Inhibition of yeast inositol phosphorylceramide synthase by aureobasidin A measured by a fluorometric assay

Wenyan Zhong, Dennis J. Murphy, Nafsika H. Georgopapadakou*

Department of Antimicrobial Research, DuPont Pharmaceuticals Company, Experimental Station, Bldg. E400, P.O. Box 80400, Wilmington, DE 19880-0400, USA

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Abstract Inositol phosphorylceramide synthase (IPC synthase) is an essential and unique enzyme in fungal sphingolipid biosynthesis and is the target of the cyclic nonadepsipeptide antibiotic aureobasidin A. As a first step towards understanding the mechanism of aureobasidin A inhibition, we developed a fluorometric HPLC assay for IPC synthase using the *Saccharomyces cerevisiae* enzyme and the fluorescent substrate analog 6-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanoyl ceramide (C_6-NBD-cer). The kinetic parameters for C_6-NBD-cer were comparable to those for the synthetic substrate *N*-acetylsphinganine used previously. Aureobasidin A acted as a tight-binding, non-competitive inhibitor with respect to C_6-NBD-cer and had a K_i of 0.55 nM.

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Key words: Fungal sphingolipid; Fluorometric assay; Inositol phosphorylceramide synthase; Aureobasidin A; 6-[*N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanoyl ceramide

1. Introduction

Sphingolipids are essential membrane components in both mammalian and fungal cells and share their biosynthetic pathway up to the formation of sphinganine [1] (Fig. 1). Subsequent to sphinganine formation, the mammalian and fungal pathways diverge. In mammalian cells, ceramide gives rise to a host of sphingolipids: sphingomyelin, cerebrosides and gangliosides. In fungi, phytoceramide gives rise mostly to inositol-containing sphingolipids: inositol phosphorylceramide (IPC), mannose-IPC (MIPC) and inositol phosphoryl-MIPC ($M(IP)_2C$) [1]. The first post-ceramide step is catalyzed by IPC synthase and involves the transfer of the phosphoinositol group from phosphatidylinositol (PI) to the 1-hydroxy group of phytoceramide to form IPC [1]. Recent studies have shown IPC synthase to be essential for fungal growth and to be the target of the antifungal aureobasidin A [2]. In addition to aureobasidin A [3–6], IPC synthase is inhibited by two structurally distinct natural products that also have antifungal activity: the lipid-like khafrefungin [7] and the macrolide galbonolide [8,9]. All three compounds inhibit IPC synthase activity at (sub)nanomolar concentrations. Their interaction with the enzyme has obvious implications for antifungal drug design.

IPC synthase activity can be assayed using radiolabeled *N*-acetylsphinganine (C_2-cer) [2] or radiolabeled PI [10]. In both assay methods, the IPC product is separated from the substrate by solvent extraction (in the case of radiolabeled PI-based assay after mild alkaline hydrolysis). Both methods generate substantial amounts of radioactive waste and neither method is adequate for the large number of assays necessary for detailed inhibition studies. We therefore examined the suitability of a fluorescent analog of ceramide as a substrate and developed an HPLC-based fluorometric assay. The assay enabled us to examine the inhibition kinetics of aureobasidin A with IPC synthase of *Saccharomyces cerevisiae*. The results are described in this paper.

2. Materials and methods

2.1. Materials

S. cerevisiae W303 (*MATa, ura3, leu2, his3, ade2, trp1*) was from the DuPont Pharmaceuticals Culture Collection. Tris base, CHAPS, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co; 6-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanoyl ceramide (C_6-NBD-cer) (Fig. 2) was from Matreya Inc.; PI was from Avanti Polar Lipids; aureobasidin A was from Panvera Corp.; acetic acid (CH_3COOH) and acetonitrile (CH_3CN) (HPLC grade) were from J.T. Baker. All other reagents were of the highest grade available commercially.

HPLC measurements were performed with a Waters 2690 Alliance System using a C_{18} reversed-phase column (ZOBAX, 25 cm × 4.6 mm i.d., 5 μm, Hewlett Packard).

