Inhibition of Tumor Necrosis Factor-induced Cell Death in MCF7 by a Novel Inhibitor of Neutral Sphingomyelinase*

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A high throughput screen for neutral, magnesium-dependent sphingomyelinase (SMase) was performed. One inhibitor discovered in the screen, GW4869, functioned as a noncompetitive inhibitor of the enzyme in vitro with an IC₅₀ of 1 μM. It did not inhibit acid SMase at up to at least 150 μM. The compound was then evaluated for its ability to inhibit tumor necrosis factor (TNF)-induced activation of neutral SMase (N-SMase) in MCF7 cells. GW4869 (10 μM) partially inhibited TNF-induced sphingomyelin (SM) hydrolysis, and 20 μM of the compound was protected completely from the loss of SM. The addition of 10–20 μM GW4869 completely inhibited the initial accumulation of ceramide, whereas this effect was partially lost at later time points (24 h). These data therefore support the inhibitory action of GW4869 on N-SMase not only in vitro but also in a cellular model. The addition of GW4869 at both 10 and 20 μM did not modify cellular glutathione levels in response to TNF, suggesting that the action of GW4869 occurred downstream of the drop in glutathione, which was shown previously to occur upstream of the activation of N-SMase. Further, whereas TNF treatment also caused a 75% increase of de novo synthesized ceramide after 20 h of incubation, GW4869, at either 10 or 20 μM, had no effect on this pathway of ceramide generation. In addition, GW4869 did not significantly impair TNF-induced NF-κB translocation to nuclei. Therefore, GW4869 does not interfere with other key TNF-mediated signaling effects. GW4869 was able, in a dose-dependent manner, to significantly protect from cell death as measured by nuclear condensation, caspase activation, PARP degradation, and trypan blue uptake. These protective effects were accompanied by significant inhibition of cytochrome c release from mitochondria and caspase 9 activation, therefore localizing N-SMase activation upstream of mitochondrial dysfunction. In conclusion, our results indicate that N-SMase activation is a necessary step for the full development of the cytotoxic program induced by TNF.

Programmed cell death is a necessary requirement during development, and an altered regulation of this process is also thought to contribute to the occurrence of serious illnesses such as cancer and neurodegenerative diseases. Significant progress has been made in the identification of crucial events that contribute to defining this process (mitochondrial dysfunction, chromatin fragmentation, and membrane blebbing) as well as in the identification of molecular mechanisms and effectors that regulate and/or execute these events (exposure of phosphatidylserine on the plasma membrane, depolarization of the outer mitochondrial membrane, formation of permeability transition pores in the mitochondrial membrane, cytochrome c release, caspase activation, and alteration of calcium homeostasis, Bcl2 family members, apoptosis-inducing factor, apoptosis activating factor-1, Smac/Diablo, IAPs, and others).

The sphingolipid ceramide has been shown to induce cellular features characteristic of programmed cell death (membrane blebbing, mitochondrial dysfunction, and nuclear fragmentation). Moreover, many agents known to cause apoptosis (such as TNF, CD95 cross-linking, daunorubicin, heat stress, growth factor withdrawal, UV-B and γ-radiation, bacterial infections, and others) have also been found to increase the intracellular levels of ceramide, with emerging evidence suggesting an important role for ceramide in regulating/mediating the apoptotic response to these agents (for reviews, see Refs. 1–3). A major current challenge is to define the role of the pathway promoted by ceramides and integrate it with other molecular mechanisms that lead to cell death.

Two main routes have been defined for the generation of ceramide: hydrolysis of sphingomyelin and de novo biosynthesis. The first occurs by the action of sphingomyelinases (SMases), which operate at different pH optima (acid or neutral SMase) and have different metal requirements (magnesium dependence) (2, 4). Activation of acid sphingomyelinase (A-SMase) has been observed after treatment with UV-A radiation (5) and stimulation of the p75 neurotropin receptor (6), CD28 (7), TNF receptor, and CD95 (8), although some of these latter conclusions have been recently questioned (9, 10). Neutral SMase (N-SMase) activation has been also observed after stimulation of the p75 neurotropin receptor (11), after ligation of CD95 and TNF receptor (12, 13), and after irradiation (14) but also upon heat stress and serum starvation (15), treatment

