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

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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 anti-cancer 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 nSMase2 (neutral sphingomyelin phosphodiesterase 3, SMPD3) in Daoy human medulloblastoma cells. Importantly, siRNA-mediated knockdown of nSMase2 prevented cyclopamine-induced ceramide generation, and protected Daoy cells from drug-induced apoptosis. Accordingly, ectopic wild type (wt) N-SMase2 caused cell death, compared to controls, which express the catalytically inactive N-SMase2 mutant. Interestingly, knockdown of smoothened (Smo), a target protein for cyclopamine, or Gli1, a down-stream 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 (fragilitas 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, upper panel) is a Smo antagonist, which inhibits growth, and induces apoptosis in various cancers 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 anti-cancer 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) (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 if this occurs, at least in part, via induction of ceramide generation by
inhibition of SHh/Smo-dependent or -independent manner in Daoy human medulloblastoma and UM-SCC-14A or UM-SCC-1 human head neck squamous cell carcinoma (HNSCC) cells.

**Materials and Methods**

**Cell culture**

The Daoy medulloblastoma line (ATCC) was grown in MEM with 10% FBS and 1% Pen/Strep. WT, +/fro, and activity-deficient fro/fro skin fibroblasts isolated from new born mice (25), were cultured in DMEM with 10% FBS and 1% Pen/Strep. UM-SCC-1 and UM-SCC-14A cells obtained from Dr. Thomas Carey (University of Michigan). Cell lines used in this study were not authenticated. Cells were treated at final concentrations of 5-20 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 using high performance liquid chromatography/ mass spectrometry (LC/MS/MS) as described (26).

**Small interfering RNAs (siRNA), and plasmids**

SiRNAs for nSMase1 and nSMase2 were obtained from Ambion (Applied Biosystems). *Gli1* and *SMO* siRNAs were custom designed by Qiagen and Invitrogen, respectively. Non-targeting (scrambled, SCR) siRNA #2 was obtained from Dhharmacon. Transfections (40 nM, 48 h) were preformed using DhharmaFECT™ as described by the manufacturer. Knockdown of *iNOS* and
**nNOS** was performed using siRNAs obtained from Dharmaco. Plasmids for wt- and mutant-N-SMase2, containing V5 tags were used as described (27).

**Detection of cell death**

Cell death was measured using various techniques: a) activation of caspase-3, measured by fluorometry using a caspase-3 activity assay kit (R&D Systems); b) loss of mitochondrial membrane potential, detected by flow cytometry using the JC-1 mitochondrial membrane potential detection kit (Cell Technology); c) depletion of cellular ATP, measured using the ATP colorimetric/fluorometric assay kit (Abcam); and d) detection of Annexin V/7-AAD staining, measured by flow cytometry using the BD Pharmingen™ PE Annexin V Apoptosis Detection Kit, as described by the manufacturers. Growth Inhibition was measured using MTT and trypan blue assay.

**Measurement of SMase activity**

N-SMase and A-SMase activities were assayed *in vitro* as described previously, utilizing **14**C-[methyl]-SM as substrate (27).

**Detection of NO by flow cytometry**

Treated and untreated cells were incubated with fluorescent dyes (1 h). Cells were trypsinsized, washed with PBS, and stained with 7AAD, before flow cytometry analysis on BD FACScan (BD Biosciences). For hCatalase transduced cells, before addition of CD34 antibody, cells were blocked in 30% human AB-serum (Gemini) for 30 min, preventing background staining. Fluorochrome-labeled antibodies were added, and cells were stained with 7AAD, and flow
cytometry analysis was performed using the BD FACScan (BD Biosciences). Data for viable (7AAD) cells were analyzed and visualized by the FlowJo software (Tree Star).

**Statistical analysis**

Data are represented as mean ± SEM, unless otherwise indicated. Data represent at least two independent trials performed as duplicates. Error bars on graphs represent standard deviations. An unpaired Student’s t-test was performed using Prism/GraphPad software; \( p < 0.05 \) was considered significant (28).

Details of chemicals, RNA isolation, Q-PCR, Western blotting, target sequences of nSMase1 and nSMase2 siRNAs, catalase expression and activity assays can be found in Supplemental Materials and Methods.

