Off-Target Function of the Sonic Hedgehog Inhibitor Cyclopamine in Mediating Apoptosis via Nitric Oxide–Dependent Neutral Sphingomyelinase 2/Ceramide Induction


Abstract

Sonic hedgehog (SHh) signaling is important in the pathogenesis of various human cancers, such as medulloblastomas, and it has been identified as a valid target for anticancer therapeutics. The SHh inhibitor cyclopamine induces apoptosis. The bioactive sphingolipid ceramide mediates cell death in response to various chemotherapeutic agents; however, ceramide’s roles/mechanisms in cyclopamine-induced apoptosis are unknown. Here, we report that cyclopamine mediates ceramide generation selectively via induction of neutral sphingomyelin phosphodiesterase 3, SMPD3 (nSMase2) in Daoy human medulloblastoma cells. Importantly, short interfering RNA-mediated knockdown of nSMase2 prevented cyclopamine-induced ceramide generation and protected Daoy cells from drug-induced apoptosis. Accordingly, ectopic wild-type N-SMase2 caused cell death, compared with controls, which express the catalytically inactive N-SMase2 mutant. Interestingly, knockdown of smoothened (Smo), a target protein for cyclopamine, or Gli1, a downstream signaling transcription factor of Smo, did not affect nSMase2. Mechanistically, our data showed that cyclopamine induced nSMase2 and cell death selectively via increased nitric oxide (NO) generation by neuronal-nitric oxide synthase (n-NOS) induction, in Daoy medulloblastoma, and multiple other human cancer cell lines. Knockdown of n-NOS prevented nSMase2 induction and cell death in response to cyclopamine. Accordingly, N-SMase2 activity-deficient skin fibroblasts isolated from homozygous fro/fro (fragilis ossium) mice exhibited resistance to NO-induced cell death. Thus, our data suggest a novel off-target function of cyclopamine in inducing apoptosis, at least in part, by n-NOS/NO-dependent induction of N-SMase2/ceramide axis, independent of Smo/Gli inhibition.

Introduction

The Sonic hedgehog (SHh) signaling pathway plays a critical role in normal cerebellar development and has been implicated in the pathogenesis of medulloblastoma and other cancers of the brain, prostate, lung, breast, and colon (1–3). SHh is regulated by the transmembrane receptor patched (Ptch), which when altered or mutated, results in SHh pathway activation and cell growth dysregulation (4–6). The SHh ligand binds Ptch, which then alleviates Ptch-mediated suppression of Smo, which activates Gli, a family of transcription factors involved in the regulation of numerous genes controlling cell division, growth, and/or apoptosis, leading to proliferation and/or inhibition of cell death (7, 8). Thus, SHh is a novel therapeutic target for the treatment of cancers, including brain tumors (9, 10). Cyclopamine (Fig. 1A, top) is a Smo antagonist, which inhibits growth, and induces apoptosis in various cancer cells, including medulloblastoma (11–15). However, off-target functions of cyclopamine in inducing apoptosis, independent of Smo/SHh inhibition, have not been clearly defined previously.

The bioactive ceramide is a precursor for the synthesis of more complex sphingolipids via multiple pathways (16–18). Stress-induced ceramide generation in response to various stimuli, such as anticancer agents, mediates cell-cycle arrest, growth inhibition, and/or apoptosis (19). Ceramide is generated mainly via de novo synthesis by...
ceramide synthases 1–6 (CerS1–6; ref. 20) or via hydrolysis of sphingomyelin (SM) by SMases with pH optima in acidic, neutral, or alkaline conditions (21). Activation of neutral (N)-SMases1–2 in response to chemotherapy has been reported to generate ceramide, thereby inducing cell death (22–24). However, whether cyclopamine induces ceramide generation has not been described previously. Therefore, our focus was to define roles and mechanisms of cyclopamine-induced apoptosis and determine whether this occurs, at least in part, via induction of ceramide generation (N-SMase2/Ceramide-dependent apoptosis).

