Inhibition of Inositol Phosphorylceramide Synthase by the Cyclic Peptide Aureobasidin A

Paul A. Aeed,† Casey L. Young,‡ Marek M. Nagiec,§ and Åke P. Elhammer*

Pharmacia Corp., 7000 Portage Rd., Kalamazoo, Michigan 49001

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By using a detergent-washed membrane preparation, the interaction of the fungal natural product inhibitor aureobasidin A (AbA) with inositol phosphorylceramide synthase (IPC synthase) was studied by kinetic analysis of wild-type and mutant enzyme-catalyzed reactions. AbA inhibited the wild-type enzyme from both Candida albicans and Saccharomyces cerevisiae in an irreversible, time-dependent manner, with apparent $K_i$ values of 183 and 234 μM, respectively. Three synthetic chemistry-derived AbA derivatives, PHA-533179, PHA-556655, and PHA-556656, had affinities 4 to 5 orders of magnitude lower and were reversible inhibitors that competed with the donor substrate phosphatidylinositol (PI). AbA was a reversible, apparently noncompetitive inhibitor, with a $K_i$ of 1.4 μM, of the IPC synthase from an AbA-resistant S. cerevisiae mutant. The $K_m$ values for both substrates (ceramide and PI) were similar when they interacted with the mutant and the wild-type enzymes. By contrast, the $V_{max}$ for the mutant enzyme was less than 10% of that for the wild-type enzyme. A comparison of the results obtained with AbA with those obtained with two other natural products inhibitors, rustmicin and khafrefungin, revealed that while rustmicin appeared to be a reversible, noncompetitive inhibitor of the wild-type enzyme, with a $K_i$ of 16.0 nM, khafrefungin had the kinetic properties of a time-dependent inhibitor and an apparent $K_i$ of 0.43 nM. An evaluation of the efficiencies of these compounds as inhibitors of the mutant enzyme revealed for both a drop in the apparent affinity for the enzyme of more than 2 orders of magnitude.

Sphingolipids are important components of eukaryotic cell membranes (8). Properties like membrane dimensions and rigidity are believed to be directly influenced by the presence of sphingolipids (5, 11, 18). Moreover, it has been demonstrated that sphingolipids are important signaling molecules (8, 26), that they are involved in the transport and targeting of membrane proteins (4, 15, 27, 29), and that the downregulation of sphingolipid biosynthesis impairs the pathogenicity of the human pathogen Cryptococcus neoformans (14, 19). Fungal cellular membranes contain significant amounts of sphingolipids (33). As much as 16% of the total lipid content in the Saccharomyces cerevisiae plasma membrane and approximately 10% of the total lipid content in the Golgi membrane are sphingolipids (17, 24). The composition of fungal sphingolipids differs from that of their mammalian counterparts, primarily in the substituent at the 1-hydroxyl of the ceramide backbone. While mammalian sphingolipids may be substituted at this position with phosphocholine or, on glycosylceramides, various carbohydrates, fungal sphingolipids mainly contain phosphoinositol and, in some species, carbohydrates. The ceramide-linked phosphoinositol moiety in fungi can be further derivatized with mannosyl, galactosyl, and an additional phosphoinositol group (8). Although the reaction steps in the assembly of the ceramide portion of sphingolipids are similar in mammalian and fungal cells, the fungal biosynthetic pathway diverges at the addition of phosphoinositol. As a consequence, the enzyme catalyzing this reaction, inositol phosphorylceramide synthase (IPC synthase), has been pursued as a target in antifungal drug discovery (16, 23, 34, 36). Supporting the validity of this concept are the facts that the IPC synthase gene (AUR1) has been shown to be essential in fungi and that potent antifungal compounds that are specific inhibitors of IPC synthase have been identified (23).