**Results**

**Cyclopamine induces cell death and increases ceramide generation/accumulation**

Cyclopamine (Fig. 1A, upper panel) 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 \( \mu \)g/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 (IC\(_{50} \sim 5 \mu \)g/ml, 48 h, and \( \sim 10 \mu \)g/ml, 24 h) compared to vehicle-treated controls (Fig. 1A, lower panel). 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 to controls (Figs. 1B and C, respectively). Pre-treatment with z-VAD (10 µg/ml) almost completely prevented caspase-3 activation and loss of mitochondrial membrane potential in response to cyclopamine (Figs. 1B and C, respectively). Thus, these data are consistent with previous studies, demonstrating 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 h) increased total ceramide ~2.5- or 3-fold, respectively, increasing total ceramide from 20 (in controls) to 50-60 pmol/nmol Pi cyclopamine-treated cells, respectively (Fig. 1D). There were no significant changes in sphingosine or S1P in response to cyclopamine (data not shown). Moreover, cyclopamine (10 µg/ml, 24 hr) induced C_{14}-, C_{16}-, C_{18}-, C_{20}-, and C_{22}-ceramide generation ~2.5-, 3.5-, 15-, 6-, or 6.5-fold, respectively, compared to vehicle-treated controls (Supplemental Fig. S1A). Comparable increases were also observed with dihydro-C_{14}-C_{22}-ceramides (Supplemental Fig. S1B). Similar data were also obtained when cells were treated with 5 µg/ml cyclopamine (24 h), which increased endogenous C_{14}-C_{22}-ceramide generation compared to controls (Supplemental Fig. S1A). Thus, these data suggest that cyclopamine induces endogenous ceramide generation, consistent with its pro-apoptotic 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 µM and 50
nM, respectively, and examined cyclopamine-induced caspase-3 activation in Daoy cells (10 µg/ml, 24 h). FB1 or MYR did not prevent cyclopamine-induced caspase-3 activation (Supplemental 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 ceramide synthase 1 (CerS1) on growth inhibition in response to cyclopamine. CerS1 is known to mainly generate C18-ceramide, and cyclopamine induced C18-ceramide generation ~15-fold compared to controls in Daoy cells (see Supplemental Fig. S1A). Down-regulation of CerS1 using siRNAs did not protect cells from cyclopamine-induced growth inhibition compared to controls (Supplemental Fig. S2B). Effectiveness of siRNAs for knockdown of CerS1 in Daoy cells was confirmed using Q-PCR. An approximately 40-60% decrease in CerS1 was observed compared to controls in the absence/presence of cyclopamine (Supplemental Fig. S2C). Similarly, ectopic expression of wild-type (wt) CerS1, or its catalytically inactive form with the H122A mutation (32), did not enhance or prevent the growth inhibitory effects of cyclopamine (Supplemental Fig. S2D). Expression of wt- and mutant CerS1-FLAG proteins were confirmed by Western blotting using anti-FLAG antibody compared to vector-transfected controls (Supplemental Fig. S1E, lanes 2-3 and 1, respectively). Actin was used as a loading control (Supplemental Fig. S2E). Thus, these data suggest 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 using Q-PCR. Cyclopamine increased nSMase2 ~3- and 6-fold (12-24 h, respectively) whereas, treatment at 6 h had no effect (Fig. 2A). Cyclopamine did not induce caspase-3 activity at 6 h (Supplemental Fig. S3A), treatment at 12 h slightly, but significantly, increased caspase-3 activity (~30%, p<0.05). Knockdown of nSMase2, but not
nSMase1 (Supplemental Fig. S3B), (~75% compared to SCR controls, as determined by Q-PCR, 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 (Figs. 2D and 2E, 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 h), 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 head neck squamous cell carcinoma cells, in which it was increased ~4- and 8-fold, or 1.5- and 5-fold at 12 and 24h, respectively (Supplemental Figs. S3C and S3D, respectively).

Overall, these data suggest 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.