Cyclopamine Induces N-SMase2/Ceramide-Dependent Apoptosis

Materials and Methods

Cell culture

The Daoy medulloblastoma line (American Type Culture Collection) was grown in minimum essential medium with 10% FBS and 1% penicillin/streptomycin. WT, +/fro, and activity-deficient fro/fro skin fibroblasts isolated from new born mice (25) were cultured in Dulbecco’s Modified Eagle’s Medium with 10% FBS and 1% penicillin/streptomycin. UM-SCC-1 and UM-SCC-14A cells were obtained from Dr. Thomas Carey (University of Michigan). Cell lines used in this study were not authenticated. Cells were treated at final concentrations of 5 to 20 µg/mL from cyclopamine stock solution (20 mg/mL; LC Laboratories) dissolved in 100% ethanol. Cyclopamine aliquots were dissolved at 55°C.

Measurement of ceramide

Endogenous ceramides were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS) as described (26).

Short interfering RNA and plasmids

Short interfering RNAs (siRNA) for nSMase1 and nSMase2 were obtained from Ambion (Applied Biosystems). Gli1 and SMO siRNAs were custom designed by
Methods. Assays can be found in Supplementary Materials and nSMase2, catalase expression, and activity (qPCR), Western blotting, target sequences of nSMase1 significant (28).

Statistical analysis

Data are presented as mean ± SEM, unless otherwise indicated. Data represent at least 2 independent trials carried out as duplicates. Error bars on graphs represent SDs. An unpaired Student t test was carried out with Prism/GraphPad software; P < 0.05 was considered significant (28).

Details of chemicals, RNA isolation, quantitative PCR (qPCR), Western blotting, target sequences of nSMase1 and nSMase2 siRNAs, catalase expression, and activity assays can be found in Supplementary Materials and Methods.

Results

Cyclopamine induces cell death and increases ceramide generation/accumulation

Cyclopamine (Fig. 1A, top) has shown some efficacy against desmoplastic medulloblastomas in preclinical and clinical studies (29–31). To confirm cyclopamine induces cell death, we treated Daoy human desmoplastic cerebellar medulloblastoma cells with increasing concentrations of cyclopamine (0–50 µg/mL) and examined its effects on cell growth and cell death; measuring survival, caspase-3 activity, and loss of mitochondrial membrane potential. Cyclopamine inhibited growth in a dose-dependent manner (IC50 ~5 µg/mL, 48 hours, and ~10 µg/mL, 24 hours) compared with vehicle-treated controls (Fig. 1A, bottom). Accordingly, cyclopamine increased caspase-3 activity around 2-fold, which was consistent with a loss of mitochondrial membrane potential, as measured by increased accumulation of cytoplasmic JC-1 (~8-fold), compared with controls (Fig. 1B and C, respectively). Pretreatment with z-VAD (10 µg/mL) almost completely prevented caspase-3 activation and loss of mitochondrial membrane potential in response to cyclopamine (Fig. 1B and C, respectively). Thus, these data are consistent with previous studies, showing that cyclopamine induces caspase-3–dependent mitochondrial apoptosis.

Next, we investigated whether cyclopamine-induced apoptosis is mediated by induction of ceramide via Smo-signaling inhibition, or via off-target functions of cyclopamine, independent of SHh/Smo inhibition. Cyclopamine (5 or 10 µg/mL, 24 hours) increased total ceramide approximately 2.5- or 3-fold, respectively, increasing total ceramide from 20 (in controls) to 50 to 60 pmol/nmol Pi cyclopamine-treated cells, respectively (Fig. 1D). There were no significant changes in sphingosine or S1P in cyclopamine-treated cells, respectively (Fig. 1A, bottom). Accordingly, cyclopamine (10 µg/mL, 24 hours) induced C14-, C16-, C18-, C20-, and C22-ceramide generation approximately 2.5-, 3.5-, 15-, 6-, or 6.5-fold, respectively, compared with vehicle-treated controls (Supplementary Fig. S1A). Comparable increases were also observed with dihydro-C14-, C22-ceramides (Supplementary Fig. S1B). Similar data were also obtained when cells were treated with 5 µg/mL cyclopamine (24 hours), which increased endogenous C14-, C22-ceramide generation compared with controls (Supplementary Fig. S1A). Thus, these data suggest that cyclopamine induces endogenous ceramide generation, consistent with its proapoptotic effects in Daoy cells.

