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Targeting Ceramide Metabolism with a Potent and Specific Ceramide Kinase Inhibitor

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ABSTRACT
Ceramide kinase (CerK) produces the bioactive lipid ceramide-1-phosphate (C1P) and appears as a key enzyme for controlling ceramide levels. In this study, we discovered and characterized adamantane-1-carboxylic acid (2-benzoylamino-benzothiazol-6-yl)amide (NVP-231), a potent, specific, and reversible CerK inhibitor that competitively inhibits binding of ceramide to CerK. NVP-231 is active in the low nanomolar range on purified as well as cellular CerK and abrogates phosphorylation of ceramide, resulting in decreased endogenous C1P levels. When combined with another ceramide metabolizing inhibitor, such as tamoxifen, NVP-231 synergistically increased ceramide levels and reduced cell growth. Therefore, NVP-231 represents a novel and promising compound for controlling ceramide metabolism that may provide insight into CerK physiological function.

Sphingolipids and their metabolizing enzymes have received increasing attention because of their participation in cellular signaling. One such enzyme, ceramide kinase (CerK), which belongs to the diacylglycerol kinase superfamily, uses ceramide to produce ceramide-1-phosphate (C1P) (Sugiura et al., 2002). Because C1P is short-lived (Boath et al., 2008) CerK activity may well serve as a means of disposing of excess ceramide. Indeed, studies in CerK-deficient [Cerk−/−] mice have demonstrated a key role for CerK in the control of C1P, ceramide, and dehydroceramide levels (Graf et al., 2008a,b). Other studies have suggested that raising ceramide levels may help in treating proliferative disorders, including cancer, by overcoming chemotherapeutic resistance (Ogretmem and Hannun, 2004). Thus, inhibition of CerK may have wide therapeutic potential. Herein we describe the discovery by high-throughput screening and the characterization of NVP-231, a diamino-benzothiazole derivative that competes with ceramide and acts as a potent and specific inhibitor of CerK.

Materials and Methods

High-Throughput Screen for CerK Inhibitors. Recombinant full-length hCerK was obtained from baculovirus-infected Sf9 cells as a glutathione transferase fusion using methods described previously and was stored frozen at −80°C (Leder et al., 2007). Both liquid chromatography/mass spectrometry analysis (LC/MS) and Coomassie-stained SDS gels showed the expected molecular weight and a purity of approximately 95%. Enzyme activity was determined as described previously (Bajjalieh and Batchelor, 2000). On each day of screening, a new aliquot was thawed and diluted to a concentration of 16 ng/μL in reaction buffer (25 mM MOPS, pH adjusted to 7.2 with NaOH, 6.25 mM CaCl₂, 62.5 mM KCl, 2.5 mM EGTA, and 1.25 mM dithiothreitol). Ceramide-containing micelles, prepared as described by Bajjalieh and Batchelor (2000), and ATP were diluted in reaction buffer to concentrations of 10% (v/v) (1 mM ceramide) and 10μM, respectively. A detection mixture containing both luciferase...
and luciferin (Kinase-Glo; Promega, Madison, WI) was reconstituted as recommended by the manufacturer and stored in the dark until use. For screening, 100 nl of a 0.5 mM intermediate dilution of compounds in 90% (v/v) DMSO was transferred into white polystyrene flat-bottomed 1536-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) by a Cartesian Hummingbird reformatting module (Genomic Solutions Ltd., Huntingdon, Cambridgeshire, UK). Wells for high- and low-activity controls received 90% (v/v) DMSO. Compound-containing assay plates were transferred to a MARK III screening system (Evotec Technologies, Hamburg, Germany) equipped with a Cartesian SynQUAD ProSys dispenser (Genomic Solutions Ltd.) and an LIL Analyst GT reader (Molecular Devices, Sunnyvale, CA). To each well, 1.6 µl of CerK at 16 ng/µl in reaction buffer (stored at 4°C) or reaction buffer only for low-activity controls (no inhibition) to 100% (full inhibition) and to calculate Z' values

Data were plotted as percentage of activity in the presence of vehicle (DMSO) and fitted with Xlfit (idbs Ltd., Guildford, Surrey, UK); IC50 and 1.6 µl of substrate mixture (stored at room temperature) was added with the use of the SynQuad dispensing module. After another 10-min incubation within the Analyst GT reader, luminescence was measured without attenuator at a height of 1 mm with a maximal integration time of 0.2 s/well and a target coefficient of variance/well of 1%. Data from control wells without compounds but with or without CerK (high controls or low controls, respectively) were used to convert luminescence photon counts into relative inhibition values ranging from 0% (no inhibition) to 100% (full inhibition) and to calculate Z' values.

