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Modulation of Ceramide-Activated Protein Phosphatase 2A Activity by Low Molecular Weight Aromatic Compounds

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ABSTRACT. Protein phosphatase 2A (PP2A) is one of the most important and abundant serine/threonine phosphatases in mammalian tissues and plays a role in gene expression, cell division, and signal transduction. PP2A is activated by ceramide, which is produced by the hydrolysis of membrane sphingomyelin in response to a variety of stress-related stimuli. To further study the role of ceramide-mediated signal transduction in cellular processes such as senescence and apoptosis, we designed and synthesized a series of low molecular weight aromatic compounds, mainly of the isoquinolone and tetralone classes, and evaluated their ability to inhibit enzymes known to be activated by ceramide. Those enzymes studied were ceramide-activated protein kinase, protein kinase C ζ and PP2A. Of these, only PP2A was found to be inhibited. A few of the compounds inhibited both ceramide-activated as well as basal PP2A activity. In addition, several of the compounds activated PP2A by up to 300% above basal enzyme activity, but only in the presence of ceramide. Thus, modulation (both inhibition and activation) of the catatylic activity of ceramide-activated PP2A is demonstrated by certain low molecular weight aromatic compounds. BIOCHEM PHARMACOL **55**;7:1105–1111, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. protein phosphatase 2A; enzyme inhibition; enzyme activation; ceramide-activated protein phosphatase; ceramide antagonists; low molecular weight aromatic compounds; isoquinoline; tetralone

The phosphorylation state of structural and regulatory proteins is a major determinant in the control of eukaryotic cell proliferation, differentiation, senescence, and apoptosis. This state is controlled by both protein kinases and protein phosphatases. The serine/threonine PP2A† is a heterotrimeric enzyme consisting of a catalytic (C) subunit and A and B regulatory subunits. PP2A is one of the major serine/threonine protein phosphatases and is found in a variety of mammalian tissues [1, 2]. PP2A plays a role in many fundamental cellular processes, including gene expression [3], cell division [4, 5], and signal transduction [6]. PP2A is activated by the lipid ceramide *in vitro* and may be an important target of ceramide acting as a second messenger *in vivo* [7]. Ceramide, a key molecule in sphingolipid metabolism, is formed by hydrolysis of abundant plasma membrane sphingomyelin by neutral or acidic sphingomyelinase. This sphingolipid pathway has been shown to regulate cell proliferation, differentiation, senescence, and apoptosis in leukemic and other cell types [8] and is activated in response to a variety of stimuli including serum withdrawal and growth factor deprivation, ionizing radiation, TNFα, IL-1, IFNγ, antimetabolites such as araC, anti-Fas antibodies, and 1,25-dihydroxyvitamin D_3 [9–12]. Recently, studies have shown that endogenous ceramide levels and sphingomyelinase activity are elevated markedly in senescent human fibroblast cells and that exogenous ceramide is able to induce a senescent phenotype in young human fibroblast cells at concentrations that mimic endogenous levels in senescent cells [13]. With these observations in mind and in an effort to further study the role of ceramide-mediated signal transduction in cell senescence and apoptosis, we synthesized a series of low molecular weight compounds and tested them for their ability to inhibit those enzymes known to be activated by ceramide, i.e. CAPK [14], PKC [15, 16], and PP2A, also called CAPP [17, 18]. The compounds were designed initially to resemble somewhat the energy-minimized structure of ceramide, wherein a rigid bicyclic aromatic ring was conceived from the nonrigid ceramide structure by hypothetical bond formation between the C-2 and C-4 carbons of the palmitoyl chain and the C-4 and C-6 carbons of the sphingosine moiety of ceramide, respectively, followed by aromatiza-

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† *Abbreviations:* PP2A, protein phosphatase 2A; araC, cytosine arabinoside; TNFα, tumor necrosis factor alpha; IL-1, interleukin-1; PKC, protein kinase C; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; C-2 ceramide, *N*-acetylsphingosine; cAMP, 3′,5′-adenosine cyclic monophosphate; MBP, myelin basic protein; and PTK, protein tyrosine kinase.