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¶¶ The abbreviations used are: TNF, tumor necrosis factor; SMase, sphingomyelinase; N-SMase, neutral sphingomyelinase; A-SMase, acid sphingomyelinase; PARP, poly(ADP-ribose) polymerase; PS, phosphatidylserine; SM, sphingomyelin; PAF, platelet-activating factor; PLC, phospholipase C; FBS, fetal bovine serum; MSA, methane sulfonic acid; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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with vitamin D (16), and CD40 ligation (17). The de novo pathway is regulated primarily by the action of serine palmitoyltransferase, the first enzyme along the biosynthetic pathway. Accumulation of ceramide via de novo biosynthesis was originally shown after treatment with retinoic acid in GH4C1 (18) and then with daunorubicin in P388 and U937 cells (19), angiotensin II stimulation of PC12W cells (20), etoposide treatment (21), IgM-induced receptor cross-linking in Ramos B cells (22), loading of palmitate in beta cells (23), and TNF/cycloheximide treatment of bovine cerebral endothelial cells (24).

The study of the de novo pathway has benefited tremendously from the availability of highly specific inhibitors: fumonisin B1 for ceramide synthase (25) and myriocin/ISP1 for serine palmitoyltransferase (26). On the other hand, very few tools have been available to dissect the neutral sphingomyelinase pathway. One molecule, scyphostatin, has been previously described to exert inhibitory activity versus a N-SMase (27, 28), and it has been used to implicate N-SMase in the outgrowth process of hippocampal neurons in response to nerve growth factor (29). We set out to develop specific inhibitors of N-SMase and to determine the role of N-SMase in apoptosis and how it interacts with other key regulators of apoptosis. A high throughput assay was developed for N-SMase, and we successfully identified a molecule, GW4869 (Fig. 1), that exhibited significant and specific inhibitory activity on N-SMase. We provide evidence of the specificity of GW4869 in vitro. Next, the effects of this inhibitor were determined in MCF7 breast cancer cells treated with TNF, which has emerged as one of the best characterized models of cytokine-induced cell death and of ceramide function. It has been previously shown that TNF induces activation of N-SMase in these cells and that this activation is a consequence of the drop of glutathione that follows the activation of the death receptor and caspase 8 (13). Also, this activation couples to processing of effector caspases (30).

Using this newly characterized inhibitor, we were able to confirm N-SMase activation upon TNF treatment of MCF7 and its effects on effector caspases and cell death. Second, we provide evidence for the first time of the participation of N-SMase in the processes that lead to cytochrome c release and mitochondrial dysfunction, indicating the requirement for N-SMase activation as a necessary step for the complete development of the cytotoxic program induced by TNF.

**EXPERIMENTAL PROCEDURES**

**Materials—** RPMI 1640 medium was from Invitrogen. Fetal bovine serum (FBS) was from Summit Technology. TNF was from Peprotech (Rocky Hill, NJ). [γ-32P]ATP, [methyl-3H]choline chloride, and ENHANCE spray were from PerkinElmer Life Sciences. [choline-13C]ISOM was provided by Alicia Bielawska (Medical University of South Carolina, Charleston, SC). SM and PS were from Avanti Polar Lipids, Inc. Silica Gel 60 thin layer chromatography plates were from Whatman. Scintillation mixture Safety Solve was from Research Products International. Poly(dI-dC) and poly(dN6) were from Amersham Biosciences. Rabbit anti-p65 NF-κB antibodies were from Rockland, monoclonal anti-cytochrome c and anti-human caspase 6 antibodies were from Becton Dickinson Corp.; and rabbit anti-PARP, mouse anti-FLAG, and anti-parvalbumin antisera were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**In Vitro Sphingomyelinase Activity Assays—** Partially purified and delipidated rat brain neutral sphingomyelinas were prepared and assayed as described (31) with variable amounts of SM or PS incorporated into the assay via solubilization into Triton X-100 micelles. Human acid sphingomyelinas, overexpressed in SF-9 insect cells using a baculoviral system, was similarly assayed in a final reaction volume of 50 μl containing 100 mM sodium acetate (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A in the absence of detergents, and the postnuclear extract was centrifuged for 1 h at 100,000 g at 4 °C. The pellet, containing the total membranes, was resuspended in lysis buffer containing 0.1% Triton X-100 and incubated on a rotating platform for 1 h at 4 °C. The suspension was then centrifuged for 1 h at 100,000 g at 4 °C, and the supernatant was applied to a HiTrap Q column equilibrated with 20 mM Tris-HCl, pH 7.4, 0.1% EDTA. The lyso-PAF-PLC was eluted with a linear salt gradient (1.5 M NaCl). Fractions were collected, N-Smase activity was measured as previously indicated (33), and the protein level was determined by Western blot analysis using anti-His6 monoclonal antibodies against the histidine-FLAG of the protein. The fraction containing peak activity (which also showed most of the protein by western) was used for determining the effect of GW4869 in vitro.

