Published in final edited form as:

Biochim Biophys Acta. 2016 February ; 1861(2): 78–90. doi:10.1016/j.bbali.2015.11.001.

The hyaluronic acid inhibitor 4-methylumbelliferone is an NSMase2 activator-role of Ceramide in MU anti-tumor activity

Jingdong Qin¹, John Kilkus¹, and Glyn Dawson^{1,2}

¹Department of Pediatrics, University of Chicago, Chicago, IL 60637

²Department of Biochemistry and molecular biology, University of Chicago, Chicago, IL 60637

Abstract

Increased synthesis of hyaluronic acid (HA) is often associated with increased metastatic potential and invasivity of tumor cells. 4-Methylumbelliferone (MU) is an inhibitor of HA synthesis, and has been studied as a potential anti-tumor drug to inhibit the growth of primary tumors and distant metastasis of tumor cells. Although several studies reported that the anticancer effects of MU are mediated by inhibition of HA signaling, the mechanism still needs to be clarified. In a previous study we demonstrated the regulation of HA synthesis by ceramide, and now show how MU activated neutral sphingomelinase2 (NSMase2), generates ceramides and mediates MU induced inhibition of HA synthesis, cell migration and invasion, and apoptosis of tumor cells. Using a HA enriched mouse oligodendroglioma cell line G26-24 we found that MU elevated the activity of NSMase2 and increased ceramide levels, which in turn increased phosphatase PP2A activity. Further, the activated PP2A reduced phosphorylation of Akt, decreased activities of HA synthase2 (HAS2) and calpains, and inhibited both the synthesis of HA, and the migration and invasion of G26-24 tumor cells. In addition, MU mediated ceramide stimulated activation of p53 and caspase-3, reduced SIRT1 expression and decreased G26-24 viability. The mechanism of the MU anticancer therefore initially involves NSMase2/ceramide/PP2A/AKT/HAS2/caspase-3/p53/SIRT1 and the calpain signaling pathway, suggesting that ceramides play a key role in the ability of a tumor to become aggressively metastatic and grow.

Keywords

4-methylumbelliferone; NSMase2; Ceramide; Hyaluronan acid; anticancer

1. Introduction

The activation of HAS2 and the over-production of HA is seen in many metastatic tumor cell lines (1-6). MU is a promising anti-cancer therapeutic agent which has been shown to

To whom correspondence should be addressed: Jingdong Qin and Glyn Dawson, Department of Pediatrics and Department of Biochemistry and molecular biology, the University of Chicago, 5841 S Maryland Ave, Wyler MC4068. Chicago, IL, USA, Tel.: (773) 702-6430; Fax: (773) 702-9234; qjingdong@peds.bsd.uchicago.edu and dawg@uchicago.edu..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

inhibit HA synthesis, reduce the metastatic potential and induce apoptosis of tumor cells (7-15). Kakizaki et al (7) previously proposed that competition for UDP-glucuronic acid (GlcUA) by UDP-glucuronyltransferases (UGT) mediated glucuronidation of MU as a likely mechanism for the inhibition of hyaluronan synthesis, *Clarkin et al.* (16) then reported that suppressed UDP-glucose dehydrogenase (UGDH) expression by MU in chick limb bud micromass culture could explain both reduced HA and sulfated-glycosaminoglycan (sGAG) production. Other studies have also reported that MU could down-regulate HAS expression (8-12). Although these previous studies have linked the anticancer effects to the inhibition of HA and the down-regulation of hyaluronan synthases (1-15), MU actually kills cancer cells, in addition to reducing HA synthesis and inhibiting cancer cell migration. Thus it is hard to explain why incomplete inhibition of HA synthesis can kill cells since many aggressive tumor cell lines such as HOG make very little HA but are readily killed by MU. Therefore, a more precise mechanism of MU anti-cancer action remains to be determined.

We have previously established the connection between sphingolipids and glycosaminoglycan metabolism and have shown that HA synthesis is regulated by NSMase2/ceramide through ceramide-activated phosphatase PP2A and Akt signaling in *fragilis ossium* (*fro/fro*) mouse fibroblasts (17). We observed that the pro-oncogenic protein Akt was activated in *fro/fro* and this was translated into activated mTOR, increased HAS2 and HA accumulation (17). We showed that ceramide levels were the trigger for these events and that elevating lipid raft ceramide levels reduced the level of p-Akt in cells by activating the PP2A phosphorylase. This finding was then confirmed by Kakoi's study on BMP-2 induced NSMase2 regulation of chondrocyte maturation (18). They found expression of Has2 protein to be decreased in NSMase2-positive hypertrophic chondrocytes in the bones of mouse embryos (18).

Ceramide is a sphingolipid bio-active second messenger and a powerful tumor suppressor that has been implicated in the regulation of tumorigenesis by activation of caspase-3 and apoptosis (19-21). Tumor cells employ a number of metabolic pathways to keep ceramide levels low, such as increased conversion to glucosylceramide and activation of ceramidase (19-21). Tumor resistance is typically associated with the activation of a glucosyltransferase to lower both ceramide levels and activate drug pumping mechanisms via the P-glycoprotein (20, 21). Thus enzymes involved in ceramide generation (NSMase2, ASMase and Ceramide Synthases 1-6) or degradation (acid and neutral ceramidases) or reutilization (ceramide kinases and glucosyltransferases) have become targets for drugs aimed at preventing cancer cells from avoiding cell death (21). Of the two major sphingomyelinases, ASMase is mainly in endosomes/lysosomes and the ASMase^{-/-} mouse shows characteristics of the human lysosomal storage disease Niemann-Pick A and B. NSMase2 is a plasma membrane-bound dynamically palmitoylated protein, and the neutral sphingomyelinase (NSMase2) deficient mouse shows multiple skeletal abnormalities (so-called *fragilis ossium*) and lung anomalies (17, 22). Nucleotide sequencing of the highly conserved *SMPD3* gene in a large panel of human cancers revealed mutations in 5% of acute myeloid leukemias and 6% of acute lymphoid leukemias which suggests that disruption of the ceramide pathway may contribute to a subset of human leukemias (23).

Oligodendroglioma cell line G26-24 expresses predominantly HAS2 and produces copious amounts of HA in culture medium (24). It was isolated from a glioma (G26) induced by methycholanthrene treatment in the C57BL/6 inbred mouse (25). In this study, we employed G26-24 to facilitate the investigation of MU mediated anti-cancer mechanisms, and a human oligodendroglioma cell line (HOG) with very low HA secretion as a comparative reference (26). Our results for the first time suggest that MU is a cell surface activator of NSMase2; NSMase2 activation produces the second messenger molecule ceramide, which induces apoptosis and cell cycle arrest in various cancer cells, and starts the anti-cancer signaling pathway (21, 27-28). Thus MU mediated NSMase2/ceramide regulation may be important in anticancer treatment.

2. Materials and methods

2.1. Materials

N-myristoyl (14:0), *N*-palmitoyl (16:0), *N*-oleoyl (18:1), *N*-stearoyl (18:0), *N*-arachidoyl (20:0), *N*-nervonoyl (24:1), *N*-lignoceroyl (24:0) sphingosines (ceramides (Cer)), *N*-palmitoyl (16:0), *N*-oleoyl (18:1), *N*-stearoyl (18:0), *N*-arachidoyl (20:0), *N*-behenoyl (22:0), *N*-nervonoyl (24:1), *N*-lignoceroyl (24:0), DHSph (dihydroceramides (DHCer)), *N*-heptadecanoyl sphingosine (17:0-Cer), and the palmitoyl (16:0), stearoyl (18:0), arachidoyl (20:0), behenoyl (22:0), nervonoyl (24:1), and lignoceroyl (24:0) coenzyme A substrates were obtained from Avanti Polar Lipids (Alabaster, AL). [³H] Palmitic acid (43 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Chloroform, methanol, and acetic acid used for high performance thin-layer chromatography (HPTLC) were of ACS grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). Silica gel HPTLC plates were obtained from Whatman (Clifton, NJ, USA). Hexamethylumbelliferyl (HMU)-phosphorylcholine was purchased from Moscerdam Substrates (Amsterdam, The Netherlands). DRAQ5 was purchased from Abcam Inc. (Cambridge, MA, USA). Sphingomyelinase from *bacillus cereus* (bNSMase), 30% (w/w) H₂O₂, staurosporine, C2-Ceramide and T-BOC-LM-CMAC was purchased from Sigma Aldrich (St. Louis, MO, USA). The antibodies for NSMase2, Akt, p-Akt (Ser473), HAS2, Calpain1 and Calpain2 and PP2A were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), caspase3 and cleaved caspase3, P53, Sirt1 were from Cell Signaling Technology, Inc. (Danvers, MA, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), beta-actin antibody and secondary antibodies anti-mouse, anti-goat and anti-rabbit were purchased from Sigma (St. Louis, MO, USA). Hyaluronic acid sandwich ELISA kit (K-4800) was purchased from Echelon Biosciences Inc. (Salk Lake, UT, USA) and CytoSelect 24 well cell migration and invasion assay kit from Cell Biolabs, Inc. (San Diego, CA, USA).