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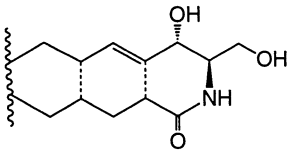
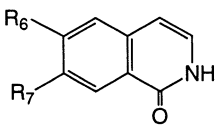
tion. The resulting general structure is represented by the substituted isoquinoline system.



Ceramide



Isoquinolines

Thus, the goal was to design analogs of ceramide as antagonists of ceramide-activated kinases and phosphatases. Initial experiments with one of the isoquinolines, designated 1I-49, which had shown activity in prolonging the survival of normal lymphocytes, failed to reveal any consistent effect on CAPK or PKC ζ enzyme activities. However, ceramide activation of PP2A was inhibited.

While PP2A is activated by ceramide and shorter chain derivatives of ceramide, such as C-2 ceramide, it is inhibited potently by okadaic acid [19], the fatty acid polyether isolated from marine sponges [20], at an IC_{50} of 1 nM. This inhibitor has proven useful for characterization of serine/threonine protein phosphatases. However, okadaic acid inhibits basal as well as ceramide-activated PP2A activity and is toxic to cells. Other well-known inhibitors of PP2A include calyculin A, microcystin, and cantharidin [21, 22]. The studies presented in this paper demonstrate that selective modulation of ceramide-activated PP2A activity is pharmacologically achievable. Indeed, we show both inhibition and activation of the catalytic activity of ceramide-activated partially purified human T cell (Jurkat) PP2A by certain small molecules of the isoquinolone and tetralone classes.

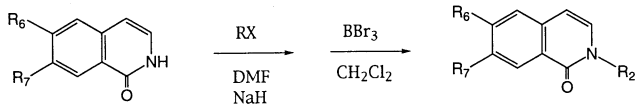
MATERIALS AND METHODS

Materials

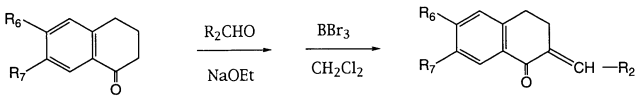
All commercial chemicals were reagent quality and used without further purification. Myelin basic protein and cAMP kinase were from Sigma. [γ-^{32}P]ATP was purchased from DuPont/NEN. DEAE-Sephacel and Mono-Q HR 5/5 columns were obtained from Pharmacia. Human T lymphoblastoid (Jurkat) cells were obtained from the American Type Culture Collection. Protein phosphatase 1 (PP1) from rabbit skeletal muscle was purchased from Upstate Biotechnology. C-2 ceramide was purchased from Biomol or from Matreya. Highly purified heterotrimeric PP2A was a gift from Dr. Marc Mumby (University of Texas Southwestern).

Preparation of Low Molecular Weight Compounds

Compounds in the isoquinoline series that are enumerated in Table 1 were prepared by direct alkylation of the preformed isoquinolone by a suitably functionalized alkylating agent followed by appropriate modifications, as outlined below.



The tetralones enumerated in Table 2 were prepared by condensation of the commercially available substituted 1-tetralone with the appropriate aldehyde in the presence of a base as illustrated here:



Some of the compounds in each series were treated with boron trifluoride to cleave the methyl ether functions to the respective alcohol group. The details of the chemical synthesis of both series of compounds and their derivatives will be reported elsewhere.†