**Specificity of GW4869 on Enzyme Inhibition—** Partially purified rat brain N-SMase and lyso-PAF PLC were incubated in the absence or presence of GW4869 and PS (100 μM), and SM hydrolysis was determined as previously described (30). In the case of Bacillus cereus N-SMase (Sigma), PS was not included in the reaction mixture, since it does not affect the bacterial enzymatic activity. B. cereus phosphatidylycerol-PLC (Sigma) was incubated in the presence or absence of GW4869 in a reaction mixture containing 100 mM Tris, pH 7.2, 25% glycerol, 20 mM p-nitrophenol/phosphorylcholine, and production of p-nitrophenol was quantified spectrophotometrically at 410 nm. Protein phosphatase 2A from bovine kidney (Calbiochem) was incubated in the presence or absence of GW4869 in buffer containing 50 mM Tris, pH 7.4, 1 mM dithiothreitol, 100 μM MnCl2, and 20% glycerol, and phosphatase activity was measured as described by Jones and Hannun (34).

**Inhibition of TNF-induced Cell Death by an N-SMase Inhibitor**

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Cell Culture and GW4869 Treatment—MCF7 human breast cancer cells were routinely cultured in RPMI 1640 containing 10% FBS at 37 °C in 5% CO2. Unless otherwise indicated, for treatment, cells were seeded at 1.7 × 106 cells/10-cm dish in 8 ml of complete growth medium; after 24 h, the medium was replaced with 7 ml of RPMI 1640 containing 2% FBS and 25 mM Hesper, pH 7.5, and the cells were rested for 2 h prior to treatment. GW4869 was routinely stored at −80 °C as a 1.5 mM stock suspension in Me2SO. Right before use, the suspension was solubilized by the addition of 5% methanol sulfonic acid (MSA) (2.5 μl of 5% MSA in sterile double-distilled H2O) were added to 50 μl of GW4869 stock suspension; therefore, the concentration of the GW4869 stock solution at the time of the experiments was 1.43 mM. The suspension was mixed and warmed up at 37 °C until clear. Cells were preincubated with the inhibitor for 30 min prior to treatment with TNF. Control cells were treated with Me2SO containing 5% MSA, similarly to the samples receiving the GW4869 solution. When different doses of GW4869 were tested, amounts of vehicle solution were added in order to equal the volume of GW4869 used for the highest dose.

**Sphingomyelin Measurement—** Cells were seeded at 0.1 × 106 cells/10-cm dish in 8 ml of complete growth medium. After 48 h, the cells were labeled with [methyl-3H]choline chloride (1 μCi/ml final concentration in 10 ml of growth medium/plate). After −60 h, the cells were washed once with 5 ml of PBS, and 7 ml of medium containing 2% FBS and 25 mM Hesper, pH 7.5, were added. After resting the cells for 1 h, preincubated with the inhibitor for 30 min, the cells were washed once with 5 ml of PBS, and TNF treatment followed. At the appropriate time points, the medium from each plate was collected, and the cells were washed once with 2 ml of ice-cold PBS. Cells were scraped off in 2 ml of PBS, and each plate was washed with an additional 2 ml of PBS. Cells and washes were pooled with the medium and centrifuged for 5 min at 4 °C. The supernatant was collected and analyzed. To the measurement, the pellets were resuspended in 600 μl of double-distilled H2O by vortexing and sonication. Aliquots of cell lysates were used for protein determination, and 250 μl in duplicate were used for SM determination as described by Andrieu et al. (35).

**Measurements of Mass Levels of Ceramide**—Cells were harvested in 10-cm dishes, and lipids were extracted from the lyophilized cells using the method of Bligh and Dyer (36). The chloroform organic phase was divided into aliquots (in duplicates) and dried down for ceramide and phosphatidylcholine measurements. Ceramide levels were evaluated using the 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine and 1,2-Di[1-14C]glycerophosphorylcholine kinase assay. Ceramide was quantitated by using external stand-
ards and normalized to phosphate content (37).