**Synthesis of NVP-231 and Related Compounds.** See supplemental Data.

**Ceramide Kinase Assays and C1P Measurements.** In vitro CerK activity was assayed exactly as described in Rovina et al. (2006), using 180 µM C8 ceramide and 500 µM [32P]ATP as substrates and 40 ng/ml glutathione transferase-CerK. Compounds were dissolved to 10 mM in DMSO and stored frozen in aliquots. Cell-based CerK activity assays were performed as described previously (Boath et al., 2008) using a cell-permeant, fluoroescently labeled, short-chain ceramide (NBD-Cer) as substrate, followed by lipid extraction and one-dimensional TLC. The full lipid extracts were used for TLC analysis. C16-C1P measurements by LC/MS, as well as analysis of [32P]-labeled lipids by two-dimensional-TLC, were performed as described previously (Boath et al., 2008; Graf et al., 2008b).

**Cell Isolation, Culture, Assays, and [32P]Orthophosphate Labeling.** COS-1 cells and hCerK-overexpressing COS-1 cells (COS-CerK), as well as elicited mouse peritoneal macrophages, mouse bone marrow-derived macrophages (BMDM), and mast cells, were ob-

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**Fig. 1. NVP-231 in vitro characterization.** A, a concentration range of compound 1 (NVP-231) was tested in an in vitro CerK assay using [32P]ATP. Data were plotted as percentage of activity in the presence of vehicle (DMSO) and fitted with Xlfit (idbs Ltd., Guildford, Surrey, UK); IC50 = 12 ± 2 nM (mean from three experiments ± S.D.). B, inhibition mechanism by NVP-231. Left, a radioactive kinase assay was performed as in A using various ATP concentrations in the presence (○) or absence (●) of 1 µM NVP-231; the results obtained typify a noncompetitive inhibition toward ATP. Right, various ceramide concentrations were used in the presence (○) or absence (●) of 0.1 µM NVP-231; the results typify a competitive inhibition of ceramide-binding to CerK by NVP-231. C, in vitro testing of compounds structurally related to NVP-231. IC50 values were obtained after testing as performed in A.
tained and maintained as reported previously (Graf et al., 2007, 2008b; Boath et al., 2008). Incorporation of BrdU in BMDM was performed exactly as described in Graf et al. (2008b). Human blood neutrophils were collected over a Ficoll gradient followed up by erythrocyte lysis according to established procedures. Neutrophil apoptosis was measured by fluorescence-activated cell sorting monitoring phycoerythrin-labeled annexin V and 7-aminoactinomycin D (BD Pharmingen, San Diego, CA). Viability in COS cells was measured with the resazurin redox indicator (Alamar Blue; Serotec, Oxford, UK) as described by Tauzin et al. (2007). For $^{33}$P orthophosphate labeling, cells were rinsed in phosphate-depleted Earle’s minimal essential medium containing 10% dialyzed serum, then preincubated in the same medium with vehicle (DMSO) or in the presence of 100 nM compound 1 (NVP-231) or 100 nM compound 7 for 6 h. Finally, cells were incubated overnight in the same conditions but in the presence of 100 μCi/ml $^{33}$P orthophosphate. At the end of the incubation, lipids were extracted and analyzed using two-dimensional TLC. Phospholipids were visualized by autoradiography.

Results

NVP-231: A Competitive CerK Inhibitor. We developed a homogeneous CerK assay for high-throughput screening based on a luminescent reaction measuring ATP consumption (Koresawa and Okabe, 2004; Klumpp et al., 2006). After miniaturization of this assay to a 1536-well plate format, we screened over a million compounds for inhibition of CerK. Primary hits active in the micromolar range were confirmed or invalidated by dose-response experiments, which also provided a preliminary ranking. The overlap to hit lists from other kinase inhibitor screens using the same assay principle was very limited, indicating specificity of the hit compounds for CerK. Although the screened libraries contained many typical protein kinase inhibitors, only a few of them inhibited CerK. Further hit validation was conducted using a direct