Preparation of [^{32}P]Phosphorylated Substrate

[^{32}P]MBP was prepared by phosphorylation with the catalytic subunit of cAMP-dependent protein kinase, as described previously [7]. The reaction contained 50 mM of Tris–HCl, pH 7.4, 10 mM of $MgCl_2$, 0.1 mM of ATP, 100 μCi of [γ-^{32}P]ATP, 0.5 mM of dithiothreitol, 10 mM of β-mercaptoethanol, 0.04% Brij, 5 mg/mL of MBP, and 250 units/mL of catalytic subunit cAMP kinase in a volume of 0.5 mL. The reaction was incubated for 2 hr at 37° and stopped with 170 μL of ice-cold 100% trichloroacetic acid. Tubes were placed on ice for 30 min and centrifuged at 15,000 *g* for 10 min at 4°. The pellet was washed with 2 × 1 mL aliquots of −20° acetone and resuspended in 1 mL of water. The concentration of labeled substrate was based on the specific activity of the [γ-^{32}P]ATP.

Partial Purification of Ceramide-Activated PP2A

PP2A was purified by a modification of the procedure of Dobrowsky *et al.* [7]. Briefly, Jurkat cells (2–10 × 10^8 cells) were washed two times with ice-cold phosphate-buffered saline (pH 7.4) and resuspended in 3 mL of isotonic homogenization buffer consisting of 20 mM of Tris–HCl, pH 7.4, 1 mM of EDTA, 0.1 mM of EGTA, 1 mM of benzamidine, 1 mM of phenylmethylsulfonyl fluoride, 0.5 mM of dithiothreitol, 2.5 μg/mL of leupeptin, and 2 μg/mL of pepstatin A. Cells were disrupted by a glass/glass Potter–Elvehjem homogenizer for 5 min at 4°, and the resulting homogenate was centrifuged at 100,000 *g* for 45 min at 4°. Cytosol (3 mg/mL, 14 mg) was applied to a 10-mL DEAE-Sephacel column (1.5 × 2.5 cm) previously equilibrated with Buffer A (20 mM of Tris–HCl, pH 7.4, 1 mM of EDTA, 0.1 mM of EGTA, 1 mM of benzamidine, 0.5 mM of dithiothreitol, 10% glycerol). The column was washed

† Shih H, Cottam H and Carson D, manuscript in preparation.

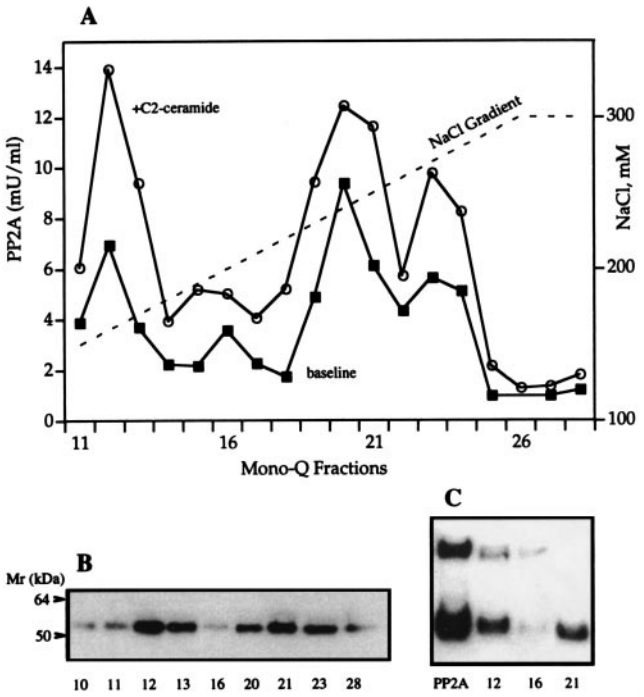


FIG. 1. Purification of PP2A from Jurkat cells. Jurkat cells were homogenized, and the cytosolic fraction was applied to a 10-mL anion exchange DEAE-Sephacel column. Elution was performed by a NaCl step gradient at a flow rate of 1 mL/min. The PP2A-enriched 300-mM NaCl fraction (15 mL volume) was concentrated, desalted, and loaded on a 2-mL anion exchange Mono-Q. (A) Mono-Q column profile. PP2A activity of control (■) and C2-ceramide-treated (20 μM) (○) fractions (1 μL of fraction) was tested (see Materials and Methods). The NaCl gradient is represented by the dotted line. Activity is expressed in mU/mL of fraction. (B) Immunoblotting at denaturing conditions of fractions obtained from a Mono-Q column (see panel A). Molecular weight markers are indicated on the left. The antibody 6G3 against the A subunit of PP2A was used to determine the presence of the enzyme. (C) Immunoblotting at native conditions of fractions obtained from a Mono-Q column (see panel A). The commercially available trimeric PP2A (UBI) was loaded in the first lane as a standard.