2.2. Generation of G26-24, HOG cell lines and drug treatment

Cell line G26-24 was isolated from glioma G26 induced by methycholanthrene treatment in the C57BL/6 inbred mouse and has been classified as an immature glial cell with oligodendroglial and some astrocyte features (25). HOG was established from a surgically removed oligodendroglioma, subcloned and maintained by continuous cell culture (26). Mouse *SMPD3* (which encodes NSMase2) was cloned in an N-terminal p3xFLAG-CMV

vector (Sigma), transfected into HOG cells and stable clones were selected for their resistance to neomycin (250µg/ml) in Dulbecco's modified Eagle's medium (DMEM). All studies involved 1mM MU and 5µM with the exception of dose response experiments. The pre-incubation time for GW4869 was 30 min before adding MU.

2.3. Hyaluronan production assay

Cells were seeded in DMEM/10% FBS, 1% Gentamycin at 10^6 /100mm dish. Cell culture conditioned media was collected after 48 h for hyaluronan quantification, and cells were quantified for protein. HA levels were determined with the competitive ELISA kit from Echelon Biosciences according to the manufacturer's instructions. Briefly, samples of conditioned media were first mixed with the detector, and then added to the HA ELISA plate for competitive binding. The colorimetric signal was detected at 450 nm with a Perkin Elmer VICTOR³_{Tm} 1420 Multilabel Counter and was inversely correlated with the amount of HA present in the samples.

2.4. Cell viability Assay

We used the MTT assay (Sigma Chem. Co) and the Alexa Fluor 488 annexin V/Dead cell apoptosis kit (Life Technologies) for flow cytometry (FACSCalibur, BD) to assess cell viability and apoptosis. In brief, cells were plated in 24-well culture plates at a density of 1×10^5 cells/cm² per well. After treatment, MTT (5 mg/ml) was added to each well of the monolayer cultures, and the cultures were incubated in a humidified 5% CO₂ incubator at 37°C. Following 2 h of incubation the cells were dissolved in 10% SDS-HCl for 6 h or overnight, and the optical density proportional to cell death was measured at 595 nm by using a microplate reader (Elx800; BioTek). All values are expressed as percentages of the control. Cell apoptosis analysis by flow cytometry was according to the manufactures experimental protocol, and the percentage of dead and dying cells (early apoptotic and late apoptotic cells) in total cells was calculated.

2.5. Analysis of MU uptake by G26-24 and HOG cells

2×10^5 G26-24 and HOG cells were seeded onto 60 mm culture dishes with DMEM/10% FBS, 1% Gentamycin overnight. Culture medium was changed with fresh serum free DMEM and 1mM MU was added. Cells were harvested at different time points by 6000 rpm 10 min centrifugation, and washed five times with serum free DMEM. Cell pellets were diluted in 300µl of serum free DMEM and lysed by sonication. Whole cell lysates and supernatants of lysed cells were used for fluorescence analysis by an Flx800 reader from BioTek with excitation 360nm and emission 460 wavelengths. 0.15 mM MU in serum free DMEM was used as a positive control. Live cell staining with DRAQ5 for nuclear DNA visualization and MU uptake was executed by laser scanning confocal microscopy. After 3 h of MU treatment, the medium and MU were replaced with a new culture medium containing 5 µM DRAQ5, gently mixed by pipetting and then incubated for 15 min. The intracellular distribution of MU was analyzed with a Marianas automated Yokogawa-type spinning disc confocal microscope (BD, Franklin Lakes, NJ).

2.6. Western blot analysis

Lysates from cell cultures were subjected to SDS-gel electrophoresis. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and Western blotting carried out with antibodies according to the manufacturer's instructions. Positive bands were detected with a chemiluminescence kit from Fisher Scientific (Pittsburgh, PA). The Western blot bands were scanned with a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA) and the images quantified using Quantity One 4.5.0 software (Bio-Rad, Hercules, CA).

2.7. Isolation of Detergent-Resistant Membranes (Lipid Rafts)

Lipid rafts (LR) were isolated by their insolubility in Triton X-100 at 4°C as described previously (17). Briefly, cell pellets were lysed in 1.5 ml of 25 mM MES, pH 6.5, 150 mM NaCl, 1.0% Triton X-100, 1 mM Na₃VO₄ (MBST) supplemented with a protease inhibitor cocktail for 1 h at 4°C. After homogenization for 10 times in a loose-fit Dounce homogenizer, lysates were mixed with 1.5 ml of 80% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl) and overlaid with 3 ml of 30% sucrose in MBST and then with 3 ml of 5% sucrose in MBST. After centrifugation for 18 h at 31,000 rpm in an SW40 rotor, 1 ml fractions were collected and analyzed. The raft fraction was typically found between fractions 3 and 4.

2.8. Lipid extraction and sample preparation for lipid quantification by LC/MS/MS and analysis of Ceramides

Cellular lipids were extracted (17) with the use of 0.1N HCl to improve phase separation. 17:0-Cer (30 pmol) was used as internal standard and was added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v), and aliquots were taken to determine the total phospholipid content. Samples were concentrated under a stream of nitrogen, re-dissolved in methanol, transferred to auto-sampler vials, and subjected to consecutive LC/MS/MS analysis of ceramides (17). Analysis of ceramides was performed by combined LC/MS/MS using an automated Agilent 1100 series liquid chromatograph and auto-sampler (Agilent Technologies, Wilmington, DE) coupled to an API4000 Q-trap hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray ionization source. Sphingolipids were ionized via electrospray ionization (ESI) with detection via multiple reactions monitoring (MRM). Analysis of the molecular species of ceramides used ESI in positive ions with MRM analysis. Standard curves for ceramide molecular species were constructed by adding increasing concentrations of the individual analyte to 30 or 40 pmol of the corresponding structural analogs used as the internal standard. Linearity and the correlation coefficients of the standard curves were obtained by a linear regression analysis. The standard curves were linear over the range of 0.0–300 pmol of ceramides with correlation coefficients (R^2) > 0.98 (17).

2.9. Analysis of lipid synthesis by HPTLC

Cells were labeled with [³H] palmitate and lipids were extracted as described previously (17). Typical labeling experiments were carried out in 100-mm Petri dishes containing 8ml of serum-free medium for 24 h. Cells (3×10^6 /100 mm plate) were harvested and washed

three times with PBS. Lipids were extracted by Chloroform-Methanol-water (2:1:0.6 v/v) partition and samples were subjected to alkaline methanolysis to remove phosphoglycerides. Lipids were applied to HPTLC plates (10 × 10 cm; LHP-K TLC plates) and developed in chloroform: methanol: glacial acetic acid: water (70: 25: 8.8: 4.5 v/v) and chloroform: methanol: acetic acid (94:1:5). Lipids were visualized in iodine vapors then scraped off for quantification by liquid scintillation counting.

2.10. Sphingomyelinase activity assay

NSMase2 and ASMase activity were determined with the fluorimetric substrate HMU-phosphorylcholine as described previously (17). Briefly, cells were harvested, the pellets were resuspended and lysed in 25 mM Tris- HCl, 150 mM NaCl, and 1% Triton X-100, pH 7.4. Protein concentration was determined by using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). 50µg protein lysates were used for SMases assay. For the ASMase activity assay, lysates were mixed with the fluorogenic substrate HMU-phosphorylcholine and the incubation carried out at pH 4.5 in 150 mM sodium acetate buffer containing 1 mM EDTA to block any NSMase activity. The NSMase2 assay was carried out at pH 7.4 in 10mM MgCl₂, 100mM Tris-HCl, and 0.1% Triton X-100, including 5mM fresh DTT to inhibit any ASMase activity. The HMU released was followed fluorometrically in a 96-well plate using a Biotek FLXmicroplate reader. The enzyme activity was calculated from the slope of the graph of intrinsic fluorescence plotted against time and standardized to µg of protein.