A functional homolog of the fungal AUR1 gene was recently identified in the protozoan Leishmania major, and the activity of the corresponding enzyme, IPC synthase, was shown to be sensitive to the fungal IPC synthase inhibitor aureobasidin A (AbA) (7). IPC synthase activities have also been identified in Trypanosoma cruzi and Trypanosoma brucei (6, 25). Moreover, since unperturbed sphingolipid synthesis appears to be essential for protozoan parasite infectivity in trypanosomes as well as in Toxoplasma gondii, IPC synthase may represent a tractable target for the development of drugs for the treatment not only of fungal infections but also of protozoan infections (10, 25, 28).

The specific IPC synthase inhibitors identified to date are natural compounds isolated from the fermentation medium of microorganisms (20, 21, 31, 32, 35). The structures of three important inhibitor compounds, AbA, rustmicin, and khafrefungin, are shown in Fig. 1.

AbA is a cyclic depsipeptide isolated from the fungus Aureobasidium pullulans (31). It is a comparatively large molecule with a number of side chains, several of which are believed to be important for activity (3, 16). Considerable efforts have been invested in attempts to map which portions of the AbA
molecule contain the elements essential for the inhibitory activity, the so-called pharmacophore, but no comprehensive conclusions have been reached. Substantial efforts have also been made to create derivatives of AbA, such as the three synthetic AbA derivatives evaluated in the investigation described here, with structures that are less complex but that retain the inhibitory properties of the native molecule. Again, these efforts have largely been unsuccessful (see reference 16 and references therein). AbA has MICs in the low- and sub-μg/ml range for *S. cerevisiae*, *Candida albicans*, and *C. neoformans*. It is considerably less effective against *Aspergillus fumigatus* (37). The in vitro 50% inhibitory concentrations (IC50s) for IPC synthase activities in various *Saccharomyces*, *Candida*, and *Aspergillus* strains have been reported to range from 0.2 to 4.9 nM (23, 37); and a *K*<sub>i</sub> value of 0.55 nM for the IPC synthase activity in *S. cerevisiae* has been reported (36).

Although AbA is an efficient inhibitor of IPC synthase, mutants resistant to the compound can be generated by chemical mutagenesis. Sequence analysis of these mutants has identified two positions, His 157 (13) and Phe 158 (12), in the IPC synthase (*AUR1*) gene sequence where mutations can generate resistant enzymes. The substituting amino acid in both reported mutants is a tyrosine. Interestingly, both sites (His 157 and Phe 158) are, to a considerable extent, removed from the amino acid residues believed to be involved in the catalytic function of the enzyme (18). Data on the kinetic properties of the mutant enzymes have not been reported.

Rustmicin (galbonolide A) is a 14-membered macrolide produced by the fungi *Micromonospora chalcea* and *Streptomyces galbus* (1, 9, 30). The molecule is a potent antifungal agent with MICs in the low-μg/ml range for both *C. albicans* and *S. cerevisiae*. It has also been reported that rustmicin is a reversible IPC synthase inhibitor and has an in vitro IC50 of about 3.8 nM (21).

Khafrefungin, an agent isolated from an unidentified fungus, is composed of an aldonic acid esterified to a linear polyketide (Fig. 1). Khafrefungin is fungicidal against both *C. albicans* and *S. cerevisiae*, and the compound reportedly inhibits the *C. albicans* IPC synthase with an IC50 of 0.6 nM (20).

This report presents the results of an in vitro kinetic evaluation of the inhibition of *C. albicans* and *S. cerevisiae* IPC synthases by native AbA. The time-dependent nature of AbA inhibition is documented, and an apparent *K*<sub>i</sub> value is presented. In addition, a comparison of the properties of AbA with those of three synthetic derivatives, as well as those of the IPC synthase inhibitors rustmicin and khafrefungin, is presented, together with data describing the altered kinetic properties of IPC synthase from an AbA-resistant (AbA<sup>r</sup>) *S. cer-
MATERIALS AND METHODS

Cells. Candida albicans (ATCC 38247) cells were cultured as described previously (2). Saccharomyces cerevisiae (SJ21R) cells were cultured, and an AbA mutant was constructed as described previously (12, 13).