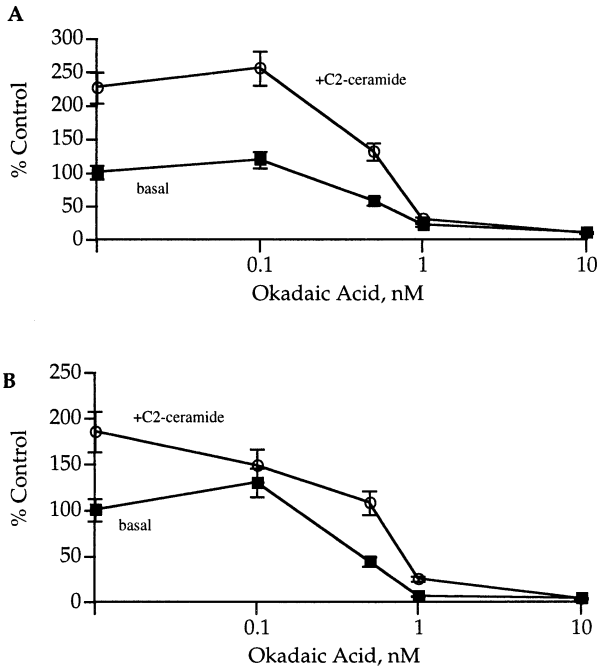


FIG. 2. Okadaic acid inhibition of the purified PP2A. The protein phosphatase activity was determined on 1 μL of Mono-Q fraction 12 (trimeric PP2A, panel A) and fraction 22 (dimeric PP2A, panel B) in the absence (■) or presence (○) of 20 μM of C2-ceramide and increasing concentrations of okadaic acid. On the y-axis, 100% represents the normalized basal activity, which was 8.1 ± 0.8 mU/mL. Data are means ± SEM from four experiments.

with Buffer A until the A_{280} returned to baseline, and the protein was eluted with a 30-mL linear gradient of 100–400 mM of NaCl in Buffer A at a flow rate of 1 mL/min. Fractions were assayed for PP2A activity and ceramide activation (20 μM) as described above. Ceramide-activated PP2A activity primarily eluted in the 300-mM salt fractions that were pooled and diluted 3-fold with Buffer A and applied to a Mono-Q HR 5/5 column (Pharmacia). Protein was eluted with a 30-mL linear gradient from 100–400 mM of NaCl at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected and assayed for ceramide-activated PP2A activity. Active fractions were divided into aliquots in 50% glycerol and stored at −80°.