Measurement of Ceramide Generated by de Novo Biosynthesis—Cells were seeded at 1.7 × 10⁶ cells/plate in complete growth medium. After 24 h, the medium was replaced with 6 ml of RPMI 1640 containing 2% FBS and 25 mM Hepes, pH 7.5. Right before the addition of GW4869, [³H]palmitate was added to the cells in 1 ml of the same medium to a final activity of 1 μCi/ml. After ~21 h of treatment with TNF, the medium was collected, and the plates were washed once with PBS that was combined with the medium and centrifuged at 2000 × g for 5 min at 4 °C to collect floating cells. Cells were scraped off the plate with methanol and combined with the floaters. Lipids were extracted by the method of Bligh and Dyer (36). One ml of the organic phase was used for ceramide determination by separation of lipids through thin layer chromatography (5, 19). Nuclei were visualized by a 5-min incubation with Hoechst dye (5 µg/ml). Coverslips were then washed and incubated for 1 h with a fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100; Jackson ImmunoResearch), respectively. Nuclei were visualized by a 5-min incubation with Hoechst dye (5 µg/ml bisbenzimide; Roche Molecular Biochemicals). The coverslips were mounted on microscope slides and stored protected from light at −20 °C. The cells stained for p65 were photographed with a Dage-MTI 100 video camera at 4°C using a modified procedure from Tietze F (39, 40). Briefly, 0.1 ml of lysate were used for protein determination using the Bio-Rad assay, and the remaining 0.3 ml of lysate were used for determination of inorganic phosphate used to normalize the ceramide values. The ceramide band was identified by comparison with an authentic standard, and radioactivity was quantified in a scintillation counter.

Electrophoretic Mobility Shift Assay of NF-κB—The assay was performed as previously described (38). Briefly, after treatments, cells were washed and harvested by scraping in PBS. The pellet was resuspended in 400 µl of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and incubated for 10 min on ice. Just prior to centrifugation, 20 µl of 10% Nonidet P-40 was added, and the suspension was mixed by pipetting up and down three times. Nuclei were pelleted by microcentrifugation at 13,000 × g for 10 min. The supernatant was removed, and the nuclei were resuspended in 20 µl of extraction buffer (20 mM Hepes [pH 7.9], 0.4 mM NaCl, 5% [v/v] glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). The suspension was mixed gently for 30 min and pelleted at 15,000 × g for 15 min. The supernatant was flash-frozen and stored at −80 °C.

Protein concentrations were determined using the Bio-Rad assay. Nuclear extracts (10 µg) were incubated in HDEK buffer (20 mM Hepes, pH 7.9, 50 mM KCl, 5% [v/v] glycerol, 1 mM EDTA, 5 mM DTT) containing 1 µg of poly(dI-dC), 1 µg of poly(dI-dN), and 10 µg of bovine serum albumin. One µl of radiolabeled oligonucleotide probe (600,000–1,000,000 cpm) was added to each reaction and incubated at room temperature for 20 min. The reaction was terminated by the addition of 6 µl of 15% Ficoll solution containing indicator dyes (bromphenol blue and xylene cyanol). Equal amounts (20 µl) of reaction mixture were loaded on a 5% non-denaturing polyacrylamide gel in 1× TBE and run at 200 V. Gels were placed onto Whatman filter paper, dried, and autoradiographed. The specificity of NF-κB activation upon TNF stimulation in the absence and in the presence of GW98948 was assayed as previously described (34).

Immunocytochemistry of NF-κB and Cytochrome c and Nuclear Staining—For immunocytochemical analysis, cells were plated at a density of 1.7 × 10⁶ cells/100-mm plate that contained 22-mm glass coverslips. Following treatment, cells were fixed for 15 min in 4% paraformaldehyde and subsequently permeabilized during a 15-min incubation in 4% paraformaldehyde and 0.2% Triton X-100 in PBS, washed, blocked and treated for 4 h with a rabbit anti-p65 NF-κB antibody (1:200) or a mouse anti-cytochrome c antibody (1:200). The coverslips were then washed and incubated for 1 h with a fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100; Jackson Immunoresearch) or a rhodamine (TRITC)-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch), respectively. Nuclei were visualized by a 5-min incubation with Hoechst dye (5 µg/ml bisbenzimide; Roche Molecular Biochemicals). The coverslips were mounted on microscope slides and stored protected from light at −20 °C. The cells stained for cytochrome c were observed with a confocal microscope (Olympus IX 70), PerkinElmer Biosciences Ultraview software, spinning disk, using a 40× oil immersion lens. Fluorescence signals were collected after single line excitation at 543 nm (red).