**Down-regulation 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 h), we measured endogenous ceramide and SM using LC/MS/MS in the absence/presence of siRNAs against nSMase1 or nSMase2 in Daoy cells. Data revealed that cyclopamine increased total ceramide ~2-3.5-fold in the presence of SCR or N-SMase1 siRNAs, and knockdown of nSMase2 prevented cyclopamine-mediated ceramide generation (Fig. 3A). Consistent with these data, total SM was significantly decreased (~70%, 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 Supplemental Figs. S4A and S4B. As observed earlier, C_{18}-ceramide was the main species generated by cyclopamine (~20-fold), and accordingly, a significant decrease in C_{18}-SM was also observed (Supplementary Figs. 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 cyclopamine induces ceramide generation by induction of nSMase2.

We then examined whether down-regulation of nSMase2, which prevented ceramide generation, also attenuated cyclopamine-mediated cell death (10 μg/ml, 24h). Data showed siRNA-mediated knockdown of nSMase2 significantly protected growth inhibition of Daoy cells (~50%, p<0.05) compared to SCR-siRNA-transfected controls in response to cyclopamine (Fig. 3C), consistent with the protective effects of down-regulation of nSMase2 on cyclopamine-mediated apoptosis (Fig. 2C-E). Taken together, these data suggest 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 to 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 using Western blotting (Fig. 3E) using anti-V5 antibody, compared to vector-transfected cells. In addition, activity of wt-compared to catalytically inactive mutant-N-SMase2-V5 or vector-transfected controls were...
confirmed (Fig. 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 down-regulation of nSMase2 or nSMase1 using siRNAs on *Smo* and *Gli* mRNAs in the absence/presence of cyclopamine (10 µg/ml, 24 h) by Q-PCR in Daoy cells. Cyclopamine reduced *Smo* and *Gli* by ~60%, but down-regulation of nSMase2 or nSMase1 had no effect on inhibition of the Smo/Gli axis by cyclopamine (Fig. 4A and 4B, respectively). Thus, these data confirmed inhibition of the Smo/Gli axis by cyclopamine is regulated independently of N-SMase2.

Then, we examined the effects of down-regulation of *Smo* or *Gli1* using siRNAs on nSMase2. We reasoned if cyclopamine-mediated nSMase2 elevation is regulated down-stream of Smo/Gli1 inhibition, knockdown of *Smo* or *Gli1* should also increase nSMase2. Interestingly, data showed while siRNAs successfully reduced *Smo* or *Gli1* by ~90 or 80%, they did not increase *N-SMase2* (Figs. 4C-D). Similarly, pharmacologic inhibitors of Gli or Smo, GANT-61 or SANT-1 (5-10 µM, 24-48h) had no effect on nSMase2 (data not shown), despite successfully reducing expression of *Gli1*, and its down-stream target *Bcl2* (Supplemental Fig. S5A-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/nitrogen species (ROS/RNS). First, we determined whether pre-treatment with NAC (0.5 mM), an anti-oxidant, altered nSMase2 in the absence/presence of cyclopamine. Interestingly, while treatment with cyclopamine induced nSMase2 around 8-fold (10 µg/ml, 24 h), pre-treatment 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 DCFDA and flow cytometry analysis. Data showed cyclopamine treatment induced ROS/RNS generation within 3 to 6 h of treatment, increasing DCFDA-fluorescence ~2-3-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 to controls (Fig. 5D, lower and upper right panels, respectively). Interestingly, there was no significant co-localization 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 using DCFDA and flow cytometry. Pre-treatment with NAC prevented the generation of ROS/RNS, shifting the DCFDA fluorescence signal to the left when compared to
cyclopamine-treated UM-SCC-14A or UM-SCC-1 cells, respectively (Fig. 5E and Supplemental Fig. S6A).

Thus, these data suggest that cyclopamine induces early ROS/RNS generation (within 3-6 h), which precedes increased nSMase2 detected at 12 h, and that cyclopamine-mediated ROS/RNS is important in induction of nSMase2.

**Cyclopamine increases nSMase-2 via induction of NO generation**

We then determined the mechanism by which cyclopamine induces ROS/RNS generation, which subsequently leads to increased nSMase2. First, to assess whether H2O2-generation was involved, we over-expressed human catalase, which increased its enzyme activity significantly (by >20-fold, p<0.001) compared to controls (Supplemental Fig. S6B-D), and then determined its effects on N-SMase2 and cell death in the absence/presence of cyclopamine (10 µg/ml, 24 h). Catalase over-expression did not prevent increased nSMase2 or Daoy-cell death in response to cyclopamine (Supplemental Fig. S6B-D), suggesting they were independent of H2O2 generation. In addition, BSO (2.5 mM, 16 h), a known inhibitor of glutathione synthesis, inducing oxidative stress via accumulation of intracellular ROS, had no significant effect on nSMase2 or apoptosis (Supplemental Fig. S6E-F).