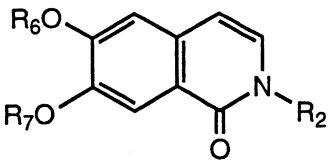
Western Blotting

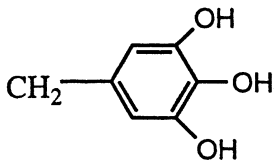
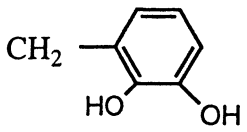
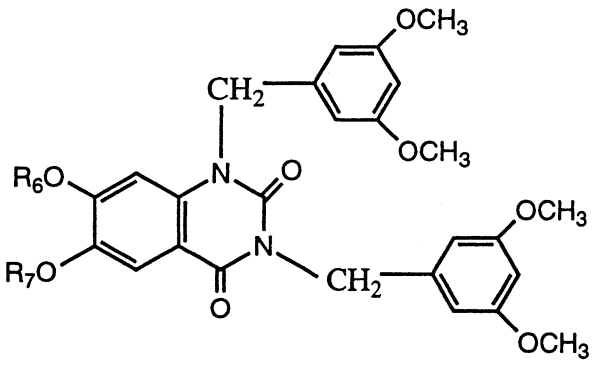
For immunodetection of PP2A, the column fractions were separated by SDS–PAGE using 10% Tris/glycine precast gels or by native PAGE using 12% Tris/glycine precast gels (Novex). Electrotransfer onto PVDF membranes was at 25 V for 1.45 hr using 50 mM of Tris containing 380 mM of glycine, 0.04% SDS, and 20% (v/v) methanol. Membrane blocking and washing, as well as antibody incubation and detection by enhanced chemiluminescence, were performed using the Tropix kit following the manufacturer's instructions. PP2A was detected using antibody 6G3. This monoclonal rat IgG recognizes the carboxy-terminal end of the A-subunit of PP2A.

Phosphatase Assay

The dephosphorylation reaction contained 50 mM of Tris–HCl, pH 7.4, 1 mM of EDTA, 2 ng of purified PP2A, and 1 μM of [^{32}P]MBP as substrate, in a final volume of 100 μL. All compounds tested were prepared fresh and dissolved in water to a concentration of 0–500 μM immediately prior to addition to the assays. Aliquots of 10 μL were added to reactions giving 0–50-μM concentrations. Reactions were run for 5 min at 37° and were terminated by the addition of 100 μL of 1 mM of KH_2PO_4 in 1 N H_2SO_4. Released $[^{32}PO_4]_i$ was quantitated by scintillation spectrometry after addition of 0.3 mL of 2% ammonium molybdate and extraction of the phosphomolybdate complex with 1 mL of toluene:isobutanol (1:1, v:v). One PP2A activity unit was defined as the liberation of 1 nmol of P_i/min.

TABLE 1. Modulation of heterotrimeric PP2A by isoquinolones in the presence of 20 μM C-2 ceramide



Compound	$R_6 = R_7$	R_2	PP2A activity (mU/mL)
1	H		6.8
2	CH_3	$(CH_2)_3COOH$	9.5
3	H		12.1
4	CH_3	$PO[N(CH_3)_2]$	40.4
5			34.7

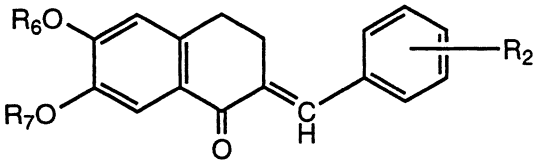
Values are expressed in mU/mL; ranges and classifications are as follows: <7 = basal inhibition; 7–15 = ceramide-induced inhibition; 15–25 = no net effect; and >25 = activation.

The kinetics of inhibition were determined with Michaelis–Menten and Lineweaver–Burk methods, using the nonlinear regression data analysis program Enzfitter.

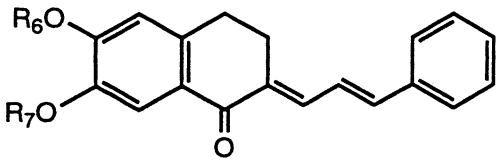
RESULTS AND DISCUSSION

The PP2A enzyme was isolated from human Jurkat cells, and the heterotrimeric and dimeric forms were separated successfully. Figure 1 shows the results of the purification process at the Mono-Q column step. The fractions with the highest PP2A activity were then analyzed by western blotting for the presence of enzyme, using the 6G3 antibody to the A subunit. Denaturing (Fig. 1B) and native (Fig. 1C) electrophoreses were used to determine the nature of the multimeric enzymatic complex. Fraction 12 showed the same pattern of migration as the commercially available purified heterotrimeric PP2A (Fig. 1C, lanes 1 and 2). Fraction 21 showed no high molecular weight band, representing probably the dimeric form of PP2A. Purified PP2A was further characterized by an inhibition assay using okadaic acid with and without C-2 ceramide present (Fig. 2). Okadaic acid inhibited the PP2A activity at an IC_{50} of 0.5 nM with or without C-2 ceramide (in agreement with reported values [21]). The purified PP2A heterotrimeric enzyme had kinetic parameters as follows using MBP as substrate: $K_m = 3.91 \pm 0.59$ μM; $V_{max} = 21.63 \pm 1.37$ μmol/min/mL. These values are the means ± one SD of four experiments. The activation of the isolated heterotri-