Glutathione Measurements—At the indicated time points, adherent cells were collected by trypsinization and combined with floaters in the medium. After centrifugation, cells were washed twice with ice-cold PBS and resuspended in 0.4 ml of ice-cold double-distilled H₂O. The following steps were performed at 4°C using a modified procedure from Tietze F (39, 40). Briefly, 0.1 ml of lysate were used for protein determination using the Bio-Rad assay, and the remaining 0.3 ml of lysate were added to 75 µl of 10% [v/v] 5-sulfosalicylic acid, mixed, and incubated on ice for 10 min to allow protein precipitation. Samples were then centrifuged, and supernatants were stored at −80 °C. For determination of the total glutathione content, 5 µl from the supernatants were added to 35 µl of a buffer containing 6.3 mM EDTA in 125 mM sodium phosphate (GAB). Then 200 µl of 0.315 mM NADPH solution prepared in GAB (final concentration, 0.21 mM), 0.35 units of glutathione reductase, and 50 µl of 12 mM 5,5'-dithiobis-(2-nitrobenzoic acid) solution prepared in GAB (final concentration, 2 mM) were added, and the reaction was followed for 5 min by reading the OD at 412 nm. Concentration values were obtained using a standard curve with known glutathione values (0.1–100 nM) from a stock solution prepared in GAB, and they were normalized to protein concentrations.
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In Vitro Caspase Activity Assay—Caspase activity was analyzed with the ApoAlert CPP32/caspase 3 assay kit according to the manufacturer’s protocol (CLONTECH, Palo Alto, CA). At the indicated time points, adherent cells were collected by trypsinization and combined with floaters in the medium. After centrifugation, cells were washed twice with ice-cold PBS and stored as a pellet at −80 °C. On the day of the analysis, cell pellets were solubilized in lysis buffer and centrifuged to remove debris, and equal amounts of protein were incubated with 50 μM N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) for 1 h at 37 °C. The samples were analyzed using an enzyme-linked immunosorbent assay plate reader with excitation of 360 ± 40 and emission of 530 ± 25.

MTT Assay—5 × 103 cells/well were seeded in a 96-well plate in 75 μl of RPMI containing 2% FBS and 25 mM Hepes, pH 7.5. After 24 h, first GW4869 was added in 15 μl of medium/well and incubated for 30 min and then TNF was added in 10 μl/well (total volume of 100 μl/well). At the indicated time points, 25 μl of MTT stock solution (5 mg/ml in PBS) were added to each well and incubated at 37 °C in 5% CO2 for 3 h. Subsequently, cells were solubilized by the addition of 100 μl of lysis buffer (20% SDS (w/v), 50% N,N-dimethylformamide (v/v), 0.8% acetic acid (v/v), pH 4.6–4.8) to each well. The production of the formazan dye was quantitated by measuring the OD at 595 nm with a multiwell plate reader. We have noticed that the efficacy of TNF to induce morphological changes was significantly slower in experiments carried out in the presence of increasing concentrations of GW8948 as described under “Experimental Procedures.”

Western Blotting—Equal amounts of protein, usually 80 μg, were resolved by 7, 12, and 15% SDS-PAGE for analysis of PARP and caspases 6 and 9, respectively. After transfer to a nitrocellulose membrane, the proteins were incubated with anti-PARP rabbit polyclonal IgG (1:2500; Santa Cruz Biotechnology), anti-human caspase 6 monoclonal IgG (2 μg/ml; Pharmingen), or anti-caspase 9 rabbit polyclonal IgG (1:500; Santa Cruz Biotechnology) and secondary anti-rabbit (1:4000) and anti-mouse (1:5000) antibodies (Santa Cruz Biotechnology). The signal was visualized by enhanced chemiluminescence (Amersham Biosciences).

Electron Microscopy Analysis—Cells were seeded at a concentration of 0.4 × 105 cells/25-cm2 flask in growth medium. After 48 h, medium was changed with RPMI 1640 containing 2% FBS and 25 mM Hepes, pH 7.5, and cells were rested for 1.5 h. Cells were pretreated with GW4869 and treated with TNF in a total volume of 4 ml. After 14 h, cells were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer for 30 min and then rinsed overnight in cacodylate buffer with 7% sucrose. Cells were then polymerized for 24 h and capped in 812 embedding resin. Samples were polymerized for 24 h and capped in a 60 °C oven, and polymerization was allowed to continue for an additional 24 h (caps off). The plastic flask was broken away from the hardened resin, and representative blocks were sawed out using a Sears electric scroll saw. The blocks were re-embedded for orientation and polymerized for 24 h at 60 °C. Thick sections were cut using the Riechert Ultramicrotome. These 0.5–μm sections were stained with toluidine blue and examined with the light microscope. Those blocks containing the appropriate monolayer of cells were chosen for thin
sectioning. Thin sections were cut using the Riechert Ultramicrotome Ultracut E. These 70-nm thin sections were picked up on copper grids and double-stained with uranyl acetate and lead citrate. Thin sections were viewed in the JEOL 1210 transmission electron microscope, and representative digital images were saved.