2.11. Detection of calpain activity and cell migration and invasion assay

For the calpain assay, cells were treated with 1 mM MU in the presence of t-BOC-LM-CMAC (30µM) for 60 min, MU induced calpain cleavage of BOC-LM-CMAC liberates CMAC and results in increased fluorescence in the supernatant of the cell lysate, which was quantified with a Biotek Synergy H1 plate reader at excitation 351 nm, emission 430 nm. Cell migration and invasion assays were executed by following the manufacturer's instruction using a CytoSelect 24-well migration and invasion assay kit from Cell Biolabs, Inc. (San Diego, CA, USA).

2.12. Caspase3 Assays

Cells were treated with MU at the concentrations and times indicated, harvested, and washed with PBS, and the pellets were re-suspended and lysed in 25 mM Tris-HCl, 150 mM NaCl and 1% Triton X-100 pH 7.4. Hydrolysis of the caspase 3 (DEVD-AFC) substrates was determined using 25-30µg of cell extract in 100 µl of 25 mM HEPES, pH 7.4 buffer containing 2 mM dithiothreitol and 5 mM EDTA. The reaction was followed for 3 h in a microplate fluorescence reader (FLX800; BioTek) at 37°C set at 400 nm excitation and 505 nm emission. Enzyme activity was calculated from the slope of intrinsic fluorescence plotted against time and standardized by µg of protein.

2.13. Statistical Analysis

The results of analyses represent mean values from multiple experiments run in duplicate or triplicate. Statistical analyses were performed by Student's *t*-test, and results were considered statistically significant when $p < 0.05$.

3. Results

3.1. MU (1mM) inhibits the proliferation and decreases HA production in G26-24 cells but does not penetrate into cells

G26-24 cells were isolated from a methycholanthrene induced mouse glioma (25), they produce large amounts of HA and express predominantly HAS2 (24). When 1mM MU was added to G26-24 cells, MU decreased HA production in G26-24 cells as expected (Fig. 1A). The reduction ranged from 3250+/- 221 ng/mg cell protein to 291+/-14 ng/mg protein after 24 h treatment, a reduction of over 90% in the G26-24 culture medium (Fig. 1A). MU also reduced cell numbers and induced G26-24 cellular morphological changes (Fig. 1B and Fig. 1C). MU decreased cell proliferation in a dose-dependent manner over the concentration range 0-1mM (Fig. 1B) and this was partially reversed by the NSMase2 inhibitor GW4869 (Fig. 1D, d and f); the treated cells showed glia cell-like processing (Fig. 1C, lower left panel) and a majority changed their appearance to stretched and adherent morphology. The morphological changes were partially reversed by the NSMase2 inhibitor GW4869 (Fig. 1C, lower right panel). By using annexin V/ PI flow cytometry analysis (Fig. 1D), we found that MU (1mM and 2 mM) induced apoptosis (Fig. 1D, c and e) and that 5μM GW4869 could partly reverse the apoptotic effect and cell death (Fig. 1D, d and f), confirming the MTT data (Fig. 1B). The effect of MU on another human oligodendroglioma cell line HOG (using as a parallel reference) which expresses very low levels endogenous HAS2 (26), showed a similar profile on MU treatment (Fig. A.1, A, B and C), namely decreased living cell numbers and HA secretion. Although there was minimal production of HA in HOG cells, the HA level decreased from 8.70+/- 0.44 to 2.39+/- 0.21ng/mg cell protein following 24 h treatment by MU. NSMase2 inhibitor GW4869 partially reversed MU induced cell stress (Fig. A.1C). Our previous studies have shown that MU hardly penetrates tumor cell lines, neither by natural uptake nor through electroporation (data not shown). In this study, we didn't observe any penetration of MU into G26-24 and HOG cells after 3 h, either as fluorescence material uptake or fluorescence staining analysis (Fig. 2). We could not detect fluorescence uptake either in whole cell lysates (following 1mM MU administration for 3 h) or in supernatants of G26-24 cells (Fig. 2A). To verify our findings we used a Marianas automated Yokogawa-type spinning disc confocal microscope, even under 500 millisecond exposure we could not find any fluorescence density difference between MU treated and untreated control cells in both G26-24 and HOG following 3 h treatment by 1mM MU (Fig. 2B and 2C). However the DRAQ5 clearly entered the cells and stained nuclear DNA. We treated HOG cells with 20μM staurosporine plus 1mM MU overnight as a positive control, and found MU in nucleus-depleted cells (Fig. 2C, bottom panel). These results are consistent with our previous studies such as in HOG cells exposed to electroporation (data not shown).

3.2. MU activates ceramide-inducible PP2A, inhibits phosphorylation of Akt and decreases HAS2 expression in G26-24 cells

Our previous study on NSMase2^{-/-} (*fro/fro*) cultured skin fibroblasts demonstrated that HA synthesis is regulated by sphingolipid mediated Akt signaling (17). We now show that reduced HA synthesis by MU treatment is accompanied by a significant elevation of ceramide, ceramide-inducible phosphatase PP2A, and reduction of phosphorylation of Akt in G26-24 cells (Fig. 3A and B). Further, by adding 5 μ M GW4869, the specific inhibitor of NSMase2, we could reverse the elevated expression of PP2A by MU treatment (Fig. 3B, lane 3). MU-mediated inhibitory effect on HAS2 (0.5, 1, 3 and 24 h) was further confirmed by Western blot analysis (Fig. 3C). As with PP2A, the expression of HAS2 was partially reversed by the specific NSMase2 inhibitor GW4869 in MU treated G26-24 cells (Fig. 3C).

3.3. MU mediated LR translocation and activation of NSMase2

We have previously elucidated that the synthesis of HA is regulated by NSMase2/ceramide though the ceramide-activated PP2A and Akt signaling pathway (17), and here we found that the reduction of HA synthesis and inhibition of HAS2 by MU are accompanied by activated PP2A and down-regulated phosphorylation of Akt in HA enriched G26-24 cell cultures. Therefore, we had reason to assume that NSMase2/ceramide might be involved in the mechanism of MU mediated HA inhibition and anticancer effects. To prove this assumption, we first investigated if MU stimulated the lipid raft (LR) translocation of NSMase2 and accelerated the expression of NSMase2. We used MU to treat HOG cells in which NSMase2 was over-expressed and Flag-tagged to permit a clear detection of the translocation and expression of NSMase2 protein by an anti-Flag M2 antibody. As expected we observed that more NSMase2 translocated into the lipid raft fraction (Fig. 4A, as shown by western blots using anti-Flag M2) in a time dependent manner and increased NSMase2 expression in whole cell lysates following 1mM MU treatment for 0.5 and 1 h (Fig. 4B). Accordingly, enzyme assay demonstrated increased NSMase2 activity in a time dependent manner (0, 0.5, 1 and 3 h) after 1mM MU treatment in HOG cells (Fig. 4C). Increased NSMase2, Caspase-3 activities and dephosphorylation of Akt with reduced HA production were also found in a breast cancer cell line MDA-MB-231 (Fig. A.2), suggesting that this is a generalized phenomenon. We then investigated the expression and activity of NSMase2 in HA enriched G26-24 cells following MU treatment. As in HOG cells, a rapid elevation of NSMase2 expression was observed. The increase in NSMase2 protein by 1mM MU stimulation was shown by Western blot at 0, 0.5, 1 and 24 h. Meanwhile, we found that the NSMase2 inhibitor GW4869 significantly reduced the increase of NSMase2 expression by MU stimulation compared to 24 h of MU alone (Fig. 4D). Enzyme assay showed that 1mM MU activated NSMase2 (Fig. 4E) but not ASMase (Fig. 4F) in G26-24 cells over a 3h period and that this increase was significantly reversed by the co-addition of 5 μ M GW4869 (Fig. 4E).

3.4. HPTLC and HPLC MS/MS analysis showed increased ceramides in G26-24 cells following MU treatment

Because of increased expression and activity of NSMase2, we investigated changes in Ceramide levels in HA enriched G26-24 cells following MU treatment. HPTLC analysis

showed that the increase in Ceramide was accompanied by a decrease in sphingomyelin (SM) as revealed by [^3H]-palmitate labeling and the ceramide/SM ratio in G26-24 cells increased in a dose (0, 0.5 and 1 mM MU with 24 h treatment) and time (0, 0.5 and 1 h with 1 mM MU) dependent manner (Fig. 5A and 5B). Once again the ceramide/SM ratio was partially restored by GW4869 compared to 1 h MU treatment alone (Fig. 5B). Similarly, an increased Ceramide/SM ratio was detected in HOG cells (Fig. A.3). Further, HPLC MS/MS analysis revealed that all fatty acid species and the total amount of Ceramide increased in G26-24 cells treated with 1 mM MU in a time responsive manner (0, 0.25, 0.5, 1 and 24 h) and that this was significantly reversed by 5 μM GW4869 the NSMase2 inhibitor compared to 24 h sole treatment with MU (Fig. 5C and D). Increased ceramide level indicates that the activation of NSMase2 occurs in less than 15 min (Fig. 5D), which is consistent with previous reports that NSMase2 activation occurs in minutes.

