased at lower temperatures and frozen at −80 °C.

HIV-1 protease activity

Hinnuliquinone (**1**) when first evaluated in the HIV-1 protease screening assay displayed an IC_{50} value of 2 μM. Testing of this compound against protease isolated from both wild-type virus and a mutant HIV-virus (A-44) that is known to be resistant to commercially available HIV-1 protease inhibitors [4] led to the data presented in Table 1.

Hinnuliquinone inhibited wild-type and mutant (A44) protease with IC_{50} values of 2.5 and 1.8 μM, respectively, and was competitive with respect to substrate exhibiting K_i values of 0.97 and 1.25 μM for

Table 1
Activity of hinnuliquinone against HIV-1 protease isolated from wild-type and mutant virus

HIV-1 protease forms	IC_{50} (μM)	K_i (μM)[a]
Wild-type	2.5	0.97
A-44 variant (V82T)	1.8	1.25

[a] K_i calculated from the relationship between IC_{50} and K_i for competitive inhibition [4].

wild-type and mutant protease, respectively. The almost equal potency of hinnuliquinone against wild-type and mutant HIV-1 protease is encouraging in that it suggests that chemical templates are available that, with a concerted medicinal chemistry effort, could ultimately generate potent chemical entities that preferentially target forms of HIV-1 protease that are resistant to current anti-viral therapy.

To determine whether the inhibition was due to the reactive nature of the quinone moiety, DMAQ-B1 (**2**), an isomeric quinone, was evaluated against wild-type HIV-1 protease and found to be essentially inactive ($IC_{50} = \sim 57\ \mu M$). This indicated that the inhibition is not simply due to the reactive quinone structure since other simple benzo-quinones also evaluated were found to be inactive (data not shown). This suggests that the inhibitory potential of hinnuliquinone is due to other structural features of the molecule other than the reactive quinone.

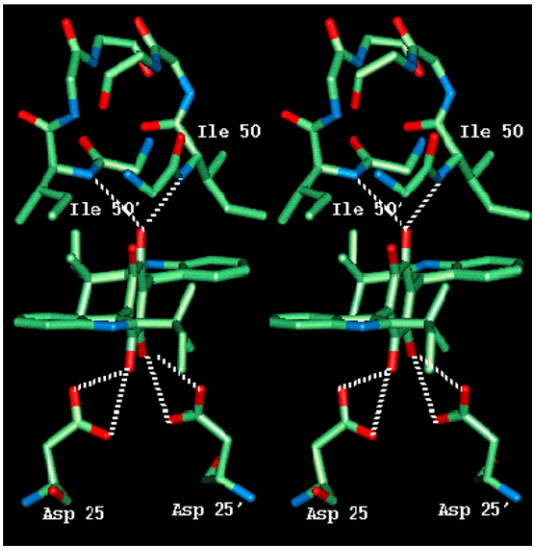

Fig. 1. Stereo view of hinnuliquinone interacting with the catalytic aspartate residues of HIV protease.

Hinnuliquinone is a C_2-symmetric compound whereas DMAQ-B1 (**2**) is not and the major structural difference between the two compounds is in the substitution pattern of one of the isoprene units in the indole ring. HIV-1 protease is a C_2-symmetrical dimeric enzyme and peptidomimetic derived C_2-symmetric dimeric structures have been shown to be potent inhibitors (e.g., cyclic sulfamides) [15]. Therefore, it is likely that the C_2-symmetry of hinnuliquinone could be responsible for binding. This is supported by the data obtained by co-crystallization of HIV-1 protease and hinnuliquinone (see below).