RESULTS

In Vitro Studies

GW4869 was discovered during a high throughput screen with a preparation of delipidated rat brain neutral SMase. The compound structure is shown in Fig. 1, and inhibition of N-SMase by the compound is shown in Fig. 2. GW4869 acted as a noncompetitive inhibitor with the substrate sphingomyelin (Fig. 2A), and the IC\textsubscript{50} for the interaction is 1 \mu M (Fig. 2B). The \( K_m \) for sphingomyelin under these conditions was found to be 13 \mu M (0.4 mol % at 0.2% Triton X-100, 0.02% PS, and 10 mM MgCl\textsubscript{2}). The compound showed competitive characteristics toward the activator PS (Fig. 3A); however, at high PS concentrations, the inhibition was more complex, since at these high PS levels, total inhibition was not observed under standard assay conditions. Since PS, an anionic lipid, could bind Mg\textsuperscript{2+}, we evaluated the effects of varied Mg\textsuperscript{2+} levels under high PS conditions. Fig. 3B shows that at higher Mg\textsuperscript{2+} levels, inhibition again became complete. The effect of the compound on the A-SMase was also determined. At up to 150 \mu M, GW4869 did not inhibit the cloned human A-SMase (Fig. 4). Additionally, the effect of GW4869 on other hydrolytic enzymatic activities was also tested. As reported in Table I, only rat brain and bacterial neutral SMases were efficiently inhibited by GW4869, whereas no inhibitory activity was observed for the bacterial phosphatidylcholine-specific PLC, and minimal inhibition was observed for the mammalian lyso-PAF PLC, an enzyme that also hydrolyzes SM in vitro. Finally, minor inhibitory effects were observed for bovine protein phosphatase 2A activity, a
target for ceramide. Overall, the results show that GW4869 is a selective inhibitor of N-SMase, especially among enzymes known to act on SM.

**Cell Studies**

**Effects of GW4869 on Cellular Activation of N-SMase**—To verify the effect of GW4869 on N-SMase activity in cells, MCF7 breast cancer cells were treated with 3 nM TNF, since in this cell line it has been previously shown that treatment with TNF would cause activation of N-SMase as evaluated by changes in SM and ceramide (13). As expected, the addition of TNF caused sphingomyelin hydrolysis (Fig. 5A) and ceramide accumulation (Fig. 5B) after 12–14 h of incubation. Importantly, the addition of 10 μM GW4869 significantly inhibited TNF-induced SM hydrolysis, whereas 20 μM of the compound protected completely from the loss of SM. In the same time frame, ceramide levels started to increase (12–14 h), and they continued to build up during the incubation as measured by the diacylglycerol kinase assay. The addition of GW4869 (10 and 20 μM) completely inhibited the initial accumulation of ceramide, whereas this effect was partially lost at later time points (24 h). These data therefore support the inhibitory action of GW4869 on N-SMase not only in vitro but also in a cellular model.

**Effects of GW4869 on TNF-induced Depletion of GSH**—To localize the site of action of GW4869 in cells, the effects of the compound on molecular events involved in the action of TNF on N-SMase were evaluated. One of the proposed mechanisms for the activation of N-SMase upon TNF signaling implicates the drop in GSH levels after induction of early caspases (1). Therefore, MCF7 cells were treated with TNF in the presence or absence of GW4869, and GSH levels were measured (Fig. 6). As expected, TNF induced a significant decrease of glutathione levels (60%), which occurred in the same time frame of the previously observed SM hydrolysis (Fig. 5A). The addition of GW4869 at both 10 and 20 μM concentrations (effective in inhibiting SM hydrolysis) did not modify glutathione levels significantly. These results suggest that the action of GW4869 occurred downstream of the drop in glutathione.

**Specificity of the Cellular Action of GW4869 to the N-SMase Pathway of Ceramide Generation**—It has been recently shown that, in MCF7 cells treated with TNF, ceramide accumulation could result from both early activation of N-SMase and later
activation of the de novo biosynthetic pathway, inhibitable by fumonisin B1 (41). Therefore, we wondered whether the inhibitory effect of GW4869 on ceramide accumulation (Fig. 5) also extended to this second ceramide-generating pathway. MCF7 cells were treated with TNF and concomitantly pulsed with radioactive palmitate, a precursor for ceramide synthesis. Incorporation of radioactivity into ceramide was evaluated in the presence or absence of GW4869. As shown in Fig. 7, TNF...
induced by TNF, is independent of activation of N-SMase. Inhibition of TNF-induced Cell Death by an N-SMase Inhibitor