3.5. Exogenous bacterial SMase (bSMase), C2-ceramide and other NSMase2 activators inhibit the synthesis of HA to the same extent as MU

To further confirm the key role of NSMase2/ceramide in MU mediated HA inhibition, we investigated the effect on HA synthesis of NSMase2 activators (bacterial SMase (bSMase), exogenous C2-ceramide and staurosporine etc.). After 5 min treatment in G26-24, we observed stimulation of activity by NSMase2 activators-250mU bSMase, 50nM staurosporine, 200 μM H_2O_2 , and exogenous 50 μM C2-ceramide, 1mM and 2mM MU (Fig. 6A). Following the activation of NSMase2, NSMase2 activators (250mU bSMase, 25nM staurosporine and 200 μM H_2O_2) and exogenous 30 μM , 60 μM and 90 μM C2-ceramide all significantly inhibited the synthesis of HA as MU (Fig. 6B), and C2-ceramide showed a dose-dependent reduction of HA in 24 h (Fig. 6B). Combining this with the effect of the specific NSMase2 inhibitor GW4869 on MU inhibited HA synthesis; we are able for the first time to propose that MU is an activator of NSMase2. Thus the pharmacological function of MU can be mediated by activation of NSMase2 and the resulting elevated sphingolipid second messenger Ceramide. This initiates a cell death signaling pathway and the anti-cancer activity of MU.

3.6. The decreased migration and invasion, and enhanced adherence of G26-24 cells occurs following the inhibition of HA and Calpains after MU treatment

As expected, we found that the migration and invasion of G26-24 was decreased following inhibition of HA after 24 h MU treatment (Fig. 7A and 7B), and both are dose dependent. They were about 24% and 42% decreased compared to control migration, following 0.5mM and 1mM MU treatment, and about 30% and 40% decreased compared to control invasion following 0.5mM and 1mM MU treatment. Corresponding to the reduction of HA, we observed that lower concentrations of MU (such as 0.5mM) enhanced the adherence of G26-24 cells, and cell morphology changes (more processes and more stretched appearance) and more cells attached to the culture dish (Fig. 7C). Similarly, other NSMase2 activators-100 μM H_2O_2 , 25 μM staurosporine and 100 μM bSMase, and exogenous 30 μM C2-ceramide all enhanced G26-24 cell attachment to variable degrees. Interestingly, bSMase treatment showed the most strong attachment effects (Fig. 7C). Recent studies have claimed that PP2A inactivates both calpain1 and calpain2, leading to suppression of migration and invasion of human lung cancer cells (29). Since MU activated NSMase2/Ceramide leads to

increased expression of Ceramide-activated PP2A in MU treated G26-24 cells (Fig. 3B), we therefore investigated the expression and activity of calpains in G26-24 cells following MU treatment. The results showed calpain activity to be decreased by 1 mM MU treatment (Fig. 7D), and both pro-calpain1 and pro-calpain2 proteins were increased in 1mM MU treated G26-24 cells in 24 h (Fig. 7E).

3.7. Ceramide induced apoptotic signaling in G26-24 cells following MU treatment

Unlike the accepted idea that suppression of tumor cell migration and invasion is mainly through the MU-mediated inhibition of HA and calpain signaling, the mechanism of inhibition of proliferation and stimulation of the apoptotic signal induced by MU in tumor cells has been less clear. Some previous studies have inferred the reasons for the inhibition of HA synthesis and reduced HAS2 expression is that the stimulatory effect of PDGF on proliferation was prevented by inhibition of HA-CD44 interaction (30). However, it seems unlikely that the complete growth block raised by MU is only due to the inhibition of HA synthesis since HA synthesis was never totally shut down. Rather, it seems more likely that the HAS expression, cell migration and proliferation are simply all targets of MU induced signals (10). Several recent studies have reported that inhibition of Akt phosphorylation, activated caspase-8, caspase-9, caspase-3 and cleaved PARP etc. (all apoptotic signaling molecules) contribute to cell death in MU treated cancer cells (11). However, it is still hard to make a direct connection between the inhibitions of HA synthesis, decreased HAS2 expression and all these explanations ignore the important role of Ceramide in tumor cell death.

A large amount of literature including our previous studies, have confirmed that Ceramide is the key mediator of oxidative stress to regulate growth and apoptosis of cells (19, 21, 27-28). Following a stress-induced apoptotic signal, Ceramide accumulates through increased NSMase2 mediated Sphingomyelin hydrolysis. The linking of cleavage of caspase-8, caspase-9 and caspase-3 with Ceramide induced dephosphorylation of Akt and its signaling molecules have been well demonstrated previously (27-28, 31). Since we have now discovered that MU induces NSMase2 activation and Ceramide accumulation, combining Ceramide specific effects on growth regulation and induction of apoptosis, the mechanism of MU mediated suppression and apoptosis of tumor cells becomes clear. In this study, we observed the expected elevated Ceramide-inducible PP2A and the reduced phosphorylation of Akt when MU activated NSMase2, together with the subsequent increase in Ceramide (Fig. 3A and B). To further support our hypothesis we showed that elevated MU-induced Ceramide also increased the phosphorylation of p53 in MU-treated G26-24 cells (Fig. 8A). Treatments were for 0, 0.5, 1 and 24 h respectively, and this was reversed by adding the NSMase2 inhibitor 5 μ M GW4869. We also observed that MU-induced Ceramide activated caspase-3 activity following MU treatment (1mM for up to 24h) in G26-24 (Fig. 8B) and increased activated caspase3 expression (Fig. 8C). The increased activation of caspase-3 was reversed by adding 5 μ M GW4869 (Fig. 8C). These results were consistent with reports that the accumulation of Ceramide triggers caspase-3 activation and p53-dependent apoptosis in cancer cells (21, 28 and 32). In addition, we found the longevity protein SIRT1 to be greatly elevated in NSMase2^{-/-} brain tissue (17) and cultured fibroblasts (Fig. 8D) and found that the expression of SIRT1 was decreased in stably transfected Smpd3 fibroblasts (Fig. 8D). It

is known that the deacetylation by SIRT1 enhances binding of Akt and PDK1 to PIP₃ and promotes Akt activation (33). Activation of p53 via SIRT1 inhibition has also been reported (34-39). Consistent with these studies, we found that 1mM MU decreased SIRT1 levels by >50% in G26-24 cells (Fig. 8E) along with activation of p-P53 (Fig. 8A) and inactivation of p-Akt (Fig. 3A). These results strongly suggest that the anti-cancer effect of MU - growth suppression and apoptotic signaling stimulation, was mediated by an NSMase2/Ceramide stimulated signaling pathway, rather than solely inhibiting nucleotide sugar homeostasis