Fig. 2. NVP-231 cell-based characterization. A, COS and COS-CerK cells were seeded into a 12-well plate with 0.8 × 10⁶ cells/well. A concentration range of compound 1 (NVP-231) was tested in a 2-h cell-based CerK assay with the use of 5 μM NBD-Cer. After lipid extraction, samples were run on a TLC followed by imaging (left) and densitometry measurements (right). B, NVP-231 (50 nM) was tested on mouse peritoneal macrophages seeded to 2 × 10⁶ cells/well in a 24-well plate and incubated for 2 h in the presence of 5 μM NBD-Cer; comparison with Cerk knockout cells. C, left, two-dimensional TLC analysis of lipid extracts from Cerk−/− BMDMs labeled with $^{33}$P orthophosphate. Cells were seeded to 2 × 10⁶ cells/ml in a six-well plate. Labeling was performed as described under Materials and Methods in the presence of 100 nM compound 1 (NVP-231) or 7. After lipid extraction, the extract was spiked with a mixture of labeled internal standards: C2-C1P, C8-C1P, C16-C1P. Right, wild-type BMDM incubated in the presence of vehicle (DMSO) or 100 nM compound 1 (NVP-231) and labeled with $^{33}$P orthophosphate. Lipid extracted and run on a two-dimensional TLC. The C16-C1P-R area is pointed with an arrow; the shorter arrow points to an endogenous longer-chained C1P (C18 or C24); bottom, same experiment on COS-CerK cells in the presence of vehicle (DMSO or 100 nM compound 1 (NVP-231) or compound 7; the C1P-R area is shown. D, LC/MS analysis showing the levels of C16-C1P measured in COS and COS-CerK cells treated with 100 nM compound 1 (NVP-231, ○) or compound 7 (●). Mean ± S.D. of quadruplicate determinations obtained from two separate experiments (statistical significance versus t = 0 measured with t tests; **, p < 0.01; ***, p < 0.001). The transient drop in C16-C1P levels observed at 1 h was also observed with vehicle alone and therefore may result from a cellular response to medium change.
enzymatic assay measuring the incorporation of radioactive phosphate into C1P.

One of the compounds identified as the benzothiazole derivative 1 (or NVP-231) showed an IC_{50} value of 12 ± 2 nM and 90% inhibition at 100 nM in the radioassay (Fig. 1A). NVP-231 did not compete with ATP but rather with ceramide, displaying an inhibition constant (K_i) of 7.4 nM (Fig. 1B). Furthermore, inhibition by NVP-231 was instantaneous and fully reversible, implying that this compound does not covalently modify CerK (Supplemental Fig. 1).

To explore structure-activity relationships, we independently modified the two amide side chains of NVP-231 (Fig. 1C). Exchanging the large and rather bulky adamantoyl residue for a long fatty acid side-chain led to a 50-fold loss in potency (compound 2). In contrast, a moderate extension of the adamantoyl moiety by an additional phenyl ring preserved the activity (compound 3). Modifications on the other side of the molecule had a more dramatic effect. The free amine (compound 4) and the bulky adamantoyl amide (compound 5) were devoid of measurable effect. We were surprised to find that the exchange of the benzamide for an acetamide function still led to moderate inhibition (compound 6). Changing the electronic properties of the aromatic ring of the benzamide by adding two methoxy substituents (compound 7) reduced potency by 170-fold. Taken together, these structure-activity relationships demonstrate the strong influence of relatively small modifications on the biological effect of the diamino-benzothiazole derivatives and indicate an excellent potential for further medicinal chemistry optimization.

Potency and Efficacy of NVP-231 in Cell Culture. We tested the cellular effects of NVP-231 in an assay based on human CerK-overexpressing COS-cells (COS-CerK) and a fluorescent ceramide derivative (NBD-Cer) (Boath et al., 2008; Graf et al., 2008b). We measured the effect of a wide concentration range of NVP-231 on all three ceramide metabolites. At 10 nM, NVP-231 inhibited C1P formation by >50%; at 100 nM, NVP-231 achieved complete inhibition (Fig. 2A). Thus the potency and efficacy of NVP-231 observed in cell culture are consistent with those found in vitro. It is noteworthy that, NVP-231 did not inhibit GlcCer and SM formation; rather, it increased these metabolites in correlation with compound concentration, demonstrating that NVP-231 does not act as a general inhibitor of ceramide metabolism (see also Table 1). Moreover, the concomitant increase in GlcCer and SM and decrease in C1P indicates that the three enzymes can compete for access to ceramide; when NVP-231 is diluted out, these metabolites return to pretreatment levels (Supplemental Fig. 2).