Cyclopamine-mediated ceramide generation is dependent on nSMase2 induction

To assess whether de novo generation of ceramide plays a role in cyclopamine-induced apoptosis, we pretreated cells with fumonisin B1 (FB1) and myriocin (MYR) at 50 µmol/L and 50 nmol/L, respectively, and examined cyclopamine-induced caspase-3 activation in Daoy cells (10 µg/mL, 24 hours). FB1 or MYR did not...
prevent cyclopamine-induced caspase-3 activation (Supplementary Fig. S2A), indicating de novo generation of ceramide might not be involved in cyclopamine-induced apoptosis. We also examined the effects of siRNA-mediated knockdown of CerS1 on growth inhibition in response to cyclopamine. CerS1 is known to mainly generate \( \text{C}_{18} \)-ceramide, and cyclopamine induced \( \text{C}_{18} \)-ceramide generation approximately 15-fold compared with controls in Daoy cells (see Supplementary Fig. S1A). Downregulation of CerS1 with siRNAs did not protect cells from cyclopamine-induced growth inhibition compared with controls (Supplementary Fig. S2B). Effectiveness of siRNAs for knockdown of CerS1 in Daoy cells was confirmed with qPCR. An approximately 40% to 60% decrease in CerS1 was observed compared with controls in the absence/presence of cyclopamine (Supplementary Fig. S2C). Similarly, ectopic expression of wt CerS1, or its catalytically inactive form with the H122A mutation (32), did not enhance or prevent the growth inhibitory effects of cyclopamine (Supplementary Fig. S2D). Expression of wt and mutant CerS1-FLAG proteins was confirmed by Western blotting using anti-FLAG antibody compared with vector-transfected controls (Supplementary Fig. S1E, lanes 2–3 and 1, respectively). Actin was used as a loading control (Supplementary Fig. S2E). Thus, these data suggest that cyclopamine-induced cell death is independent of CerS1 activation in these cells.

Because activation of N-SMase2 is known to mediate apoptosis (33, 34), we determined whether cyclopamine affects nSMase2 with qPCR. Cyclopamine increased nSMase2 approximately 3- and 6-fold (12–24 hours...
respectively), whereas treatment at 6 hours had no effect (Fig. 2A). Cyclopamine did not induce caspase-3 activity at 6 hours (Supplementary Fig. S3A), treatment at 12 hours slightly, but significantly, increased caspase-3 activity (~30%, P < 0.05). Knockdown of nSMase2, but not nSMase1 (Supplementary Fig. S3B), (~75%) compared with SCR controls, as determined by qPCR, Fig. 2B), prevented cyclopamine-induced caspase-3 activation (Fig. 2C). These data were also consistent with studies in which siRNA-mediated knockdown of nSMase2 abrogated cyclopamine-induced cell death, as measured by increased Annexin V staining or depletion of cellular ATP (Fig. 2D and E, respectively). Importantly, increased nSMase2 was also consistent with increased enzyme activity of N-SMase2 (~1.7-fold) in response to cyclopamine (10 μg/mL, 12 hours), which was prevented by knockdown of nSMase2 using siRNAs (Fig. 2F). Cyclopamine had no effect on A-SMase activity in these cells (data not shown). Increase in nSMase2 by cyclopamine (10 μg/mL) was also observed in UM-SCC-14A or UM-SCC-1 human HNSCC cells, in which it was increased approximately 4- and 8-fold, or 1.5- and 5-fold at 12 and 24 hours, respectively (Supplementary Fig. S3C and S3D, respectively).
Overall, these data suggest that cyclopamine induces nSMase2 in multiple human cancer cell lines, and knockdown of nSMase2 prevents cell death in response to cyclopamine, indicating an important role for N-SMase2 in this process.