We next examined whether RNS generation plays a role in this process. To determine RNS generation, effects of cyclopamine on DAF fluorescence, a probe for peroxynitrite (ONOO(-)) and NO generation, was examined in Daoy cells using flow cytometry. As shown in Fig. 6A, cyclopamine (10 µg/ml, 4 h) enhanced DAF and DCFDA, but not DHE (which detects superoxide anion), fluorescence compared to controls, suggesting cyclopamine induces RNS, but not ROS, via generation of peroxynitrite (ONOO(-)) and/or NO. Pre-treatment with MnTBAP, a
ONOO(-) scavenger, did not prevent nSMase2 up-regulation (Supplemental Fig. S7A), whereas L-NAME pre-treatment, an inhibitor of NO synthase (NOS), almost completely blunted cyclopamine-induced nSMase2 (Fig. 6B).

In reciprocal experiments, DETA, a well known 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 inducible-, endothelial-, or neuronal-nitric oxide synthase (i-NOS, e-NOS, or n-NOS, respectively). Cyclopamine (10 µg/ml, 24 h) increased (~2-fold, measured by Q-PCR) n-NOS (Supplemental 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 Q-PCR, Supplemental Fig. S7C) prevented nSMase2 induction and cell death (Figs. 6D and 6E, 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 to 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 wild-type (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 non-cancerous 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/anti-cancer 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 exists. Although ceramide was reported to play roles in sodium nitroprusside- (a 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 demonstrated 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 glutathione (GSH) in human airway epithelial cells (HAE) (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 non-cancerous 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 eNOS, iNOS or nNOS (47). Interestingly, our novel data suggest that cyclopamine 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-retionic-acid (ATRA) 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 demonstrate a novel off-target function of cyclopamine by inducing n-NOS/NO-dependent nSMase2 induction, 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.

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Figure Legends

Fig. 1. Cyclopamine induces cell death via caspase-3 activation and loss of mitochondrial membrane potential in Daoy cells. (A) Effects of increasing concentrations of cyclopamine (upper panel) at 0, 2.5, 5, 10, 25 and 50 µg/ml (24-48 h) on cell viability were measured using MTT assay (lower panel). (B-C) Activation of caspase-3 (B) or loss of mitochondrial membrane potential (C) in response to cyclopamine (10 µg/ml, 24 hr), as compared to controls, were measured. (D) Effects of cyclopamine on total ceramide accumulation were measured using LC/MS/MS, normalized to inorganic phosphate (Pi).

Fig. 2. Cyclopamine induces nSMase2, and down regulation of nSMase2 protects cells from drug-induced apoptosis. (A) Effects of cyclopamine on nSMase2 (0, 6, 12, and 24 h) were determined in Daoy cells using Q-PCR. Ribosomal RNA was used as a control. (B) Roles of siRNA-mediated knockdown of nSMase2 versus nSMase1 in cyclopamine-induced caspase-3 activity (12 h) were assessed. Non-targeting scrambled (SCR) siRNA-treated cells were used as controls. (C) Effectiveness of siRNAs for targeting/knockdown of nSMase2 in the absence/presence of cyclopamine (10 µg/ml) compared to SCR-siRNA-transfected controls was confirmed using Q-PCR. (D-E) Effects of siRNA-mediated knockdown of nSMase2 on cyclopamine-induced cell death were measured by AnnexinV/7-AAD staining (D), or ATP depletion (E) in Daoy cells. (F) Effects of down-regulation of nSMase2 on cyclopamine-mediated increase in N-SMase enzyme activity (n=2) were determined.