We also found that NVP-231 is a potent inhibitor of mouse CerK. When mouse peritoneal macrophages and mouse bone marrow-derived mast cells were treated with NVP-231, C1P formation dropped to background levels similar to those found in CerK (−/−) cells (Fig. 2B and Supplemental Fig. 3) (Boath et al., 2008; Graf et al., 2008b).

Effect of NVP-231 on C1P Levels. Endogenous levels of C1P are controlled by CerK. Therefore, we labeled mouse BMDMs with [33P]orthophosphate and examined phosphorylated endogenous ceramides in lipid extracts after NVP-231 treatment. NVP-231 prevented the phosphorylation of endogenous ceramides, whereas the structurally related but inactive compound 7 had no effect (Fig. 2C); similar observations were made in COS-CerK cells (Fig. 2C, bottom). Next, we monitored the levels of C16-C1P, a major naturally occurring species of C1P, with the use of LC/MS. In COS-CerK cells, levels of C16-C1P are >15-fold up-regulated compared with COS cells. At 100 nM, NVP-231 rapidly and efficiently reduced C16-C1P to <20% of its control level (Fig. 2D), but no significant reduction was observed with compound 7. Neither increasing NVP-231 concentration to 1 μM nor prolonging incubation up to 24 h resulted in more than a 90% reduction of C1P levels (data not shown). This finding may indicate other enzyme activity producing C1P, as already suggested by residual C1P levels in CerK (−/−) mice (Graf et al., 2008b).

Testing the Specificity of NVP-231. Although sphingosine kinases are close homologs of CerK (Spiegel and Milstien, 2007), NVP-231 did not inhibit these enzymes (Table 1). Diacylglycerol, however, shares structural homology with ceramide. Thus, when we tested NVP-231 against diacylglycerol kinase α, we found that it was 500-fold less potent.

### Table 1: Specificity of NVP-231

Sphingosine kinase inhibition was assayed using purified recombinant enzymes according to the method described by Billich and Ettmayer (2004). Diacylglycerol kinase activity was assayed as described (Kanoh et al., 1983) in COS cells lysates after transient expression of recombinant human diacylglycerol kinase α. Phosphoinositide kinase assays were performed using purified recombinant enzymes. Glucosylceramide synthase and sphingomyelin synthase 1 were assayed upon recombinant expression in COS-1 cells using NBD-Cer as a substrate. Ceramide transfer protein activity was assayed using fluorescence microscopy as readout to follow transport of a fluorescent ceramide derivative (NBD-Cer) from the endoplasmic reticulum to the Golgi complex (Kumagai et al., 2005).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC_{50} μM</th>
<th>Specificity Window</th>
<th>Assay Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCerK</td>
<td>0.01</td>
<td>1</td>
<td>In vitro: purified enzyme</td>
</tr>
<tr>
<td>hSphK1</td>
<td>0.01</td>
<td></td>
<td>In vitro: cell lysate</td>
</tr>
<tr>
<td>hSphK2</td>
<td>0.01</td>
<td></td>
<td>Cell-based/lipid extraction</td>
</tr>
<tr>
<td>hDAGKα</td>
<td>&gt;100</td>
<td>&gt;10,000</td>
<td>In vitro: purified enzyme</td>
</tr>
<tr>
<td>hPI3Kα</td>
<td>&gt;25</td>
<td>&gt;2500</td>
<td>In vitro: purified enzyme</td>
</tr>
<tr>
<td>hPI4Kβ</td>
<td>&gt;25</td>
<td>&gt;2500</td>
<td>In vitro: purified enzyme</td>
</tr>
<tr>
<td>hVps34</td>
<td>&gt;25</td>
<td>&gt;2500</td>
<td>In vitro: purified enzyme</td>
</tr>
<tr>
<td>hGCS</td>
<td>&gt;10</td>
<td>&gt;1000</td>
<td>Cell-based/lipid extraction</td>
</tr>
<tr>
<td>hSMS-1</td>
<td>&gt;10</td>
<td>&gt;1000</td>
<td>Cell-based/lipid extraction</td>
</tr>
<tr>
<td>CERT</td>
<td>&gt;10</td>
<td>&gt;1000</td>
<td>Live cell microscopy</td>
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hSphK1, human sphingosine kinase; hDAGKα, human diacylglycerol kinase α; hPI3Kα, human phosphoinositide-3 kinase; hPI4Kβ, human phosphatidylinositol 4-kinase; hGCS, human glucosylceramide synthase; hSMS-1, human sphingomyelin synthase 1; CERT, ceramide transfer protein; DMB-Cer, N-(5-(5,7-dimethyl BODIPY)-1-pentanoyl)-a-erythro-sphingosine.
compared with CerK (Table 1). NVP-231 also did not inhibit other lipid kinases, including phosphatidylinositol-3 kinase α, phosphatidylinositol-4 kinase β, and Vps34. Testing GlcCer synthase and SM synthase-1 after overexpression in COS-1 cells confirmed the lack of inhibitory effect of NVP-231 on these activities. Finally, we tested the recently described ceramide transfer protein, which is required for ceramide transport to the Golgi complex and for SM synthesis (Hanada et al., 2003) and found no inhibition by NVP-231 (Table 1).