Enzymes. Detergent-washed membranes from C. albicans, S. cerevisiae, and S. cerevisiae AbAr cells were prepared as described previously (2).

Inhibitors. AbA was from TaKaRa Biomedicals. PHA-533179, PHA-556655, and PHA-556656 (Fig. 1) were synthesized at Pharmacia. Khaferefungin was isolated from Mycelia sterilia (ATCC 74305) by previously published procedures (20, 22). Rustmicin (galbonolide A) was a gift from Hans Achenbach (University of Erlangen, Erlangen, Germany).

IPC synthase assay. IPC synthase assays were performed essentially as described previously (2). Briefly, A 28-μl enzyme mixture containing 10 μg (protein) of detergent-washed membranes, phosphatidylinositol (PI), and potassium phosphate buffer (pH 7.0) was preincubated for 30 min in a 96-well plate. The standard enzymatic reaction was started by addition of a substrate mix containing C6-NBD (7-nitro-2-1,3-benzoxadiazol-4-yl)-ceramide (Avanti Polar Lipids) in ethanol or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) diluted to 12 μl with water to the preincubated membranes. For inhibitor studies, the substrate mix also contained 2 μl inhibitor solution in dimethyl sulfoxide (DMSO). The final assay volume was 40 μl, and the final concentrations were 50 mM potassium phosphate (pH 7.0), 0.25 mg membrane protein/ml, 100 μM PI, 5 μM C6-NBD-ceramide, 0.6 mM CHAPS, 0.3% ethanol, and 5% DMSO.

Following incubation at room temperature, the reaction was stopped by addition of 200 μl of 96% methanol, and the reaction product was isolated on ion-exchange resin and quantified as outlined previously (20).

Data analysis. The inhibition of C. albicans and S. cerevisiae wild-type enzymes by AbA was analyzed as two-step irreversible inhibition. Progress curves were consistent with first-order kinetics, and Ki and inactivation constant (k_inact) values were calculated from the first-order rate constant (k_obs) with the following equation:

$$k_{\text{obs}} = \frac{k_{\text{inact}}}{K_i + \frac{S}{K_m}}$$

where [S] and [I] denote the substrate and the inhibitor concentrations, respectively.

Kinetic parameters for both substrates were calculated by using the Michaelis-Menten equation.

Competitive, reversible inhibitors were analyzed by measuring the initial reaction velocities (v) and calculating K_i by using the following equation:

$$v = \frac{V_{\text{max}}[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

AbA inhibition of the S. cerevisiae AbAr enzyme was analyzed as noncompetitive, reversible inhibition by measuring the initial reaction velocities and calculating K_i by using the following equation:
RESULTS AND DISCUSSION

As a first step in the analysis of the effect of AbA on IPC synthase, a determination was made as to whether the interaction between the enzyme and the inhibitor was time dependent. Product formation was plotted as a function of time at several different inhibitor concentrations. Figure 2A and B shows that both the C. albicans and S. cerevisiae enzymes clearly interact with the inhibitor in a time-dependent manner. Particularly at higher inhibitor concentrations, the product formation reaches a plateau 10 to 15 min into the reaction, indicating that product formation has ceased, that the substrate cannot compete with the inhibitor, and that the inhibitor is essentially titrating the enzyme. Figure 2C and D shows that AbA inhibited the C. albicans and S. cerevisiae enzymes with very similar apparent $K_i$ values of 183 pM and 234 pM, respectively. The apparent $k_{inact}$ values for these two enzymes were 2.5 ms$^{-1}$ and 1.8 ms$^{-1}$, respectively, hence confirming the potency of this inhibitor observed in the experiments described above.

To confirm the irreversible nature of the interaction between IPC synthase and AbA, an experiment was performed in which the C. albicans enzyme was preincubated with AbA prior to the initiation of the reaction. Figure 3 shows that this clearly resulted in a time-dependent inactivation of the enzyme. There was a significant difference in the amount of product formed in the reaction with preincubation compared to that formed in the reaction without preincubation.