Fig. 3. N-SMase2-generated ceramide is important in cyclopamine-induced cell death in Daoy cells. (A-B) Ceramide (A) and SM (B) in response to cyclopamine (10 µg/ml), in the
absence/presence of siRNAs targeting nSMase1 or nSMase2, were measured in Daoy cells using LC/MS/MS. SCR-siRNA-treated cells were used as controls. (C) Effects of down-regulation of nSMase2 versus nSMase1 using siRNAs on cell viability were determined using trypan blue exclusion assay. (D) Roles of nSMase2/ceramide in the induction of apoptosis were examined using trypan blue exclusion assay after transfection of Daoy cells with vectors containing cDNAs of the wt- or catalytically inactive mutant of N-SMase2, compared to vector-only-transfected cells. (E-F) Ectopic expression of wt- or mutant-N-SMase2-V5 was confirmed by measurement of SMase activity (E) and Western blotting (F) using anti-V5 antibody (as duplicates) in Daoy cells as compared to vector-transfected controls. Actin was used as a loading control in F (lower panel).

Fig. 4. Up-regulation of nSMase2 and ceramide generation in response to cyclopamine is independent of the inhibition of Smo/Gli of the SHh pathway in Daoy cells. (A-B) Effects of down-regulation of nSMase2 or nSMase1 using siRNAs on the inhibition of Smo (A) or Gli1 (B) in the absence/presence of cyclopamine (10µg/ml, 24 h) were determined by Q-PCR. SCR-siRNA-transfected cells were used as controls. (C-D) Potential roles of Smo or Gli1 inhibition on the regulation of nSMase2 were examined after siRNA-mediated knockdown of Smo (C) or Gli1 (D) using Q-PCR. Effectiveness of siRNAs on knockdown of Smo or Gli1 were also confirmed using Q-PCR (C and D, respectively). SCR-siRNA-transfected cells were used as controls.

Fig. 5. Cyclopamine-mediated increased nSMase2 is regulated by ROS/RNS generation. (A-B) Effects of oxidative stress via ROS/RNS generation on cyclopamine-mediated
overexpression of nSMase2 were examined in the absence/presence of anti-oxidant NAC (500 µM) using Q-PCR in Daoy (A) and UM-SCC-14A (B) cells. (C) ROS/RNS induction in response to cyclopamine (10 µg/ml) at 0, 3, 6, 9, 12, and 15 h was measured using DCFDA by flow cytometry in Daoy cells. (D) Effects of cyclopamine (10 µg/ml, 4 h) on ROS/RNS generation (left panel) and potential mitochondrial co-localization (middle and right panels) in Daoy cells were visualized by confocal microscopy using DCFDA (green) and mitotracker (red) as compared to vehicle-treated controls (lower and upper panels, respectively). (E) Effects of NAC (500 µM) on prevention of ROS/RNS generation in response to cyclopamine (10 µg/ml, 6 h) in UM-SCC-14A cells were measured using DCFDA by flow cytometry.

**Fig. 6. Cyclopamine-mediated induction of nSMase2 is regulated selectively by NO generation in Daoy cells.** (A) Generation of NO/ONOO(-) in response to cyclopamine (10 µg/ml for 4 h) was measured using DAF, DCFDA, or DHE by flow cytometry in Daoy cells. (B-C) Effects of L-NAME (50 µM) (B) versus DETA (3 mM, 12 h) (C) on nSMase2 in the absence/presence of cyclopamine (10 µg/ml, 24 h) were measured by Q-PCR. (D-F) Effects of siRNA-mediated knockdown of n-NOS on nSMase2 (D), or cell death in Daoy (E) and UM-SCC-14A (F) cells were measured by Q-PCR, or trypan blue exclusion assay, respectively. (G) Cells isolated from wt or fro/fro mice were treated with increasing concentrations of cyclopamine, and effects on cell growth were measured by trypan blue exclusion assay. Vehicle-treated cells were used as controls.
Figure 1

A

Cyclopamine

![Chemical structure of cyclopamine]

B

Fold Caspase-3 Activation

* = p<0.0001

DMSO

10ug/mL zVAD

Cyclopamine (ug/mL)

0 2.5 5 10 25 50

Fold Cell Viability

* = p<0.01

24hrs

48hrs

C

Fold Cytoplasmic/ Monomeric JC-1

* = p<0.0001

DMSO

10ug/mL zVAD

Untreated

10ug/mL Cyclopamine

D

Total Ceramide Level (nmol/mmol Pi)