Lack of Effect of GW4869 on TNF-induced Activation of NF-κB—Several molecular events triggered by TNF stimulation have been suggested to be independent of TNF-induced accumulation of ceramide. The rapid and sustained activation of NF-κB after TNF treatment and its translocation to the nucleus appears to be a major ceramide-independent effect (42). To further verify that the effects observed during TNF treatment in the presence of GW4869 were due to specific interaction of the compound with the ceramide-mediated pathway, the effects of GW4869 on TNF-induced NF-κB translocation were studied. MCF7 cells were treated with TNF for 30 min in the absence or presence of GW4869 (10 and 20 μM), and NF-κB activation and translocation to the nucleus were monitored by immunocytochemical methods (Fig. 8B). As shown in the figure, control cells exhibited a diffuse NF-κB localization both in the cytosol and in the nucleus, and the presence of the N-SMase inhibitor at both concentrations did not affect this pattern. Thirty-minute treatment with TNF induced a robust translocation of the cytosolic fraction to the nucleus, and the presence of the N-SMase inhibitor did not interfere with this phenomenon.

TNF-induced translocation of NF-κB was also evaluated by electrophoretic mobility shift assay (Fig. 8C). Cells were treated with TNF for 30 min with or without GW4869, and nuclei and nuclear proteins were extracted and processed as described under “Experimental Procedures.” As shown in the gel (left panel), the amount of NF-κB in the nuclei greatly increased upon TNF treatment, and the presence of GW4869 did not significantly impair it. Supershift assays (right panel) demonstrate the specificity of this translocation in the presence or absence of GW6948 and the involvement of the p65 subunit of NF-κB. Therefore, these results show that GW4869 does not interfere with key TNF signaling effects, and they further suggest that the activation of NF-κB by TNF is independent of N-SMase.

Biological Implications of N-SMase Activation in TNF-induced Cell Death—the above results suggest specific cellular effects of GW4869 on activation of N-SMase but not on other key cellular effects such as activation of NF-κB or depletion of GSH or even de novo generation of ceramide. Since both the drop of GSH and the elevation in ceramide have been correlated with TNF-induced cell death, the effects of GW4869 on cell viability were next examined. Initially, the effects of GW4869 on TNF-induced cytotoxicity were evaluated by trypan blue exclusion assay. As shown in Fig. 9A, the presence of the N-SMase inhibitor at 10 and 20 μM effectively reduced the number of trypan blue-positive floating cells. Similarly, GW4869 was also able, in a dose-dependent manner, to significantly protect from nuclear condensation after TNF treatment, as evaluated by DNA staining with Hoechst (Fig. 9B). In fact, after 20 h of treatment with TNF, 81% of the cells were positive for chromatin condensation, whereas the presence of 10 and 20 μM GW4869 during the incubation reduced this percentage to 57.2 and 34%, respectively.

To further verify the action of GW4869 on N-SMase, we investigated whether exogenous ceramide can bypass the effects of the inhibitor. Therefore, MCF7 cells were treated with either TNF or C6-ceramide, in the presence or absence of GW4869, and cell viability was evaluated using the MTT assay. As shown in Fig. 10A, TNF treatment significantly affected cell viability, reducing it by 35% after 48 h. The addition of GW4869 efficiently protected cell viability at both 10 and 20 μM. MCF7 cells were then treated with C6-ceramide. In response to treatment with C6-ceramide, cell viability was significantly impaired (34%), but in this case the presence of GW4869 was unable to prevent this effect (Fig. 10B). Therefore, these results show that the GW4869 does not interfere with molecular events leading to cell death downstream of ceramide formation, further supporting its action at the N-SMase level.

To gain more insight on the specific components of the apoptotic pathway regulated by GW6948, cell morphology was evaluated by EM studies conducted in cells treated with TNF in the absence and in the presence of the N-SMase inhibitor (Fig. 11). As shown in the figure, cells treated with TNF (Fig. 11, E and F) showed massive chromat condensation compared with control cells (A and B), whereas the presence of the inhibitor...
on activation of effector caspases induced by TNF treatment. MCF7 cells (1.7 × 10⁶) were preincubated with GW4869 (10 or 20 μM) for 30 min, and treatment with TNF (3 μM) followed. Cytosolic proteins (80 μg) were resolved by 7% (PARP cleavage) or 12% (caspase 6) SDS-PAGE (upper panels). Western blot analysis was performed using anti-PARP rabbit polyclonal IgG or anti-human caspase 6 monoclonal IgG. The effect of GW4869 on activation of DEVD-cleaving caspases was also evaluated in vitro (lower panel). Equal amounts of proteins from cell lysates were incubated with 50 μM N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) for 1h at 37 °C in a 96-well plate, and samples were analyzed using an enzyme-linked immunosorbent assay plate reader with excitation of 360 ± 40 nm and emission of 530 ± 25 nm. The results are representative of two independent experiments in quadruplicate.