The compounds PHA-533179, PHA-556655, and PHA-556656 (Fig. 1) are derived from the native AbA structure. The molecules are the result of a synthetic chemistry effort to generate smaller, less complicated molecules that retain at least a significant portion of the inhibitory properties of native AbA. Evaluation of the compounds with the C. albicans enzyme revealed that although these compounds retained significant inhibitory activity, they were all at least 3 orders of magnitude less potent than native AbA (Fig. 4D, inset). Figure 4A shows that, in contrast to AbA, PHA-533179 do not inhibit IPC synthase in a time-dependent manner. The amount of product formed increased with time at all inhibitor concentrations tested. Moreover, determinations of the kinetic parameters at increasing substrate concentrations in the presence of increasing concentrations of inhibitor revealed that while the $K_m$ values for PI increased significantly with increasing inhibitor concentrations, the $V_{max}$ values remained essentially constant (Fig. 4B). By contrast, no such increase was observed when the ceramide concentrations were varied (Fig. 4C). These observations suggest that PHA-533179 (as well as PHA-556655 and PHA-556656, with which similar results were obtained; data not shown) competes with the donor substrate PI. Analysis of the three compounds as reversible, competitive inhibitors resulted in $K_i$ values of 11.8, 10.5, and 7.9 nM, respectively (Fig. 4D, inset). These values are more than 4 orders of magnitude higher than the $K_i$ value of 183 pM determined for native AbA (compare this result with those described above), suggesting that the derivatives retain only a minor fraction of the potency of the parent molecule. It appears likely that these molecules, which in essence are fragments of the native AbA molecule, are lacking several of the binding functionalities required for high-affinity binding to the IPC synthase enzyme. Previous investigations have shown that mere manipulations of side chains on AbA can have drastic effects on the inhibitory effect of the compound (16). Conceivably, at least some of these effects may have been caused by a loss of affinity for the enzyme. Interestingly, all three AbA derivatives appeared to compete with the donor substrate PI. Since the native molecule is a time-dependent inhibitor, it was not possible to
determine whether it interacts with the substrate binding site(s) on the enzyme. Nonetheless, given the observations discussed above and assuming that the three AbA derivatives still inhibit IPC synthase in a manner similar to that of native AbA, although they are less potent than native AbA, it is possible that the inhibitory effect of the native compound is the result of binding to or at the PI binding site on the enzyme. Consequently, AbA may in fact be a competitive inhibitor, albeit a very potent one. Interestingly, however, this conclusion is not consistent with the data generated for the AbAr enzyme (see below).

To gain additional insight into the interaction(s) between IPC synthase and AbA, the kinetic properties of IPC synthase from an AbA-resistant S. cerevisiae mutant were studied. The IPC synthase in this strain contained the previously described F158Y mutation (12). As a first step in the characterization of this enzyme, the kinetic parameters of both substrates were determined for the wild-type S. cerevisiae enzyme. This resulted in a $K_m$ value of 3.0 μM for C6-NBD-ceramide, which is quite similar to that of the C. albicans enzyme (3.3 μM), and a $K_m$ value for PI of 555 μM, which is approximately four times higher than that of the C. albicans enzyme (Table 1). The $V_{max}$ values for the S. cerevisiae enzyme were 367 and 824 pmol min$^{-1}$ mg protein$^{-1}$ for C6-NBD-ceramide and PI, respectively. Both these values are a little less than 40% of the values obtained with the C. albicans enzyme (884 and 1864 pmol min$^{-1}$ mg protein$^{-1}$, respectively [2]). Taken together, the data indicate that slight differences in the catalytic properties of the C. albicans and S. cerevisiae enzymes may exist. However, it appears more likely, given that both enzyme prepara-