* = p<0.05

Untreated

5ug/mL Cyclopamine

10ug/mL Cyclopamine
Figure 3

A. Fold Ceramide Level (pmol/nmol PI)

- SCR RNAi
- nSMase1 RNAi
- nSMase2 RNAi

* = p<0.001
** = p<0.05

B. Fold SM Level (pmol/nmol PI)

- SCR RNAi
- nSMase1 RNAi
- nSMase2 RNAi

* = p<0.001
** = p<0.05

C. % Cell Viability

- SCR RNAi
- nSMase2 RNAi

* = p<0.001
** = p<0.05

D. % Cells

- Live Cells
- Trypan Blue Positive Cells

* = p<0.001

E. nSMASE Activity (pmol ceramidase activity/nmol protein)

- Vector
- WT nSMase2
- Mut nSMase2

* = p<0.01

F. V5

Actin
Figure 4

A

Fold mRNA Levels (RQ normalized to rRNA)

* = p<0.05

- Untreat
- 10ug/mL Cyclopamine

SCR RNAi | nSMase1 RNAi | nSMase2 RNAi

SMO mRNA

B

Fold mRNA Levels (RQ normalized to rRNA)

* = p<0.01

- Untreat
- 10ug/mL Cyclopamine

SCR RNAi | nSMase1 RNAi | nSMase2 RNAi

Gli1 mRNA

C

Fold mRNA Level (RQ normalized to rRNA)

* = p<0.05

- Gli1 mRNA
- SMO mRNA
- nSMase2 mRNA

SCR RNAi | SMO RNAi

96hrs RNAi Txn

D

Fold mRNA Level (RQ normalized to rRNA)

* = p<0.05

- Gli1 mRNA
- SMO mRNA
- nSMase2 mRNA

SCR RNAi | 100nM Gli1
Figure 5

A

* = p<0.01
** = p<0.05

Fold mRNA Level (RQ normalized to rRNA)

Daoy Medulloblastoma: nSMase2 mRNA

Untreat
500uM NAC

B

* = p<0.05

Fold mRNA Level (RQ normalized to rRNA)

UM-SCC-14A: nSMase2 mRNA

Untreat
500uM NAC

C

ROS/RNS induction via Cyclopamine time course

(Cells stained with DCFDA)

Units

Daoy Medulloblastoma

Time with 10ug/mL Cyclopamine (Hrs)

D

DCFDA (Green) - ROS
Mitotracker Red
Control - Untreated
Overlay

E

Counts

FL1-H

10^3 10^4

SCC-14A No Txt - Unstained
SCC-14A No Txt - DCFDA
SCC-14A Cyclopamine - DCFDA
SCC-14A NAC - DCFDA
SCC-14A NAC + Cyclopamine - DCFDA

4hrs 10ug/mL Cyclopamine

DCFDA (Green) - ROS
Mitotracker Red
Overlay
Figure 6

A

Unstained

DAF

DCFDA

DHE

Log FL

B

Fold mRNA Level

(RQ normalized to rRNA)

* = p<0.001
** = p<0.01

Untreat

50μM L-NAME

Daoy Medulloblastoma: nSMase2 mRNA

C

Fold mRNA Level

(RQ normalized to rRNA)

* = p<0.001

Untreat

10ug/mL Cyclopamine

3mM DETA

Daoy Medulloblastoma: nSMase2 mRNA

D

Fold nSMase2 mRNA Level

(RQ normalized to rRNA)

* = p<0.05

Untreated

10ug/mL cyclopamine

SCR RNAi

nNOS RNAi

E

% Live cells

* = p<0.05

Untreated

10ug/mL cyclopamine

SCR RNAi

nNOS RNAi

F

% Live cells

* = p<0.01

Untreated

10ug/mL Cyclopamine

SCR RNAi

nNOS RNAi

G

% Live Cell

* = p<0.01

WT

fro/fro

Concentration 12hr DETA (mM)

0

0.25

0.75

1.5
Off-Target Function of the Sonic-Hedgehog Inhibitor Cyclopamine in Mediating Apoptosis via Nitric Oxide-Dependent Neutral Sphingomyelinase 2/Ceramide Induction

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