**Downregulation of nSMase-2 prevents cyclopamine-induced ceramide generation and cell death**

To determine whether increased nSMase2 plays a role in cyclopamine-mediated ceramide generation (10 μg/mL, 24 hours), we measured endogenous ceramide and SM by LC/MS-MS in the absence/presence of siRNAs against nSMase1 or nSMase2 in Daoy cells. Data revealed that cyclopamine increased total ceramide approximately 2- to 3.5-fold in the presence of SCR or nSMase1 siRNAs, and knockdown of nSMase2 prevented cyclopamine-mediated ceramide generation (Fig. 3A). Consistent with these data, total SM was significantly decreased (~20%, $P < 0.05$) in response to cyclopamine in the absence/presence of SCR or nSMase1 siRNAs (Fig. 3B). Concentrations of ceramides and SM in response to cyclopamine in the absence/presence of nSMase1 or nSMase2 siRNAs were depicted in Supplementary Fig. S4A and S4B. As observed earlier, C18-ceramide was the main species generated by cyclopamine (~20-fold), and accordingly, a significant decrease in C18-SM was also observed (Supplementary Fig. S4C and S4D). Interestingly, although knockdown of nSMase2 prevented ceramide generation (Fig. 3A), it did not attenuate decrease in SM (Fig. 3B), suggesting the hydrolysis of SM without increased ceramide generation by an unknown mechanism, or inhibition of SM synthases. These data suggest that cyclopamine induces ceramide generation by induction of nSMase2.

We then examined whether downregulation of nSMase2, which prevented ceramide generation, also attenuated cyclopamine-mediated cell death (10 μg/mL, 24 hours). Data showed that siRNA-mediated knockdown of nSMase2 significantly protected growth inhibition of Daoy cells (~50%, $P < 0.05$) compared with SCR-siRNA–transfected controls in response to cyclopamine (Fig. 3C), consistent with the protective effects of downregulation of nSMase2 on cyclopamine-mediated apoptosis (Fig. 2C–E). Taken together, these data suggest that increased ceramide generation by elevation of nSMase2 mRNA and activity is involved, at least in part, in caspase-3 activation, and cell death in response to cyclopamine.

Accordingly, wt N-SMase2 expression significantly decreased cell growth, and increased apoptosis (~30%, $P < 0.05$) in Daoy cells compared with cells transfected with the catalytically inactive form of N-SMase2 (Fig. 3D), or vector-transfected controls (data not shown). Expression of wt and mutant N-SMase2-V5 (27) was confirmed by Western blotting using anti-V5 antibody, and activity of wt compared with catalytically inactive mutant N-SMase2-V5...
or vector-transfected controls were confirmed (Fig. 3E and 3F), supporting that the N-SMase2/ceramide axis plays an important role for inducing cell death in these cells.

Cyclopamine-induced nSMase-2 is independent of Smo inhibition

Because cyclopamine is an antagonist of Smo, it was important to determine whether cyclopamine-induced nSMase2 is dependent or independent of Smo inhibition. First, we examined the effects of downregulation of nSMase2 or nSMase1 using siRNAs on Smo and Gli mRNAs in the absence/presence of cyclopamine (10 μg/mL, 24 hours) by qPCR in Daoy cells. Cyclopamine reduced Smo and Gli by approximately 60%, but downregulation of nSMase2 or nSMase1 had no effect on inhibition of the Smo/Gli axis by cyclopamine (Fig. 4A and B, respectively). Thus, these data confirmed that inhibition of the Smo/Gli axis by cyclopamine is regulated independently of N-SMase2.

Then, we examined the effects of downregulation of Smo or Gli1 using siRNAs on nSMase2. We reasoned whether cyclopamine-mediated nSMase2 elevation is regulated downstream of Smo/Gli1 inhibition, knockdown of Smo or Gli1 should also increase nSMase2. Interestingly, data showed while siRNAs successfully reduced Smo or Gli1 by approximately 90% or 80%, they did not increase N-SMase2 (Fig. 4C and D). Similarly, pharmacologic inhibitors of Gli or Smo, GANT-61 or SANT-1 (5–10 μmol/L, 24–48 hours) had no effect on nSMase2 (data not shown), despite successfully reducing expression of Gli1, and its downstream target Bcl2 (Supplementary Fig. S5A and S5B). Taken together, these data indicate that induction of nSMase2 in response to cyclopamine is independent of the Smo/Gli axis of SHh signaling in Daoy cells.
Cyclopamine-induced nSMase-2 is regulated by oxidative stress