**TABLE 1. Kinetic properties of C. albicans wild-type, S. cerevisiae wild-type and S. cerevisiae AbAr IPC synthasea**

<table>
<thead>
<tr>
<th>Organism</th>
<th>PI</th>
<th>Ceramide</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (pmol min$^{-1}$ mg protein$^{-1}$)</td>
</tr>
<tr>
<td>C. albicans wt</td>
<td>130</td>
<td>1,864</td>
</tr>
<tr>
<td>S. cerevisiae wt</td>
<td>555</td>
<td>824</td>
</tr>
<tr>
<td>S. cerevisiae AbAr</td>
<td>330</td>
<td>69</td>
</tr>
</tbody>
</table>

a The assays were performed as outlined in the text. Ceramide, C6-NBD-ceramide.

FIG. 4. Inhibition of Candida albicans IPC synthase with PHA-533179. IPC synthase assays were performed and the data were analyzed as outlined in Materials and Methods. (A) Progress curves generated in the absence (0) and in the presence of increasing concentrations (5, 10, 25, and 50 μM) of the inhibitor; (B) kinetic parameters for the PI substrate determined in the absence (0) and in the presence of increasing concentrations (5, 10, and 25 μM) of the inhibitor; (C) kinetic parameters for the ceramide substrate determined in the absence (0) and in the presence of increasing concentrations (5, 10, and 20 μM) of the inhibitor; (D) $K_i$ calculated for a competitive inhibitor.
tions are made from detergent-washed membranes, that differences in the overall protein, lipid, and/or carbohydrate compositions of the two cell types, as well as in the amounts of the enzyme preparations recovered (again, likely caused by structural differences in the two cell types), are the true reasons for the observed differences. A more accurate comparison must await purification of the two enzymes. Nonetheless, the data generated strongly suggest that the catalytic properties of IPC synthase are quite similar in *C. albicans* and *S. cerevisiae*. This conclusion is further supported by the very similar sensitivities of the two enzymes to AbA (Fig. 2 and data not shown).

Determination of kinetic parameters (for the two substrates) for the *S. cerevisiae* AbAr mutant enzyme (Table 1) yielded *K_m* values similar to those for the wild-type enzyme: approximately 2.0 and 330 μM for C_{6}-NBD-ceramide and PI, respectively. In contrast, the *V_max* values, at approximately 25 and 69 pmol min^{-1} mg protein^{-1} for C_{6}-NBD-ceramide and PI, respectively, were less than 10% of those obtained for the wild-type *S. cerevisiae* enzyme. Clearly, the amino acid substitution in IPC synthase conferring AbA resistance has a considerable impact on the catalytic performance of the enzyme. Nonetheless, the affinities for the two substrates appeared to be essentially unaltered for the mutant enzyme. This is in stark contrast to the affinity of the mutant enzyme for AbA, which was reduced by more than 3 orders of magnitude. A kinetic evaluation revealed that AbA is a reversible inhibitor of the *S. cerevisiae* AbAr mutant enzyme, with an apparent *K_i* of approximately 1.4 μM (Fig. 5).

Determinations of the values of the kinetic parameters at several substrate concentrations in the presence of increasing concentrations of the inhibitor showed that in assays with the mutant enzyme, AbA does not appear to compete with the binding of either PI (Fig. 5B) or ceramide (Fig. 5C). Although the finding that native AbA does not compete with the binding of PI in the mutant enzyme is in contrast to the mechanism of action of AbA-derived compounds PHA-533179, PHA-556655, and PHA-556656, this finding is consistent with the fact that the mutations cause a drop in the affinity of AbA for the mutant IPC synthase of more than 3 orders of magnitude. Since the mutations have virtually no effect on the affinity of AbA for either substrate (Table 1) but cause a drastic drop in the reaction velocity, the mutations may alter the enzyme such that although
the substrates are still capable of binding to the enzyme with an affinity similar to that of the wild-type enzyme, their orientation or intermolecular distance(s) in the active site is compromised. AbA may bind to a site that is related to or part of the active site. Alternatively, the compound may bind to a site that is removed from the near vicinity of the active site but that still influences (by steric means or otherwise) the catalytic function of the enzyme. It is noteworthy, in this context, that the amino acid substitutions of the AbAr mutants are located at positions believed to be quite remote from the enzyme active site (12, 13, 18).