4. Discussion

HA is a large, nonsulfated glycosaminoglycan composed of repeating D-glucuronic acid, (β 1-3) *N*-acetyl-D-glucosamine (β 1-4) units (40, 41), which is produced by three membrane hyaluronan synthases (HAS1/2/3) in mammals. Knock-out mouse developmental studies show that HAS2 is essential but that HAS1 and HAS3 have lesser roles (42, 43). It has been shown that HA levels are elevated in various cancer cells (44) and HA is believed to form a less dense matrix to enhance the cancer cells' motility and invasive ability into other tissues (45). The use of MU as a hyaluronic acid inhibitor with anti-cancer effects has been widely reported recently (8-15), but its working mechanism still has not been well elucidated. Based on studies with HAS2-overexpressed in rat fibroblasts 3Y1, Kakizaki et al (7) previously proposed that MU-mediated inhibition of HA synthesis involved the glucuronidation of MU by endogenous UGT, resulting in a depletion of UDP-GlcUA, a substrate precursor for HA synthesis by HAS2 (7). They found the production of MU-glucuronic acid (MU-GlcUA) identified by MS was consistent with the inhibition of HA synthesis in 3Y1 HAS transfectants (7). Kawizaki et al further suggested that MU post-transcriptionally inhibited HAS2 activity at low or moderate concentrations of MU (<100 μ M). In contrast a high concentration (>300 μ M) of MU suppressed HAS2 function both transcriptionally and post-transcriptionally (7). Although they proposed that the post-transcriptional inhibition of HAS2 activity was from a negative feedback though a MU-GlcUA initiated glucuronidation, they did not further discuss the transcriptional regulation of HAS2 under the high dose of MU (1mM) usually used for anticancer treatment (7). Recently, the efficiency of MU as a glucuronate scavenger has been questioned since Wei et al showed that 1 μ M MU could inhibit glucuronidation in a prostate cancer cell line (46). Based solely on the Km value of the glucuronyltransferase, at such a low concentration, MU would not be expected to function as a competitive inhibitor of glucuronidation or HA synthesis. Another great concern with these explanations is that many drugs such as Morphine, Oxazepam, Bilirubin, Paracetamol, Androsterone and Lamotrigine etc. are all substrates for glucuronidation as part of their metabolism, and there is no evidence showing any competitive effect for HA synthesis (47). Lokeshwar et al (11) therefore proposed that the inhibitory potential of MU may be some other effect rather than competitive inhibition of glucuronidation on HA synthesis, or cell-type dependent expression of specific UGT isozymes. In our study, we found that MU hardly penetrated into cells. Thus we could not find MU uptake by a variety of cells in 3 h (Fig. 2) even following electroporation. Interestingly, we did find that MU initiated the elevation of ceramide (Fig. 5) and this might eventually initiate some MU uptake by cells since accumulated ceramide kill cells and could change the stability or permeability of the cell membrane. However, as a second messenger we believe MU-

induced Ceramide will quickly execute the regulation of signaling before penetrating of MU into cells. In other studies, although Kultti et al (10) proposed that all of the HAS expression, cell migration and proliferation could be targets of MU induced signaling, they did not further verify the signal molecules. Clarkin et al (16) have also reported that UGDH, the regulating enzyme of HA essential precursor UDP-GlcUA, was suppressed by MU and reduced both HA and sGAG production. However, in G26-24 the changes in this enzyme were ambiguous. Previous studies on the anti-cancer effects of MU focused on the inhibition of HA synthesis and HAS2 expression, but any mechanism for the anticancer action of MU has to include both induction of apoptosis and inhibition of metastasis. Our previous research has shown that NSMase2 regulates HAS2 expression (17), and in this study we lay out a novel mechanism for MU action that begins with the activation of NSMase2 and the release of Ceramides.

In our previously work, we found that primary cultured fibroblasts from the *fro/fro* mouse (with a deletion in the *Smpd3* gene coding for the active site of NSMase2) produced increased amounts of HA. We showed that NSMase2 and Ceramide are the key mediators of the regulation of HA synthesis via a Cer/PP2A/Akt/mTOR/HAS2 signal pathway (17). NSMase2, a palmitoylated protein on the surface of the cell, is well-known to be stress-sensitive and rapidly activated (48-50), and typically results in an increase in ceramides (21, 27). The increased Ceramide can turn off HAS2 expression through a suppressed Akt signal, and the regulation by Ceramide was verified directly by adding exogenous ceramides to cells and transfecting *Smpd3* to produce a concomitant reduction in HA secretion (17). Since HAS2 is predominantly in plasma membrane microdomains and thus co-migrates with Ceramide enriched “lipid rafts”, the increased Ceramide might also lower the stability of lipid rafts and affect the activity of HAS2. In support of our findings Kultti et al showed that disruption of lipid rafts by treatment with methyl-beta-cyclodextrin (M β CD) suppressed secretion of HA by down-regulation of PI3K/Akt/mTOR pathway in a breast cancer cell line MCF7 (15). Based on the characteristics of MU on HA synthesis and growth suppression, we theorized that NSMase2 and Ceramide were involved in the cell stress and HA regulation, leading to MU mediated anti-cancer effects. In this study, we clearly show that exogenous MU rapidly activates NSMase2 and elevates Ceramide levels in the HA overproducing glioma cell line G26-24. The expression of HAS2 and HA production were decreased in parallel to the stimulation of NSMase2/Cer, and the increase of NSMase2/Cer could be partially reversed by the specific NSMase2 inhibitor GW4869. Consistently, the inhibition of HAS2 expression and HA synthesis by MU treatment could also be partially reversed by GW4869. Activated PP2A and dephosphorylated Akt were observed along with activated NSMase2 and elevated ceramide by MU in G26-24 cells. In addition, other activators of NSMase2 demonstrated a similar action to that of MU on HA synthesis and cell migration.

Tumor metastasis and invasion requires multi-steps which include alterations in cell adhesion, the expression and activation of motility factors and proteolytic enzyme degradation of the extracellular matrix (51). Besides the role of HA, calpains have also been reported to enhance cancer cell motility and invasivity (52-54). The calpains are a family of calcium-dependent, non-lysosomal, neutral cysteine endopeptidases (52-54). They localize

to focal adhesions and cleave focal adhesion-related proteins including integrin receptors, focal adhesion kinase and talin (55, 56). Calpain1 and 2 are two widely characterized isoforms and show increased expression and activity in various tumor cells (52, 53, 57-60). PP2A suppresses the migration and invasion of tumor cells through dephosphorylation of calpain1 and calpain2 (61). In this study we observed that the suppression of calpain1 and calpain 2 following increased PP2A by the Ceramide generated by MU action, further illustrates the role of Ceramide in the metastasis of tumor cells.

Besides inhibiting metastasis of tumor cells, increased Ceramide levels are likely to induce apoptosis of cancer cells as described in many previous studies (19-21). Ceramide can induce the activation of p53 and the increase of Bax/Bcl-2 ratio followed by release of cytochrome c, leading to caspase-9 and caspase-3 activation in neuroblastoma SKN-SH cells and C6 glioma cells (62, 63). This is consistent with previously reported apoptotic signals attributed to MU, including increased levels of activated caspase-8, caspase-9, caspase-3, and cleaved PARP etc. All these inhibit Akt signaling. SIRT1 also plays an important role in protecting cells from cellular oxidative stress and DNA damage (35, 36), and it has been shown that SIRT1 levels are elevated in cancer tissues (64-67). SIRT1 physically interacts with p53 and deacetylates the Lys382 residue of p53 in a NAD⁺- dependent manner, which in turn decreases p53-mediated transcriptional activation and reduces downstream proteins such as p21 and PUMA levels (68), suggesting a role in tumor suppression (34). In addition, SIRT1 was shown to promote cell migration by deacetylation of cortactin (69). Conversely, SIRT1 is sensitive to NSMase2 since it significantly increases in NSMase2 deficient cells and tissues and decreases in cells overexpressing Smpd3, which encodes NSMase2 protein (17). Consistently, we show that the expression level of SIRT1 was decreased following increased ceramide by MU treatment in G26-24 cells.

In conclusion, our mechanism gives the first clear elucidation of the effects of MU on both cell migration and apoptosis. Thus, MU activates NSMase2 and elevates Ceramide levels in the plasma membrane microdomain. Ceramide then translocates to stimulate the activation of PP2A. Though dephosphorylation of Akt by PP2A and regulation of downstream signals, the expression of HAS2 and synthesis of HA are then reduced. Much of this can happen quickly because it can take place on the cell surface. In addition, PP2A also inhibits the activation of calpains, which, combined with the inhibition of HA, decreases migration and invasivity of cancer cells, thus reducing metastasis by tumor cells. The accumulation of Ceramide by MU may induce mitochondrial oxidative stress and stimulate the activation of apoptotic signaling molecules, such as Caspase3 and p53 etc., to induce apoptosis of tumor cells. Consistently, decreased SIRT1 by MU, which has effects on cell survival and migration, was observed following increased Ceramide and this is the opposite of what we observed in NSMase2 depleted *fro/fro* fibroblasts. Therefore, this new mechanism is the first to provide an integrated elaboration of the anti-cancer properties of MU. It further indicates that Ceramide-generating drugs (such as NSMase2 activators, glucosyltransferase inhibitors and other Ceramide inducers) can be potentially useful for both inducing apoptosis and metastasis inhibitory as part of anti-cancer therapy (Fig. 9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Fibroblasts were derived from *fro/fro* mice generously provided by Dr. Christophe Poirier; MDA-MB-231 cell line was kindly provided by Dr. Olufunmilayo I. Olopade. Mass spectrometric analyses were performed by Dr. Evgeny Berdyshev, Director of Lipidomics Research Facility in University of Illinois at Chicago. We thank Dr. Vytas Bindokas, Director of Microscopy Core Facility in University of Chicago for confocal microscopy analysis. We also thank Dr. Miriam Domowicz and Nancy B. Schwartz for helpful discussions regarding the analysis of hyaluronic acid. We thank Sylvia Dawson for excellent technical assistance in primary cell cultures.