TABLE 2. Modulation of heterotrimeric PP2A by tetralones in the presence of 20 μM C-2 ceramide



Compound	$R_6 = R_7$	R_2	PP2A activity (mU/mL)
6	H	3,5-di-OH	1.2
7	CH_3	3,4-di-OCH_3-6-OH	4.3
8	H	2,6-di-OH	6.5
9	H	3,4,5-tri-OH	11.2
10	CH_3	2,4-di-OCH_3	12.4
11	CH_3	3-COOH, 4-OH	16.3
12	CH_3	2,3-di-OCH_3	19.2
13	CH_3	(cinnamyl)	21.0
14	CH_3	3,5-di-OCH_3 (indanone)	21.6
15	CH_3	4-OCH_3	27.7
16	CH_3	2,6-di-CH_3	30.9
17	CH_3	2,5-di-OCH_3	30.9
18	5,7-dimethyl	3,5-di-OCH_3	33.3
19	CH_3	2,5-di-CH_3	35.6
20	CH_3	3,5-di-OCH_3	38.7
21	CH_3	4-pyridine-*N*-oxide	44.5



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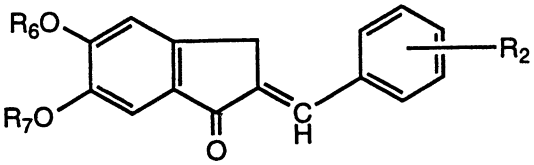

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Values are expressed in mU/mL; ranges and classifications are as follows: <7 = basal inhibition; 7–15 = ceramide-induced inhibition; 15–25 = no net effect; and >25 = activation.

meric form of PP2A (fraction 12) by 20 μM of C2-ceramide was consistent and ranged from a 2- to 4-fold increase of the specific activity. On the other hand, the intensity of activation by C2-ceramide of the freshly isolated heterodimeric PP2A fractions 21 and 23 never exceeded a 2-fold increase. In addition, we were never able to obtain ceramide activation after freezing and thawing aliquots of those fractions, even by utilizing numerous freezing conditions and buffers. For those reasons, we evaluated the compounds against the fractions that contained the heterotrimeric PP2A. Thus, compounds listed in Tables 1 and 2 were evaluated for their effects on purified heterotrimeric PP2A activity, each at a concentration of 20 μM in the presence of 20 μM of C-2 ceramide. Figure 3 summarizes