Two other natural products, rustmicin and khafrefungin, are both specific inhibitors of IPC synthase. However, they differ significantly from AbA in their interaction with the enzyme. In contrast to AbA, rustmicin is a reversible inhibitor with an apparent $K_i$ almost 2 orders of magnitude higher than that of AbA (Fig. 6; Table 2). The data also suggest that this inhibitor does not compete with either of the two substrates. As discussed above, since the interaction of AbA with wild-type IPC synthase is time dependent, it is difficult to determine whether this compound interacts with the enzyme in a competitive or a noncompetitive manner. Interestingly, the IC$_{50}$ of rustmicin for the AbAr enzyme shows the same increase as that of AbA of approximately 3 orders of magnitude compared to that for the wild-type enzyme (Table 2). This indicates a possible over-

FIG. 6. Inhibition of C. albicans IPC synthase by rustmicin. IPC synthase assays were performed and the data were analyzed as outlined in Materials and Methods. (A) Progress curves generated in the absence (0) and in the presence of increasing concentrations (5, 10, 20, 30, and 40 nM) of the inhibitor; (B) kinetic parameters for the PI substrate determined in the absence (0) and in the presence of increasing concentrations (10, 20, and 30 nM) of the inhibitor; (C) kinetic parameters for the ceramide substrate determined in the absence (0) and in the presence of increasing concentrations (5, 10, 20, and 30 nM) of the inhibitor.

TABLE 2. Comparison of the inhibitory properties of AbA, khafrefungin, and rustmicin$^a$

<table>
<thead>
<tr>
<th>Organism</th>
<th>Aureobasidin A</th>
<th>Khafrefungin</th>
<th>Rustmicin</th>
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<tbody>
<tr>
<td></td>
<td>$K_i$ (nM)</td>
<td>$IC_{50}$ (nM)</td>
<td>$K_i$ (nM)</td>
</tr>
<tr>
<td>C. albicans wild type</td>
<td>0.18 T-d</td>
<td>0.43</td>
<td>T-d</td>
</tr>
<tr>
<td>S. cerevisiae wild type</td>
<td>0.23 T-d</td>
<td>43</td>
<td>ND</td>
</tr>
<tr>
<td>S. cerevisiae AbA</td>
<td>$1.4 \times 10^3$ Non</td>
<td>$11.3 \times 10^3$</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ The assays were performed as outlined in the text. Abbreviations: Inh., inhibition type; T-d, time dependent; Non, noncompetitive; ND, not determined.
with the AbAr enzyme) that AbA does not compete with the reaction, consistent with the possibility (supported by the data generated herein) that the substrate binding sites for AbA and rustmicin do not overlap between the binding sites for AbA and rustmicin. It is also possible that the compounds differ in their abilities to fit into the substrate binding site(s) on the wild-type enzyme. Despite significant differences in the structures of khafrefungin and AbA, the inhibitory activity of AbA is not mediated by a specific portion of the structure, but rather, the entire structure appears to be essential for both the affinity and the specific inhibitory mechanism. Hence, the inhibitory activity of AbA may not be mediated by a specific portion of the structure, a pharmacophore, but rather, the entire structure appears to be essential for full biological activity. All fragments of the structure evaluated were comparatively inactive, and compounds comprising other portions of the AbA structure have even less potency (data not shown). The data are consistent with previously published data that suggest that the range of structural alterations that can be made to AbA but that result in the retention of its inhibitory activity is quite limited (summarized in reference 16). Two other inhibitors, rustmicin and khafrefungin, show some similarities to AbA in their modes of action with the IPC synthase, with the most significant difference being their lower affinity for the enzyme (particularly for rustmicin). Evidence for a competitive inhibition mechanism was not found for any of the three inhibitors.

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REFERENCES