Crystallographic analysis reveals that hinnuliquinone bound to the HIV-1 protease in a symmetrical fashion. Although, the inhibitor was symmetrical the conformation of the protein dimer interacting with the inhibitor was asymmetric. The two catalytic aspartate residues (Asp-25 and Asp-25′) interact almost symmetrically with the adjacent keto and hydroxy groups of the dihydroxy-quinone group (C-1 and C-2 or C-4 and C-5). The other half of the quinone ring faces the flap with the hydroxy (or keto) group hydrogen bonding with the backbone amide nitrogen of the Ile-50 and Ile-50′ (Fig. 1). The indole ring interacts with the residues Val-32, Asp-29, and Asp-30. The isoprene group is in contact with residues Leu-23, Pro-80, and Ile-84. Based on the interactions of the hinnuliquinone (**1**), it is tempting to suggest that in DMAQ-B1 (**2**), the isoprene moiety at the position C-8′ will result in short contacts with the side chains of residues Asp-29 and Asp-30 (Fig. 2). This could also break the two salt bridges Asp-29 makes with Arg-87 and Arg-8, thus disrupting the binding pocket. However, any substitution at positions C-6 and/or C-7 of the indole ring can also lead to undesirable short contacts with the protein residues. Hence, it is not obvious

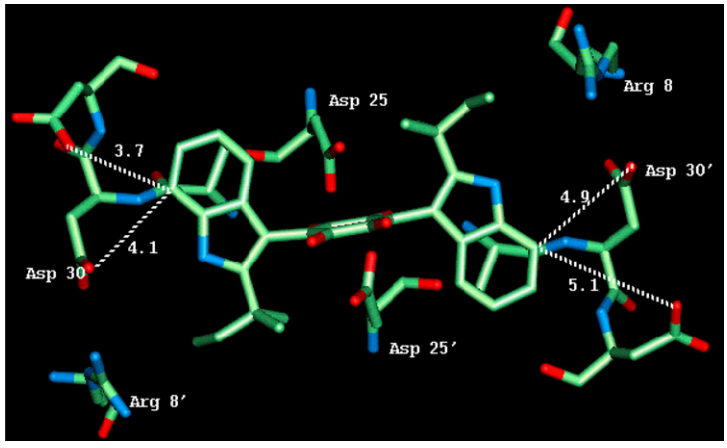

Fig. 2. Interaction of the indole ring of hinnuliquinone with the Asp-29 and Asp-30. Modifications at C-8′ of indole ring would be too close to Asp-29 and Asp-30.

why compounds **4–6** are more potent HIV protease inhibitors than DMAQ-B1.

Bis-indolyl-quinones **3–6** were also reported to inhibit HIV-1 protease with IC_{50} values of 180–>500 nM [14]. Comparison of the activities of these compounds with hinnuliquinone and DMAQ-B1 suggests that the substitution at C-8 is detrimental for protease activity.

In 1999, DMAQ-B1, a fungal metabolite, was reported as an oral anti-diabetic agent, which manifested its activity by mimicking insulin. It resulted in significant lowering of blood glucose in two mouse models [5,16]. This study generated several reports on DMAQ-B1 describing various related mechanistic activities [17–22]. Hinnuliquinone was inactive in these assays and did not display any anti-diabetic activity. This indicates that the activities of hinnuliquinone and isomeric natural product DMAQ-B1 are distinct and mutually exclusive.

The literature contains several reports of natural products that are inhibitors of HIV-1 protease. These inhibitors have been isolated from microbes, terrestrial plants, and marine organisms. Examples include cytochalasins ($IC_{50} = 3\ \mu M$) from the fungus *Hypoxylon fragiforme* [2], tetronic acid homologs ($IC_{50} = 84$–$135\ \mu M$) from *Actinomycetes* sp. [23], peptides, e.g., Mer-N5075A ($IC_{50} = 17.8\ \mu M$) from *Streptomyces chromofuscus* [24], ellagic acid derivatives ($IC_{50} = 10\ \mu M$) from the branches of *Combretum yunnanensis* [25], Curcumin ($IC_{50} = 100\ \mu M$) from *Curcuma longa* [26], triterpenoid saponins ($IC_{50} = 35$–$50\ \mu M$) from the seeds of *Aesculus chinensis* [27], and didemnaketals A ($IC_{50} = 2\ \mu M$) and B ($IC_{50} = 10\ \mu M$) from ascidian *Dimemnum* sp. [28].

In this paper, we have described the details of isolation and HIV-1 protease inhibitory activity as well as crystallographic data of the C_2-symmetric dimeric bis-indolyl-quinone hinnuliquinone bound to enzyme. The activity of hinnuliquinone is disparate and mutually exclusive from an isomer that has been extensively reported on, namely, non-C_2-symmetric bis-indolyl-quinone, DMAQ-B1.

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