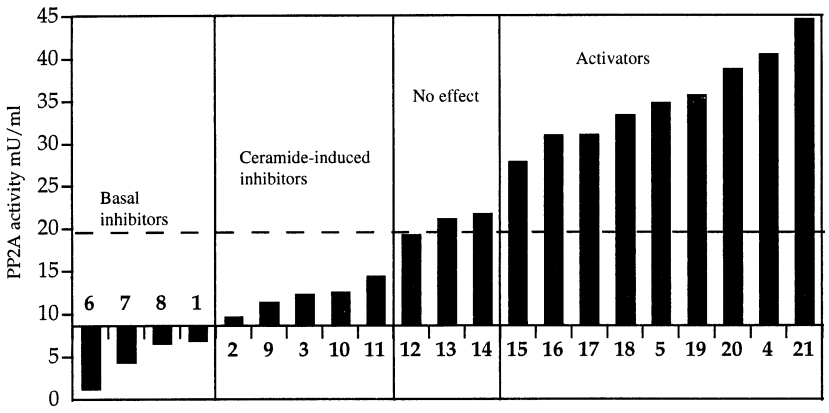

FIG. 3. Effects of compounds on PP2A enzymatic activity. The compounds were tested at 20 μM against partially purified trimeric PP2A from Jurkat cells (see Fig. 1). The enzymatic activity was measured in the presence of 20 μM of C2-ceramide. The x-axis is placed at 8.6 mU/mL, which is the PP2A basal activity in the absence of C2-ceramide. The dotted line (ceramide) is placed at 19.5 mU/mL and represents the PP2A activity in the presence of 20 μM of C2-ceramide. Data are representative of results from four experiments.

the results, with the compounds arranged in order from most inhibitory listed first, to most activating listed last. The first nine compounds listed were found to be inhibitors of ceramide-activated PP2A activity. Interestingly, the first four inhibited not only the ceramide-activated PP2A activity but also the basal PP2A activity as well. A distinct pattern of structure–activity is now apparent. The basal inhibitors, without exception, have one or more hydoxyl functions on either the bicyclic ring, the substituent ring, or both. If the hydroxyls are methylated, as in the case of the other twelve compounds, the compounds appear to enhance PP2A activity in the presence of C-2 ceramide, although compounds **12–14** were neutral—they neither enhanced nor inhibited enzyme activity under these conditions. Compound **6**, for example, was a potent inhibitor, whereas compound **20** was a potent activator of PP2A activity. Yet **6** was derived from **20** by a simple methyl ether cleavage reaction described above. It is possible, therefore, to modulate, both positively and negatively, the ceramide-activated PP2A activity within a series of compounds by simple functional group modification.

Compounds **6** and **21** were studied further as examples of potent inhibitors and activators of PP2A, respectively (Fig. 4). Compound **6** was shown to have a K_i of 14.32 ± 1.91 μM and is best described by a non-competitive model of inhibition. Interestingly, the inhibition was observed in the presence or absence of C-2 ceramide, suggesting that the inhibition by these polyhydroxy aromatics is independent of ceramide. To date, no simple polyhydroxy aromatic compounds have been reported to inhibit serine/threonine phosphatases. Other polyhydroxy aromatics that resemble structurally the type of compounds studied in this paper, such as the trihydroxyisoflavone genistein and the pentahydroxyflavone quercetin, are good inhibitors of PTK [23, 24] but are not inhibitors of serine/threonine protein phosphatases, namely PP2A. Also, the tyrphostins, most of which are simple polyhydroxylated aromatics, are selective PTK inhibitors [25]. In this regard, it is known that the catalytic subunit of PP2A can be phosphorylated by PTK, which leads, at least *in vitro*, to deactivation of PP2A [26]. In our studies, deactivation of PP2A by this mechanism can be ruled out because the same results were obtained with these compounds using the partially purified PP2A preparation or the highly purified sample (obtained from M. Mumby), in which the absence of PTK was confirmed (data not shown).

With regard to activation, compound **21** at 25 μM was shown to enhance PP2A activity by as much as 300% above the basal activity level. As with all of the activators, the presence of C-2 ceramide was required for the enhancement of activity. Alone, **21** had no effect on PP2A activity (Fig. 4B). Indeed, with the exception of the four basal inhibitors, none of the compounds studied had any effect on PP2A activity in the absence of ceramide. The mechanism by which the activators enhance PP2A activity is unknown, but may be due to the ability of the compounds