Financial support for this project was provided by National Institutes of Health Grant NS36866-37 from the United States Public Health Service to Dr. Glyn Dawson.

The abbreviations used are

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
fro	fragilis ossium
HA	hyaluronic acid
HAS	HA synthase
HPTLC	high performance thin-layer chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MES	2-(N-Morpholino) ethanesulfonic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MU	4-methylumbelliferone
MβCD	methyl-beta-cyclodextrin
NSMase2	neutral sphingomyelinase2
PP2A	protein phosphatase 2A
sGAG	sulfated-glycosaminoglycan
UDP-GlcUA	UDP-glucuronic acid
UGT	UDP-glucuronyltransferases

References

1. Lokeshwar V, Mirza S, Jordan A. Targeting hyaluronic Acid family for cancer chemoprevention and therapy. *Adv Cancer Res.* 2014; 123:35–65. [PubMed: 25081525]
2. Bharadwaj G, Kovar L, Loughman E, Elowsky C, Oakley G, Simpson A. Spontaneous metastasis of prostate cancer is promoted by excess hyaluronan synthesis and processing. *American Journal of Pathology.* 2009; 174(3):1027–1036. [PubMed: 19218337]
3. Li Y, Li L, Brown J, Heldin P. Silencing of hyaluronan synthase 2 suppresses the malignant phenotype of invasive breast cancer cells. *International Journal of Cancer.* 2007; 120(12):2557–2567.

4. Ropponen K, Tammi M, Parkkinen J, Eskelinen M, Tammi R, Lipponen P, Agren U, Alhava E, Kosma VM. Tumor cell-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. *Cancer Res.* 1998; 58:342–7. [PubMed: 9443415]
5. Ichikawa T, Itano N, Sawai T, Kimata K, Koganehira Y, Saida T, Taniguchi S. Increased synthesis of hyaluronate enhances motility of human melanoma cells. *J Invest Dermatol.* 1999; 113:935–9. [PubMed: 10594733]
6. Itano N, Sawai T, Miyaishi O, Kimata K. Relationship between hyaluronan production and metastatic potential of mouse mammary carcinoma cells. *Cancer Res.* 1999; 59:2499–2504. [PubMed: 10344764]
7. Kakizaki I, Kojima K, Takagaki K, Endo M, Kannagi R, Ito M, Maruo Y, Sato H, Yasuda T, Mita S, Kimata K, Itano N. A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. *J Biol Chem.* 2004; 279:33281–9. [PubMed: 15190064]
8. Yoshihara S, Kon A, Kudo D, Nakazawa H, Kakizaki I, Sasaki M, Endo M, Takagaki K. A hyaluronan synthase suppressor, 4-methylumbelliferone, inhibits liver metastasis of melanoma cells. *FEBS Lett.* 2005; 579:2722–6. [PubMed: 15862315]
9. Arai E, Nishida Y, Wasa J, Urakawa H, Zhuo L, Kimata K, Kozawa E, Futamura N, Ishiguro N. Inhibition of hyaluronan retention by 4-methylumbelliferone suppresses osteosarcoma cells in vitro and lung metastasis in vivo. *Br J Cancer.* 2011; 105:1839–49. [PubMed: 22045192]
10. Kultti A, Pasonen-Seppänen S, Jauhainen M, Rilla KJ, Kärnä R, Pyörä E, Tammi RH, Tammi MI. 4-Methylumbelliferone inhibits hyaluronan synthesis by depletion of cellular UDP-glucuronic acid and downregulation of hyaluronan synthase 2 and 3. *Exp Cell Res.* 2009; 315(11):1914–23. [PubMed: 19285976]
11. Lokeshwar VB, Lopez LE, Munoz D, Chi A, Shirodkar SP, Lokeshwar SD, Escudero DO, Dhir N, Altman N. Antitumor activity of hyaluronic acid synthesis inhibitor 4-methylumbelliferone in prostate cancer cells. *Cancer Res.* 2010; 70:2613–23. [PubMed: 20332231]
12. Nakazawa H, Yoshihara S, Kudo D, Morohashi H, Kakizaki I, Kon A, et al. 4-Methylumbelliferone, a hyaluronan synthase suppressor, enhances the anticancer activity of gemcitabine in human pancreatic cancer cells. *Cancer Chemotherapy and Pharmacology.* 2006; 57(2):165–170. [PubMed: 16341905]
13. Nagy N, Kuipers HF, Frymoyer AR, Ishak HD, Bollyky JB, Wight TN, Bollyky PL. 4-Methylumbelliferone treatment and hyaluronan inhibition as a therapeutic strategy in inflammation, autoimmunity, and cancer. *Front Immunol.* 2015; 6:123. [PubMed: 25852691]
14. Piccioni F, Malvicini M, Garcia MG, Rodriguez A, Atorrasagasti C, Kippes N, et al. Antitumor effects of hyaluronic acid inhibitor 4-methylumbelliferone in an orthotopic hepatocellular carcinoma model in mice. *Glycobiology.* 2012; 22(3):400–410. [PubMed: 22038477]
15. Twarock S, Freudenberger T, Poscher E, Dai G, Jannasch K, Dullin C, et al. Inhibition of oesophageal squamous cell carcinoma progression by in vivo targeting of hyaluronan synthesis. *Molecular Cancer.* 2011; 10:1–14. [PubMed: 21205300]
16. Clarkin E, Allen S, Wheeler-Jones P, Bastow R, Pitsillides A. Reduced chondrogenic matrix accumulation by 4-methylumbelliferone reveals the potential for selective targeting of UDP-glucose dehydrogenase. *Matrix Biol.* 2011; 30(3):163–8. [PubMed: 21292001]
17. Qin J, Berdyshev E, Poirer C, Schwartz NB, Dawson G. Neutral sphingomyelinase 2 deficiency increases hyaluronan synthesis by up-regulation of Hyaluronan synthase 2 though decreased ceramide production and activation of Akt. *J Biol Chem.* 2012; 287(17):13620–32. [PubMed: 22383528]
18. Kakoi H, Maeda S, Shinohara N, Matsuyama K, Imamura K, Kawamura I, Nagano S, Setoguchi T, Yokouchi M, Ishidou Y, Komiya S. Bone morphogenic protein (BMP) signaling up-regulates neutral sphingomyelinase 2 to suppress chondrocyte maturation via the Akt protein signaling pathway as a negative feedback mechanism. *J Biol Chem.* 2014; 289(12):8135–50. [PubMed: 24505141]
19. Yoshimura S, Banno Y, Nakashima S, Takenaka K, Sakai H, Nishimura Y, Sakai N, Shimizu S, Eguchi Y, Tsujimoto Y, Nozawa Y. Ceramide formation leads to caspase-3 activation during hypoxic PC12 cell death. Inhibitory effects of Bcl-2 on ceramide formation and caspase-3 activation. *J Biol Chem.* 1998; 273:6921–7. [PubMed: 9506997]