Because oxidative stress is known to induce N-SMase activity, and ceramide-mediated apoptosis (35–37), we then examined whether cyclopamine enhances nSMase2 via induction of reactive oxygen species (ROS)/nitrogen species (RNS). First, we determined whether pretreatment with NAC (0.5 mmol/L), an antioxidant, altered nSMase2 in the absence/presence of cyclopamine. Interestingly, while treatment with cyclopamine induced nSMase2 around 8-fold (10 μg/mL, 24 hours), pretreatment with NAC almost completely prevented cyclopamine-mediated nSMase2 in Daoy (Fig. 5A) and also in UM-SCC-14A cells (Fig. 5B), suggesting a role for cyclopamine-induced ROS/RNS in increased nSMase2, which was observed not only in Daoy cells, but was also observed in other human cancer cells, such as HNSCCs. Induction of ROS/RNS in response to cyclopamine (10 μg/mL) at various time points was also confirmed by staining the cells with dichlorofluorescin diacetate (DCFDA) and flow cytometry analysis. Data showed cyclopamine treatment induced ROS/RNS generation within 3 to 6 hours of treatment, increasing DCFDA fluorescence approximately 2- to 3-fold, respectively (Fig. 5C). Cyclopamine-mediated ROS/RNS generation was also detected by confocal microscopy, in which green fluorescence of DCFDA, a probe for ROS/RNS generation (38, 39), was visualized in Daoy cells compared with controls (Fig. 5D, bottom and top right, respectively). Interestingly, there was no significant colocalization of the green fluorescence of DCFDA with the red mitotracker in these cells in response to cyclopamine (Fig. 5D). Thus, these data suggest cyclopamine induces ROS/RNS generation, which is not selectively induced in mitochondria. Protective effects of NAC on cyclopamine-induced ROS/RNS generation were confirmed by DCFDA and flow cytometry. Pretreatment with NAC prevented the generation of ROS/RNS, shifting the DCFDA fluorescence signal to the left when compared with cyclopamine-treated UM-SCC-14A or UM-SCC-1 cells, respectively (Fig. 5E and Supplementary Fig. S6A).

These results were also consistent when wt versus activity-deficient skin fibroblasts isolated from newborn homozygous fro/fro (fragilitas ossium) mice (25) were treated with DETA. Treatment of wt cells with increasing concentrations of NO donor, significantly increased nSMase2 (~15-fold, P < 0.05; Fig. 6C). Thus, these novel data indicate that cyclopamine-induced nSMase2 is selectively regulated via NO, but not via ONOO(−), generation in Daoy cells. To define the mechanism by which NO generation induces nSMase2 and cell death in response to cyclopamine, we examined the roles of i-NOS, endothelial NOS (e-NOS), or n-NOS. Cyclopamine (10 μg/mL, 24 hours) increased (~2-fold, measured by qPCR) n-NOS (Supplementary Fig. S7B), but it had no significant effect on e-NOS or i-NOS (data not shown). Importantly, siRNA-mediated knockdown of n-NOS (~70%, measured by qPCR; Supplementary Fig. S7C) prevented nSMase2 induction and cell death (Fig. 6D and E, respectively). Similar data were also obtained in UM-SCC-22A cells, in which knockdown of n-NOS using siRNAs significantly blocked cyclopamine-induced cell death, compared with SCR-siRNA–transfected controls (Fig. 6F), suggesting a role for n-NOS in cyclopamine-mediated nSMase2 induction and cell death.

These results were also consistent when wt versus activity-deficient skin fibroblasts isolated from new born homozygous fro/fro (fragilitas ossium) mice (25) were treated with DETA. Treatment of wt cells with increasing concentrations of NO donor DETA elevated N-SMase2 (data not shown), and inhibited cell viability (Fig. 6G), whereas fro/fro skin fibroblasts with inactive N-SMase2, exhibited resistance to DETA-mediated cell death (Fig. 6G), supporting the role of N-SMase2 in DETA/NO-mediated cell death. Interestingly, in contrast to human cancer cells, these noncancerous wt and fro/fro skin fibroblasts were equally sensitive to cyclopamine-mediated cell death, suggesting that SHh/Smo plays an important role for the growth and/or proliferation of these cells, regardless of their N-SMase activity (data not shown).
Overall, these data suggest that increased nSMase2/ceramide and cell death in response to cyclopamine is regulated, at least in part, by the n-NOS/NO axis, in Daoy medulloblastoma and HNSCC cells.