**NVP-231 As a Tool Compound to Elucidate CerK Biology.** CerK biology is still poorly understood. The discovery that C1P could activate the cytosolic phospholipase A2 α isoform (PLA2G4A) in A549 human lung adenocarcinoma cells (Pettus et al., 2003, 2004) stimulated a search for CerK inhibitors that might have anti-inflammatory properties (Kim et al., 2005; Munagala et al., 2007). However, despite significant progress in characterizing in vitro the interaction of C1P and PLA2G4A (Stahelin et al., 2007), evidence that a C1P-PLA2G4A interaction plays a role in the cell or in vivo is lacking. Most striking was our observation that cells from CerK-deficient animals can readily signal to PLA2G4A as wild-type cells do (Graf et al., 2008b). In line with these findings, when we tested NVP-231 on PLA2G4A-dependent processes in either neutrophils, macrophages, or fibroblasts, measuring arachidonic or prostaglandin E2 release after stimulation with phorbol 12-myristate 13-acetate/ionomycin, interleukin-1β, zymosan, or lipopolysaccharide, we did not detect any inhibitory effect (data not shown).

The participation of CerK and C1P in the control of cell growth and survival has been reported by different groups. The emerging consensus is that CerK and C1P have proliferative/prosurvival effects (for review, see Gómez-Muñoz, 2006). However, given the phenotype observed in CerK-deficient mice (Graf et al., 2008b), it is clear that either CerK is dispensable or that efficient compensatory mechanisms may work. In addition, our understanding is limited for the following reasons. The first relates to the use of high C1P concentrations, applied exogenously to cells (e.g., Gangoiti et al., 2008). Furthermore, the transmembrane signaling of...
C1P is unknown and may not necessarily recapitulate CerK-dependent C1P signaling. The use of C1P in conjunction with a dodecane-based solvent mixture that is not inert has somewhat complicated the picture in previous years (Tauerz et al., 2007). Second, the number of cell systems used to characterize CerK/C1P biology is still very limited. Third, in models relying on ectopic expression of CerK, exogenously added short-chain Cers are potent inducers of apoptosis (Graf et al., 2007), which was rather unexpected given the known effects of short-chain C1P on fibroblast cell growth (Gomez-Munoz et al., 1995). Perhaps the most convincing study was reported by Mitra et al. (2007), whose conclusions were supported by both exogenously applied C1P and inhibition of CerK by RNA interference. However, in our hands, silencing CerK in A549 cells did not significantly affect cell viability, thereby contrasting with the findings from the Mitra et al. (2007) study. All together, the conditions under which and the extent to which cell proliferation and survival may depend on CerK, and what may be the compensatory mechanism(s), is currently unknown.

To characterize the effect of NVP-231 on cell growth and survival, we selected three cellular systems that we identified to have significant CerK activity. We first applied NVP-231 to freshly isolated human blood neutrophils and found no acceleration in their spontaneous apoptosis rate despite significantly reduced NBD-C1P (Fig. 3A). We then asked whether mouse BMDM survival in the absence or presence of M-CSF would be sensitive to CerK inhibition by NVP-231 and found no significant effect on NBD-Cer metabolism, either alone (Fig. 4A, c), or in combination with tamoxifen (d), consistent with the very low endogenous CerK activity levels in these cells. In COS-CerK cells, steady-state levels of NBD-Cer, NBD-GlcCer, and NBD-SM were significantly lower than in normal COS-cells (compare a and a'). This results from increased ceramide metabolism, competition with formation of NBD-C1P (as seen above), and a high C1P turnover rate (Boath et al., 2008). In COS-CerK cells, tamoxifen inhibited NBD-GlcCer accumulation by 50% (Fig. 4A, compare a and b). At the same time, we found a 25% increase in NBD-Cer and a 70% increase in NBD-SM synthesis. NVP-231 had no significant effect on NBD-Cer metabolism, either alone (Fig. 4A, c), or in combination with tamoxifen (d), consistent with the very low endogenous CerK activity levels in these cells. In COS-CerK cells, steady-state levels of NBD-Cer, NBD-GlcCer, and NBD-SM were significantly lower than in normal COS-cells (compare a and a'). Conversely, NVP-231 abolished NBD-C1P formation, increased NBD-GlcCer and NBD-SM formation by