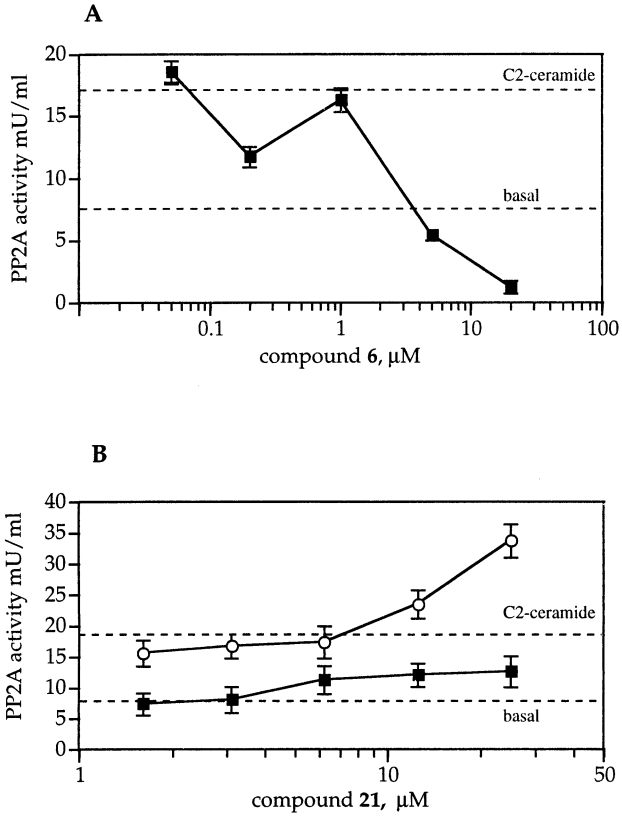

FIG. 4. (A) Inhibition of PP2A activity by compound 6. Various concentrations of the compound were tested on Jurkat partially purified trimeric PP2A in the presence of 20 μM of C2-ceramide. The PP2A activity in the absence of C2-ceramide (basal dotted line) was 7.7 ± 1.1 mU/mL, and the PP2A activity in the presence of C2-ceramide (C2-ceramide-labeled dotted line) was 17.0 ± 1.3 mU/mL. (B) Activation of PP2A activity by compound 21. Various concentrations of the compound were tested on Jurkat partially purified trimeric PP2A in the absence (■) and presence (○) of 20 μM of C2-ceramide. PP2A activity in the absence of C2-ceramide (basal dotted line) was 8.0 ± 0.9 mU/mL; PP2A activity in the presence of C2-ceramide (C2-ceramide dotted line) was 18.9 ± 1.1 mU/mL. Data are means ± SEM from five experiments. On the y-axis, 0% represents the normalized basal activity in the absence of C2-ceramide, which was 8.6 ± 0.7 mU/mL; 100% (dotted line) represents the normalized PP2A activity in the presence of C2-ceramide, which was 19.5 ± 1.0 mU/mL. The specific activities for compounds 6 and 21 were 3.4 ± 0.4 and 43.5 ± 1.5 mU/mL, respectively. Data are representative of results from four experiments.

to stabilize the interaction of ceramide with the multimeric PP2A enzyme.

Finally, the specificity of the compounds for modulation of PP2A versus PP1 activity was studied. The polyhydroxy compounds, **1**, **6**, **7** and **8**, as in the case of PP2A, were observed to inhibit basal PP1 activity, although the magnitude of inhibition was less than one-sixth that of PP2A (data not shown). There was no structure–activity pattern noted for inhibition or activation of PP1 for the other compounds, as only very slight effects were observed in the presence or absence of ceramide. As expected, ceramide alone activated PP1 by only about 10%.

In summary, low molecular weight aromatic compounds designed as ceramide antagonists have been prepared and evaluated as inhibitors of partially purified PP2A from Jurkat cells in the presence and absence of ceramide. A few of the compounds, particularly those containing hydroxyl groups, were found to inhibit both basal and ceramide-mediated enzyme activity. Several others were shown to inhibit only the ceramide-activated enzyme activity. Still others were observed to enhance PP2A activity, but only in the presence of ceramide. Simple changes in the functional groups present on the small molecules, such as methyl ether cleavage to OH groups, resulted in the conversion of activators to inhibitors or vice versa. The modulatory activity of these compounds was shown to be considerably more specific for PP2A than for PP1.

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