20. Lavie Y, Cao H, Bursten SL, Giuliano AE, Cabot MC. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J. Biol. Chem.* 1996; 271:19530–19536. [PubMed: 8702646]
21. Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat Rev Cancer.* 2004; 4:604–16. [PubMed: 15286740]
22. Poirier C, Berdyshev EV, Dimitropoulou C, Bogatcheva NV, Biddinger PW, Verin AD. Neutral sphingomyelinase 2 deficiency is associated with lung anomalies similar to emphysema. *Mamm Genome.* 2012; 23(11-12):758–63. [PubMed: 22945695]
23. Kim J, Okimoto A, Purton E, Goodwin M, Haserlat M, Dayyani F, Sweetser A, McClatchey I, Bernard A, Look T, Bell W, Scadden T, Haber A. Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias. *Blood.* 2008; 111(9):4716–22. [PubMed: 18299447]
24. Philipson LH, Schwartz NB. Subcellular localization of hyaluronate synthetase in oligodendroglioma cells. *J Biol Chem.* 1984; 259:5017–23. [PubMed: 6425282]
25. Sundarraj N, Schachner M, Pfeiffer SE. Biochemically differentiated mouse glial lines carrying a nervous system specific cell surface antigen (NS-1). *PNAS.* 1975; 72:1927–1931. [PubMed: 168583]
26. Post GR, Dawson G. Characterization of a cell line derived from a human oligodendroglioma. *Mol Chem Neuropathol.* 1992; 16:303–17. [PubMed: 1329795]
27. Bartke N, Hannun Y. Bioactive sphingolipids: metabolism and function. *J Lipid Res.* 2009; 50(Suppl):S91–S96. [PubMed: 19017611]
28. Morad S, Cabot M. Ceramide-orchestrated signaling in cancer cells. *Nat Rev Cancer.* 2013; 13(1): 51–65. [PubMed: 23235911]
29. Xu, Lijun; Deng, Xingming. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of μ - and m-calpains. *J Biol Chem.* 2006; 281:35567–35575. [PubMed: 16982626]
30. Li L, Asteriou T, Bernert B, Heldin CH, Heldin P. Growth factor regulation of hyaluronan synthesis and degradation in human dermal fibroblasts: importance of hyaluronan for the mitogenic response of PDGF-BB. *Biochem. J.* 2007; 404:327–336. [PubMed: 17324121]
31. Hannun Y, Obeid L. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Molecular cell biology.* 2008; 9(2):139–150. [PubMed: 18216770]
32. Pruschy M, Resch H, Shi YQ, Aalame N, Glanzmann C, Bodis S. Ceramide triggers p53-dependent apoptosis in genetically defined fibrosarcoma tumour cells. *Br J Cancer.* 1999; 80(5-6): 693–8. [PubMed: 10360645]
33. Sundaresan NR, Pillai VB, Wolfgeher D, Samant S, Vasudevan P, Parekh V, Raghuraman H, Cunningham JM, Gupta M, Gupta MP. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci Signal.* 2011; 4(182):ra46. [PubMed: 21775285]
34. Lee JT, Gu W. SIRT1: Regulator of p53 Deacetylation. *Genes Cancer.* 2013; 4(3-4):112–7. [PubMed: 24020002]
35. Luo J, Nikolaev AY, Imai S, et al. Negative control of p53 by Sir2 α promotes cell survival under stress. *Cell.* 2001; 107(2):137–48. [PubMed: 11672522]
36. Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2 (SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell.* 2001; 107(2):149–59. [PubMed: 11672523]
37. Motta MC, Divecha N, Lemieux M, et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell.* 2004; 116(4):551–63. [PubMed: 14980222]
38. Nemoto S, Fergusson MM, Finkel T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1 α . *J Biol Chem.* 2005; 280(16):16456–60. [PubMed: 15716268]
39. Picard F, Kurtev M, Chung N, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR- γ . *Nature.* 2004; 429(6993):771–6. [PubMed: 15175761]
40. Bastow R, Byers S, Golub B, Clarkin E, Pitsillides A, Fosang J. Hyaluronan synthesis and degradation in cartilage and bone. *Cell. Mol. Life Sci.* 2008; 65:395–413. [PubMed: 17965830]

41. Tammi H, Passi G, Rilla K, Karousou E, Vigetti D, Makkonen K, Tammi I. Transcriptional and post-translational regulation of hyaluronan synthesis. *FEBS J.* 2011; 278:1419–1428. [PubMed: 21362137]
42. Weigel PH, DeAngelis PL. Hyaluronan synthases, a decade-plus of novel glycosyltransferases. *J. Biol. Chem.* 2007; 282:36777–36781. [PubMed: 17981795]
43. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J. Clin. Invest.* 2000; 106:349–360. [PubMed: 10930438]
44. Lin G, Stern R. Plasma hyaluronidase (Hyal-1) promotes tumor cell cycling. *Cancer Letters.* 2001; 163:95–101. [PubMed: 11163112]
45. Hill A, McFarlane S, Johnston PG, Waugh DJJ. The emerging role of CD44 in regulating skeletal micrometastasis. *Cancer Lett.* 2006; 237(1):1–9. [PubMed: 15979783]
46. Wei Q, Galbenus R, Raza A, Cerny RL, Simpson MA. Androgen-stimulated UDP-glucose dehydrogenase expression limits prostate androgen availability without impacting hyaluronan levels. *Cancer Research.* 2009; 69(6):2332–2339. [PubMed: 19244115]
47. Wikipedia. 2015. <http://en.wikipedia.org/wiki/Glucuronidation>
48. Wiegmann K, Schutze S, Machleidt T, Witte D, Kronke M. Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell.* 1994; 78:1005–1015. [PubMed: 7923351]
49. Filosto S, Fry W, Knowlton AA, Goldkorn T. Neutral sphingomyelinase 2 (nSMase2) is a phosphoprotein regulated by calcineurin (PP2B). *J Biol Chem.* 2010; 285(14):10213–22. [PubMed: 20106976]
50. Clarke CJ, Guthrie JM, Hannun YA. Regulation of neutral sphingomyelinase-2 (nSMase2) by tumor necrosis factor- α involves protein kinase C- δ in lung epithelial cells. *Mol Pharmacol.* 2008; 74(4):1022–32. [PubMed: 18653803]
51. Chen B, Tang J, Guo YS, Li Y, Chen ZN, Jiang JL. Calpains are required for invasive and metastatic potentials of human HCC cells. *Cell Biol Int.* 2013; 37(7):643–52. [PubMed: 23733271]
52. Storr SJ, Carragher NO, Frame MC, Parr T, Martin SG. The calpain system and cancer. *Nat Rev Cancer.* 2011; (5):364–74. [PubMed: 21508973]
53. Franco SJ, Huttenlocher A. Regulating cell migration: calpains make the cut. *J Cell Sci.* 2005; 118:3829–38. [PubMed: 16129881]
54. Carragher NO, Fonseca BD, Frame MC. Calpain activity is generally elevated during transformation but has oncogene-specific biological functions. *Neoplasia.* 2004; 6:53–73. [PubMed: 15068671]
55. Chan KT, Bennin DA, Huttenlocher A. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). *J. Biol. Chem.* 2010; 285:11418–11426. [PubMed: 20150423]
56. Franco SJ, et al. Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nature Cell Biol.* 2004; 6:977–983. [PubMed: 15448700]
57. Yamaguchi R, Maki M, Hatanaka M, Sabe H. Unphosphorylated and tyrosine-phosphorylated forms of a focal adhesion protein, paxillin, are substrates for calpain II in vitro: implications for the possible involvement of calpain II in mitosis-specific degradation of paxillin. *FEBS Lett.* 1994; 356:114–116. [PubMed: 7988702]
58. Franco S, Perrin B, Huttenlocher A. Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp. Cell Res.* 2004; 299:179–187. [PubMed: 15302585]
59. Wang H, et al. PKA-mediated protein phosphorylation protects ezrin from calpain I cleavage. *Biochem. Biophys. Res. Commun.* 2005; 333:496–501. [PubMed: 15950939]
60. Raynaud F, et al. The calpain I- α -actinin interaction. Resting complex between the calcium-dependent protease and its target in cytoskeleton. *Eur. J. Biochem.* 2003; 270:4662–4670. [PubMed: 14622253]

61. Xu L, Deng X. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. *J Biol Chem*. 2006; 281(46):35567–75. [PubMed: 16982626]
62. Pruschy M, Resch H, Shi YQ, Aalame N, Glanzmann C, Bodis S. Ceramide triggers p53-dependent apoptosis in genetically defined fibrosarcoma tumour cells. *Br J Cancer*. 1999; 80(5-6): 693–8. [PubMed: 10360645]
63. Sawada M, Nakashima S, Banno Y, Yamakawa H, Hayashi K, Takenaka K, Nishimura Y, Sakai N, Nozawa Y. Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells. *Cell Death Differ*. 2000; 7(9):761–72. [PubMed: 11042671]
64. Blander G, Guarente L. The Sir2 family of protein deacetylases. *Annual Review of Biochemistry*. 2004; 73:417–435.
65. Huffman DM, Grizzle WE, Bamman MM, et al. SIRT1 is significantly elevated in mouse and human prostate cancer. *Cancer Research*. 2007; 67:6612–6618. [PubMed: 17638871]
66. Jang KY, Kim KM, Hwang SH, et al. Expression and prognostic significance of SIRT1 in ovarian epithelial tumours. *Pathology*. 2009; 41:366–371. [PubMed: 19404850]
67. Jang KY, Hwang SH, Kwon KS, et al. SIRT1 expression is associated with poor prognosis of diffuse large B-cell lymphoma. *American Journal of Surgical Pathology*. 2008; 32:1523–1531. [PubMed: 18724249]
68. Yamakuchi M, Lowenstein CJ. MiR-34, SIRT1 and p53. *Cell Cycle*. 2009; 8:712–715. [PubMed: 19221490]
69. Zhang Y, Zhang M, Dong H, Yong S, Li X, Olashaw N, Kruk PA, Cheng JQ, Bai W, Chen J, Nicosia SV, Zhang X. Deacetylation of cortactin by SIRT1 promotes cell migration. *Oncogene*. 2009; 28(3):445–60. [PubMed: 18850005]