2.2. IPC synthase activity assay

Typically, C_6-NBD-cer (stock solution, 2 mM in DMSO; assay concentration, 0.1 mM) and PI (stock solution, 10 mM in assay buffer; assay concentration, 2 mM) were added to assay buffer (50 mM Tris–HCl, pH 7.0, 10 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM CHAPS) and the reaction was initiated by the addition of microsomal membranes (prepared by a slight modification of a published procedure [11]; final protein concentration 0.1 mg/ml). The reaction mixture (total volume 50 μl) was incubated at 30°C for various times and the reaction was quenched with 10% acetic acid. After centrifugation at 10 000 × *g* for 3 min, a 10–20 μl aliquot of the supernatant was injected with an automatic sample injector into an HPLC instrument. Fluorescent substrate and product (λ_{ex} = 465 nm, λ_{em} = 530 nm) were separated on a C_{18} reversed-phase column with a gradient of 50% CH_3CN–50% H_2O (0.1% CH_3COOH) to 90% CH_3CN–10% H_2O (0.1% CH_3COOH) at a flow rate of 1 ml/min and quantitated by integrating the peak areas. The gradient was stable for about 60 cycles, after which period the column was washed with 100% CH_3CN. Fractions from the two HPLC peaks with retention times of 5.2 and 12.2 min respectively were collected. After evaporating the solvent, samples were analyzed by electrospray LC/MS and MS/MS using a Micromass Q-TOF hybrid mass spectrometer operated in the positive ion electro-

*Corresponding author. Fax: (1)-302-695 7407.
E-mail: nafsika.h.dakou@dupontpharma.com

Abbreviations: IPC, inositol phosphorylceramide; PI, phosphatidylinositol; C_6-NBD-cer, 6-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanoyl ceramide; DMSO, dimethylsulfoxide

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spray mode with an HP1100 liquid chromatograph and autosampler.

In preliminary experiments, the linear range of IPC product formation as a function of protein concentration was determined. Assay mixtures contained a fixed concentration of substrates (2 mM PI (four times the apparent K_m) and 0.1 mM C_6-NBD-cer) and samples were incubated for a fixed time (20 min). The linear range of IPC product formation was then determined as a function of incubation time. Assay mixtures contained a fixed concentration of substrates (2 mM PI and 0.005 mM C_6-NBD-cer) and 0.1 mg/ml of membrane protein.

The initial velocity of IPC synthase was next determined at different concentrations of C_6-NBD-cer with a fixed PI concentration of 2 mM and membrane protein concentration of 0.1 mg/ml. Data were plotted and fitted to the Michaelis–Menten equation using the Kaleidagraph fitting program (Synergy Software) and K_m and V_{max} were determined.

2.3. Inhibition by aureobasidin A

To investigate the mechanism of aureobasidin A inhibition, the IPC synthase concentration was determined first at a protein concentration of 1.0 mg/ml and C_6-NBD-cer concentration of 10 μM. Various aureobasidin A concentrations (19.8–0.5 nM; stock solution, 1 mM in DMSO) were preincubated with microsomal membranes for 5 min prior to the addition of substrates. IPC product formation was subsequently analyzed by HPLC and the fractional velocity of the reaction was plotted as a function of inhibitor concentrations. The curve was fitted to Eq. 1, allowing both $[E_t]$ and K_{i-app} to float [12,13]. A value of 5.6 nM for $[E_t]$ was thus obtained. To determine the inhibition pattern of IPC synthase by aureobasidin A, the above measurements were repeated at various substrate concentrations (2.5–80 μM) using a diluted protein concentration of 0.1 mg/ml (corresponding to $[E_t]$ of 0.56 nM). Under these assay conditions, the values of K_{i-app} and $[E_t]$ are close together and error in the estimation of $[E_t]$ can have a significant effect on the estimation of K_{i-app}. $[E_t]$ was therefore fixed at 0.56 nM to examine the dependence of K_{i-app} as a function of C_6-NBD-cer concentration. Data were fitted to Eq. 1 and K_{i-app} was calculated and plotted as a function of substrate concentration.

$$\frac{v_i}{v_0} = 1 - \frac{([E_t] + [I] + K_{i-app}) - \sqrt{([E_t] + [I] + K_{i-app})^2 - 4[E_t][I]}}{2[E_t]} \quad (1)$$

3. Results and discussion

The recent emergence of IPC synthase as an essential and unique fungal enzyme has provided a new target for antifungal drug discovery. The absence of a mammalian counterpart reduces the odds for mechanism-associated host toxicity. Further, blocking this step not only depletes the fungal cell of essential sphingolipids but also causes accumulation of ceramide, known to induce apoptosis in mammalian cells [14,15]. The fungicidal action of aureobasidin A, a feature highly desirable for the treatment of opportunistic fungal infections, has been suggested to be due to ceramide accumulation [6]. Significantly, the IPC synthase (*AUR1*) gene has been identified in a wide range of fungi including two key opportunistic human pathogens: *Candida albicans* and *Aspergillus fumigatus* [16]. Thus, IPC synthase inhibitors are potentially broad-spectrum antifungals.