![Image](image_url)

**Fig. 4.** Synergistic effects of NVP-231 and tamoxifen on ceramide levels and growth inhibition. A, COS and COS-CerK cells were seeded to 2 x 10^4 cells/well in a 24-well plate. Pretreatment with vehicle (a, a'), 5 μM tamoxifen (b, b'), 100 nM NVP-231 (c, c'), or both compounds (d, d') for 1 h followed by 2 h in the presence of 5 μM NBD-ceramide before lipid extraction and analysis on TLC (left). Densitometry (right) is expressed as a percentage of ceramide levels in lane a (i.e., COS cells, vehicle treated). The diameter of the circle is proportional of the total amount of ceramide and metabolites measured. B, treatment with compounds (NBD-Cer was not used in this experiment) was performed as in A but for 24 h, and a viability assay was used as final readout. Mean ± S.D. from three experiments run in quadruplicates (*, p < 0.05; ***, p < 0.001).
70% and 80% respectively, but also increased NBD-Cer levels by 90% (c'). In the presence of the two inhibitors, NBD-C1P was not detectable, NBD-GlCer was marginally reduced; remarkably, NBD-SM and NBD-Cer levels reached 300 and 250% of control levels, respectively (d'). It is noteworthy that the absolute level of every metabolite was comparable in both cell types after treatment with the two inhibitors (compare d and d').

Because an increase in ceramide leads to growth inhibition, we evaluated the influence of treatment with NVP-231 and tamoxifen on cell growth in a second set of experiments (Fig. 4B). Growth-inhibition induced by tamoxifen alone was more pronounced in COS cells (compare a' and b') compared with COS-CerK cells (compare a and b). NVP-231 had a marginal effect alone on either cell population (c and c'). When tamoxifen and NVP-231 were combined, no further growth inhibition was observed in COS cells compared with tamoxifen alone (d). However, in COS-CerK cells, the two compounds synergized to reduce growth (d'), yielding viability scores similar to those observed in COS cells treated with tamoxifen alone (compare d' and b).

Discussion

In this work, we have discovered and characterized the first potent inhibitor of CerK and one of the most potent lipid kinase inhibitors to date. NVP-231 features a number of remarkable properties: 1) it competes with ceramide binding to CerK by a mechanism that does not rely on ceramide mimicry (Fig. 1, 2) it is as potent and efficacious in cell based assays as it is in vitro (Figs. 1 and 2), 3) it is highly specific for CerK (Fig. 2 and Table 1), and 4) its action is fully reversible (Supplemental Figs. 1 and 2). Based on these characteristics, we anticipate that the discovery of NVP-231 has provided a powerful tool to elucidate CerK biology and probe whether its regulation may have therapeutic potential.

Our experiments based on a mechanistic cellular model (Fig. 4) suggest that CerK inhibition can be detrimental to cell growth provided a concomitant stress signal is applied. We found that inhibition of glucosyl ceramide synthase by tamoxifen allows for synergy with NVP-231 to increase NBD-Cer levels and reduce cell growth. Further work, including in particular the profiling of endogenous Cer and C1P species, will be required to thoroughly test this hypothesis. In the studies performed by others previously, the critical stress may have been the removal of M-CSF in macrophages derived from CD-1 mice—these animals cannot synthesize this growth factor (Gomez-Munoz et al., 2004)—or the use of a low serum-containing culture medium (Pettus et al., 2003; Mitra et al., 2007; Tsuchi et al., 2008); both conditions are known to increase Cer levels. All together, this illustrates the key role played by CerK in disposing of Cer, thereby in keeping with the higher Cer levels measured in CerK-deficient mice (Graf et al., 2008a,b). The proposed role for CerK in controlling cell growth and survival via regulation of Cer levels is summarized in Fig. 5. Our finding that a combination of ceramide metabolism inhibitors can synergistically increase ceramide levels and reduce cell growth may provide a valuable rationale for the treatment of proliferative disorders.

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References


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