Discussion

Here, possible roles and mechanisms of interaction between the SHh inhibitor/anticancer drug cyclopamine, and the bioactive sphingolipid ceramide, in apoptosis of Daoy and HNSCC cells were examined. Unexpected and novel data revealed that cyclopamine induces apoptosis in part via selective induction of nSMase2/increased ceramide. Mechanistically, induction of nSMase2 was regulated selectively by n-NOS/NO in response to cyclopamine, which was independent of Smo/Gli1 inhibition.

Cyclopamine was shown to induce apoptosis (1, 9, 14). Another Smo antagonist GDC-0449 (40) is currently in clinical trials against medulloblastomas and various other cancers. In fact, amino acid substitution at a conserved aspartic acid residue of SMO, which interrupted GDC-0449 binding, was reported to cause drug resistance in the clinic (41). Ceramide mediates drug-induced apoptosis. However, any involvement of ceramide in cyclopamine-induced apoptosis has not previously been reported. Our novel data revealed that cyclopamine results in robust ceramide generation via induction of nSMase2, which is at least partly critical in cyclopamine-induced cell death in Daoy and HNSCC cells. These data are somewhat in agreement with a previous study, in which cyclopamine-induced apoptosis was partially rescued by Gli...
overexpression in Daoy cells, indicating an involvement of other mechanisms in this process (42). Unexpected data additionally suggested cyclopamine-induced N-SMase2/ceramide generation is independent of SHh/Smo/Gli inhibition, but is regulated by NO stress/signaling in these cells.

To our knowledge, no previous data involving cyclopamine-induced NO generation in cancer cells exist. Although ceramide was reported to play roles in sodium nitroprusside (an NO donor)-induced apoptosis (43), any role of NO in inducing nSMase2 has not been previously reported. However, many excellent previous reports exist (35–37), which showed the involvement of ROS/RNS in SMase regulation and cell death in various cell types. For example, H$_2$O$_2$ was shown to activate N-SMase2, which is prevented by GSH in human airway epithelial cells (HAE; ref. 44). In aging rat hepatocytes, however, decreased GSH induced N-SMase2, whereas in young hepatocytes, inhibition of GSH synthesis activated N-SMase (45), suggesting that ROS/N-SMase2 regulation might be context dependent. This was also consistent with the roles of RNS in SMase regulation. It was shown that ONOO$^-$ induced A-SMase, but not N-SMase, in HAE cells (35). However, our data indicate a role for n-NOS/NO in the activation of N-SMase but not A-SMase in Daoy and HNSCC cells. These data together suggest that SMase regulation by RNS is also context dependent, and it might be differentially regulated in noncancerous HAE versus some cancer cell types. The cell type/context-dependent regulation of ceramide metabolism by NO is also consistent with earlier studies, in which NO donor induced apoptosis in cultured fibroblasts but not in keratinocytes (46). Moreover, L-NAME, inhibitor of NOS, increased ceramide formation and apoptosis in keratinocytes, but not in fibroblasts (46).

NO generation is regulated mainly by e-NOS, i-NOS, or n-NOS (47). Interestingly, our novel data suggest that cycloamine increased n-NOS mRNA, and knockdown of n-NOS (48) prevented cyclopamine-induced N-SMase2 and cell death, indicating its involvement in this process. However, specific mechanisms involved in n-NOS/NO generation in response to cyclopamine remain unknown. Moreover, mechanisms by which cyclopamine-induced n-NOS/NO results in increased nSMase2 are unknown. It was reported previously that daunorubicin activated the nSMase2 promoter via Sp1/Sp3 transcription factors in MCF-7 human breast cancer cells, increasing ceramide accumulation and cell death (49). Recently, all-trans retinoic acid was also shown to induce nSMase2, resulting in MCF-7 growth arrest (50). These studies are in agreement with our data, suggesting that various anti-cancer drugs, including cyclopamine, induce nSMase2, leading to increased ceramide generation and apoptosis.

In summary, our data show a novel off-target function of cyclopamine by inducing n-NOS/NO-dependent nSMase2 expression, and ceramide generation, which, in part, was necessary for drug-induced apoptosis. These data may have important implications for the Smo-independent apoptotic roles of cyclopamine in the treatment of various human cancers, in which the n-NOS/NO/N-SMase2/ceramide axis is intact, but SHh activation might be partly dispensable.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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