Although the essential role of IPC synthase has been firmly established, its interactions with substrates and inhibitors have not been fully investigated. To facilitate this task, we developed a fluorometric HPLC assay based on an analog of phytoceramide, C_6-NBD-cer. This analog has been successfully employed previously to study the intracellular transport and metabolism of dihydroceramide, an intermediate in mammalian sphingolipid synthesis [17–20].

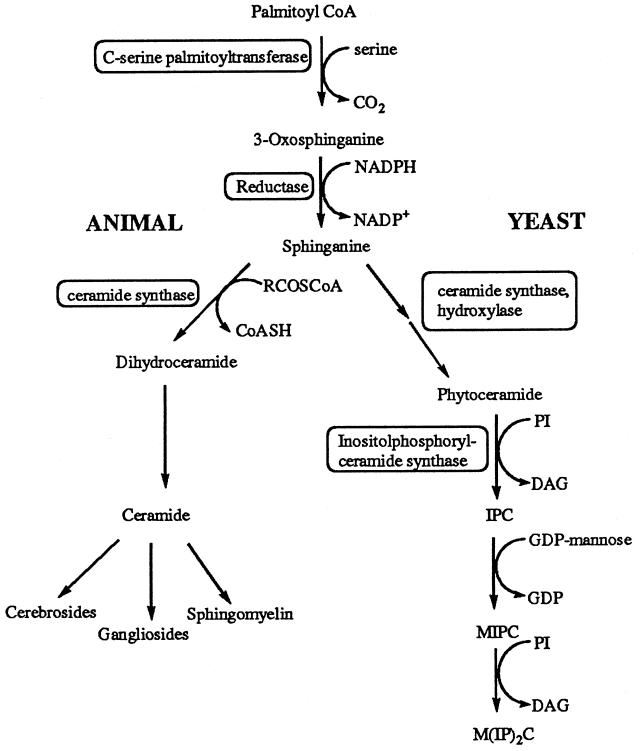


Fig. 1. Sphingolipid biosynthesis in animals and yeast. Abbreviations: DAG, diacylglycerol; MIPC, mannose inositol phosphoryl-ceramide; $M(IP)_2C$, mannose (inositol phosphoryl)$_2$-ceramide.

3.1. IPC synthase activity assay

The IPC synthase reaction with C_6-NBD-cer is shown in Fig. 2. The mass spectra of the two fluorescent peaks on the HPLC trace of the reaction mixture displayed $(M+H)^+$ at *m/z* 576 (12.2 min HPLC peak) and at *m/z* 818 (5.2 min HPLC peak). The former corresponds to the predicted mass of the C_6-NBD-cer substrate and the latter to the predicted mass of the C_6-NBD–IPC product. In addition, the MS/MS spectrum of *m/z* 818 showed a fragment ion at *m/z* 547, corresponding to the loss of the phosphoinositol head group and providing further structural evidence for the identity of the 5.2 min peak as C_6-NBD–IPC. The peak area of the C_6-NBD–IPC product had a detection limit of 0.1 pmol and was linear with product concentration up to at least 25 pmol.

IPC synthase activity was linear with incubation time at least up to 30 min and with protein concentration up to 2 mg/ml (data not shown). All subsequent studies were performed within the linear range.

Initial velocities of the IPC synthase reaction were next measured at various C_6-NBD-cer concentrations (2.5–80 μM) with a fixed PI concentration of 2 mM ($K_m = 0.5$ mM) and an enzyme concentration of 0.1 mg/ml. Data were fitted to the Michaelis–Menten equation and the apparent K_m and V_{max} were estimated to be 9.8 ± 0.1 μM and 0.50 ± 0.02 nmol/min/mg of protein respectively (Fig. 3). These compare favorably with the K_m and V_{max} values for the radiolabeled C_2-cer substrate [2], estimated to be 30 μM and 0.26 nmol/min/mg of protein respectively (M. Jeffries and N.H. Georgopapadakou, unpublished data). The attractive kinetic parameters of the C_6-NBD-cer, together with the high sensitivity of C_6-NBD–

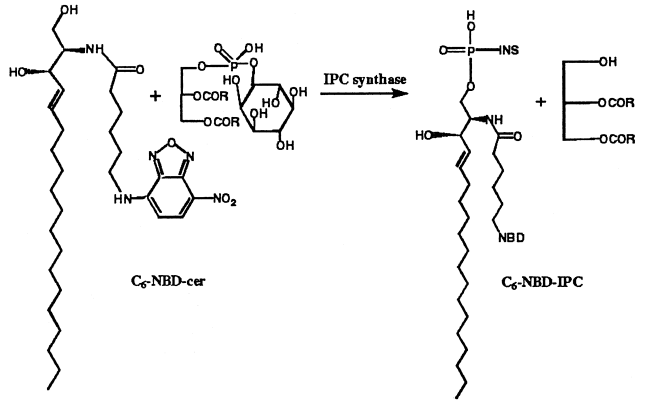


Fig. 2. The IPC synthase reaction with C_6-NBD-cer as substrate.