Fig. 1. The effects of MU on HA production, cell viability and morphology changes in G26-24 cells

A, 1 mM MU significantly blocks synthesis of HA (24 h) in G26-24 cells. B, MTT assay shows dose-respondering decreases of cell viability induced by MU in G26-24 cells (0, 0.25, 0.5, 0.75 and 1 mM MU). C, Morphological changes of G26-24 cells after treatment with 1mM MU for 24 h are blocked by NSMase2 inhibitor GW4869. Magnification, X200. D, Dead and apoptosis cell analysis by flow cytometry. a-f, Shows cell distributions after MU treatment for 24 h (con, GW4869, 1mM MU, 5 μ M GW and 1mM MU, 2mM MU, 5 μ M GW and 2mM MU); g, Shows the percentage of dead and dying cells (early and late apoptotic

cells). GW4869 clearly reverses cell death induced by MU. The procedures are as described in the text and results are representative of three independent experiments. Each experiment includes triplicate treatment samples. Data are means $\pm$ SEM, statistically significant when $p < 0.05$. Scale bar represent 100 μ M.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. MU is not significantly taken up by G26-24 and HOG cells

A, Fluorescence analysis shows no uptake of MU by G26-24 cells. When compared to the negative control (without MU administration) and the positive control (0.15 mM MU in serum free DMEM), no fluorescence uptake was detected either in whole cell lysate or in supernatant (with 1mM MU administration for 3 h). Ex/Em is 360/460 (+/-20). B and C, Fluorescence staining analysis (Ex 405 for MU and Ex 633 for DRAQ5) shows no uptake of MU by G26-24 (B) and HOG cells (C) after 1mM MU for 3 h using a Marianas confocal microscope (X100 oil objective Lens). No significant fluorescence density differences were

detected in 1mM MU treated G26-24 and HOG cells compared to their control (except the staurosporine treated positive control). The procedures are as described in the text and results represent one of three independent experiments; each experiment includes triplicate treatment samples.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. The effects of MU on phosphorylation of Akt, activation of PP2A and inhibition of HAS2 expression in G26-24

A, Western blot shows de-phosphorylation of Akt in G26-24 cells after 24 h treatment with 1mM MU. B, Western blot shows increased expression of PP2A after treatment with 1mM MU 24 h in G26-24 cells and GW4869 inhibits MU induced activation of PP2A. C, Western blots show that MU blocks HAS2 expression in G26-24 cells (0.5, 1, 3 and 24 h). The NSMase2 inhibitor 5 μ M GW4869 partially reversed the inhibition. The figure represents one of three independent experiments; each experiment including triplicate treatment samples. Column graphs show quantification of the blots corrected for protein. The procedures are as described in the text.

Fig. 4. MU initiated translocation and activity of NSMase2 in HOG and G26-24 cells

A, Lipid raft analysis shows that NSMase2 expression increased and NSMase2 moved into the lipid raft fraction after 1 mM MU treatment in 1 h in Smpd3(encoding NSMase2) transfected HOG cells. B, Western blots show increased expression of NSMase2 at 0.5 and 1 h after 1 mM MU treatment in Smpd3 transfected HOG cells. C, Enzyme analysis shows that 1 mM MU activates NSMase2 in HOG cells for up to 3 h. D, Western blots show MU increases NSMase2 for up to 24 h and this is partially reversed by 5μM GW4869 (compared to 24 h treatment). E and F, Enzyme analysis shows that 1 mM MU activates NSMase2 in G26-24 cells linearly for 3 h, E) but there is no corresponding increase in ASMase activity (3 h) in G26-24 (F). MU activated NSMase2 is partially reversed by GW4869 in G26-24 cells (E, compared to 3 h). The procedures are as described in the text and results are representative of three independent experiments. Each experiment includes triplicate treatment samples.

Fig. 5. MU induced elevation of Ceramide in HA enriched G26-24 cells

A and B, [^3H]palmitate labeling and HPTLC analysis shows that MU increases the Ceramide/SM ratio in a dose (A, 0, 0.5 and 1 mM for 24 h treatment) and time (B, 0, 0.5 and 1 h with 1mM MU) dependent manner. The increased Ceramide/SM ratio was reversed by GW4869 in G26-24 cells (B, compared to 1 h treatment). C, HPLC/MS/MS Analyses showed time responsively increased total Ceramide (0, 0.25, and 0.5, 1 and 24 h) and this was partially reversed by 5 μM GW4869 in G26-24 cells (compared to 24 h). D, HPLC/MS/MS analysis shows that 1mM MU induces a broad increase in Ceramides and this can be reversed by GW4869 in G26-24 cells (compared to 24 h). The procedures are as described in the text, the figure represents one of three independent experiments. Each experiment includes triplicate treatment samples. Data are means $\pm$ SEM, statistically significant when $p < 0.05$.

Fig. 6. Multi-NSMase2 activators and exogenous C₂Ceramide reduce the synthesis of HA

A, Enzyme analysis shows that multi-NSMase2 activators (Staurosporine, bSMase, H₂O₂ and MU) and exogenous C₂Ceramide activate NSMase2 in G26-24 cells. B, The reduction of HA synthesis is mediated by multi-NSMase2 activators (Staurosporine, bSMase, H₂O₂) and exogenous C₂Ceramide (30, 60, 90μM) in 24 h. The procedures are as described in the text. The figure represents one of three independent experiments, and each experiment includes triplicate treatment samples. Data are means±SEM, statistically significant when $p < 0.05$.

Fig. 7. Decreased migration and invasion, enhanced adherence of G26-24 cells, and inhibited activation of calpain1 and 2 are mediated by NSMase2 activators and exogenous C₂Ceramide
 A and B, Migration (A) and invasion (B) analysis show that the level of migration and invasion of G26-24 cells is significantly decreased by 0.5mM and 1mM MU treatment after 24 h. C, Morphological changes of G26-24 cells after treatment with multi-activators of NSMase2 (Staurosporine, bSMase, H₂O₂ and MU) and exogenous C₂Ceramide for 24 h, all show increased adherence of cells. D, The activity of calpains is significant decreased in G26-24 cells treated with 1mM MU (1h). E, Western blots show that 1mM MU induced PP2A inhibits activation of capain1 and calpain2, expression of pro-calpain1 and pro-

calpain2 are increased in 24 h. The right column graphs shows quantification of the blots corrected for protein (left panel, pro-calpain1; right panel, pro-calpain-2). The procedures are as described in the text and results are representative of three independent experiments. Each experiment includes triplicate treatment samples. Data are means $\pm$ SEM, statistically significant when $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 8. The activation of apoptotic signaling molecules by MU induced NSMase2/ceramide
 A, Western blot shows p-P53 activation after 0.5 h (lane 2), 1h (lane 4) and 24h (lane 7). 5μM GW4869 inhibits the activation (lanes 3 and 5). B and C, Pro-caspase3 is cleaved (C) and Caspase3 is activated after 24h treatment by MU (B). D, Western blot shows SIRT1 is increased in fibroblasts from *fro/fro* mouse which lacks NSMase2 (lane 2), the activation of SIRT1 is reversed by transfection with the *Smpd3* gene for NSMase2 (lane 3). E, SIRT1 expression is decreased by 1mM MU treatment after 24 h in G26-24 cells. SIRT1 is decreased by MU treatment suggesting that it is regulated by Ceramide. The figure represents one of three independent experiments. The right column graphs show quantification of the blots corrected for protein.

Fig. 9. A scheme to explain the anticancer mechanism of MU on glioblastoma G26-24 cells following its initial action on plasma membrane neutral sphingomyelinase2
Like other activators of NSMase2, MU initially stimulates NSMase2 expression and activation and hydrolyses SM to Ceramide. The increased ceramide activates PP2A, dephosphorylating p-Akt and turning off HAS2 expression. PP2A also inhibits calpains, thereby combining reduced HA with decreasing tissue migration and reduced invasivity of tumor cells. The increased Ceramide caused by MU also stimulates the activation of p53 and caspase3 etc. apoptotic molecules and inhibits Sirt1 expression to initiate arrest of cell proliferation and induce apoptosis. This is a new mechanism to explain the anti-cancer properties of MU, indicating that Ceramide-generating drugs can be potentially useful for both inducing growth suppression and inhibiting metastasis of tumor cells.