IPC detection, make the fluorometric assay more suitable than the radiochemical assay for inhibition studies, where detection of low product levels is necessary.

3.2. Inhibition by aureobasidin A

The activity of IPC synthase was measured in the presence of various aureobasidin concentrations. Microsomal enzyme was preincubated with aureobasidin A for 5 min before the addition of the two substrates to allow for completion of any slow-binding phase and the reaction was initiated by the addition of PI and C_6-NBD-cer. A fixed PI concentration of 2 mM and a range of C_6-NBD-cer concentrations (2.5–80 μM) were employed. The plot of fractional velocity as a function of inhibitor concentration (Fig. 4) strongly suggested that aureobasidin A acts as a tight-binding inhibitor of IPC synthase with an apparent K_i in the subnanomolar range. With tight-binding inhibitors, where K_{i-app} is at or lower than $[E_t]$, the Henri–Michaelis–Menten equations are not adequate (since they assume $K_{i-app} \gg [E_t]$) and the Morrison equation [12,13] (Eq. 1) is used to determine K_i values. Therefore, the data were fitted using Morrison's equation and K_{i-app} at various substrate concentrations was obtained. The behavior of K_{i-app} as a function of substrate concentration is diagnostic of competitive, uncompetitive, or non-competitive inhibition with respect to the varied substrate. The data in Fig. 4 show that K_{i-app} is independent of the concentration of C_6-NBD-cer (above and below the K_m) implying non-competitive inhibition (i.e. $K_i = K_{i-app}$). This finding, along with evidence that aureobasidin A inhibits IPC synthase activity in a variety of fungi, including *C. albicans* and *A. fumigatus* [21], suggests that aureobasidin A may bind to a distinct, hydrophobic site on IPC synthase that is conserved among different fungal species.

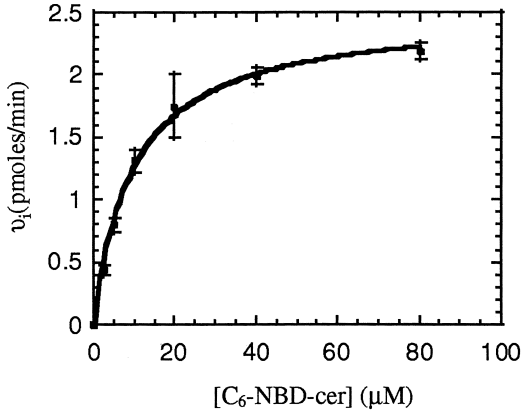


Fig. 3. Determination of K_m and V_{max} for C_6-NBD-cer. Solid circles represent experimental data while the solid line represents the nonlinear curve fitting of the experimental data to the Michaelis–Menten equation using the Kaleidagraph fitting program.

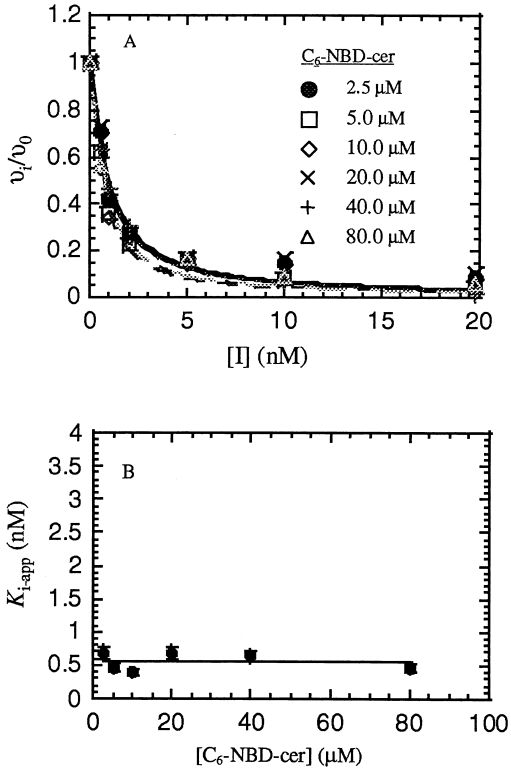


Fig. 4. Inhibition of IPC synthase activity by aureobasidin A. A: Plot of fractional velocity as a function of inhibitor concentration for aureobasidin A at different C_6-NBD-cer substrate concentrations (indicated). B: Plot of apparent K_i, calculated from A, as a function of C_6-NBD-cer substrate concentration. Assay conditions are described in Section 2.

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