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FIG. 12. Protective effects of GW4869 onactivation of effector caspases induced by TNF treatment. MCF7 cells were treated with TNF in the presence or absence of GW4869 (10 or 20 μM) for 30 min, and treatment with TNF (3 μM) followed. Cytosolic proteins (80 μg) were resolved by 7% (PARP cleavage) or 12% (caspase 6) SDS-PAGE (upper panels). Western blot analysis was performed using anti-PARP rabbit polyclonal IgG or anti-human caspase 6 monoclonal IgG. The effect of GW4869 on activation of DEVD-cleaving caspases was also evaluated in vitro (lower panel). Equal amounts of proteins from cell lysates were incubated with 50 μM N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) for 1h at 37 °C in a 96-well plate, and samples were analyzed using an enzyme-linked immunosorbent assay plate reader with excitation of 360 ± 40 nm and emission of 530 ± 25 nm. The results are representative of two independent experiments in quadruplicate.

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In order to verify that these morphological changes were part of an ongoing apoptotic process, we evaluated the effect of TNF treatment on activation of late/effector caspases, thought to be responsible for nuclear fragmentation. As shown in Fig. 12 and in agreement with previous reports (13, 30, 43, 44), treatment of MCF7 cells with TNF caused cleavage of PARP, in MCF7 a substrate for active caspase 7, with the formation of the characteristic 85-kDa fragment. The presence of the N-SMase inhibitor prevented, in a dose-dependent manner, the TNF-induced PARP cleavage. In fact, after 12 h of treatment, preincubation with 10 μM GW4869 was able to partially block the appearance of the 85-kDa fragment, whereas 20 μM of the inhibitor completely prevented the cleavage. These results clearly suggest a role for ceramide generated through N-SMase peripheral to the nuclei (H).

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To further investigate the effects of GW4869 late/effector caspases, we evaluated the effect of pretreatment with the N-SMase inhibitor on caspase 6 processing. Therefore, MCF7 cells were treated with TNF in the presence or absence of 20 μM GW4869. After 14 h, cells were collected, and Western blotting was performed on cytosolic proteins using antibodies recognizing the full-length caspase 6. As shown in Fig. 12, TNF induced processing of caspase 6, whereas the addition of the N-SMase inhibitor effectively blocked it. These results suggest that inhibition of N-SMase activation prevents activation of late caspases. Next, in vitro activity of DEVDase caspases was assayed after treating cells with TNF in the presence or absence of the N-SMase inhibitor in order to evaluate a more generalized activation of effector caspases. As shown in Fig. 12, the presence of increasing concentrations of GW4869 (10 and 20 μM) resulted in almost complete inhibition of activation of DEVDase caspases induced by TNF, confirming and extending the results obtained from the Western blots.

One of the receptor-mediated mechanisms for the activation of late caspases involves mitochondrial dysfunction, characterized by release of cytochrome c and subsequent activation of caspase 9 (45–48). Thus, we wondered if N-SMase activity was involved in mediating these events. Therefore, MCF7 cells were treated with TNF in the presence or absence of GW4869, and its effects on cytochrome c release were evaluated by immunocytochemistry and confocal microscopy. As shown in Fig. 13A, untreated cells exhibited a punctate pattern characteristic of the mitochondrial network, whereas treatment with TNF induced the appearance of a diffuse staining characteristic of cytosolic localization. Importantly, the presence of the N-SMase inhibitor significantly and in a dose-dependent manner protected the release of cytochrome c from mitochondria into the cytosol; 10 and 20 μM of GW4869 reduced by 46 and 62% the number of positive cells, respectively. In support of the effects on cytochrome c release, the presence of the N-SMase inhibitor also protected from activation of caspase 9 (Fig. 13B). Treatment with TNF resulted in the appearance of the characteristic proapoptotic fragment and the presence of the N-SMase inhibitor efficiently prevented processing of caspase 9.

DISCUSSION

Here we report the identification of GW4869 as a novel inhibitor of the Mg²⁺-dependent N-SMase and its characterization and biological effect in a cellular system of cytokine-induced apoptosis. Importantly, evidence is provided of specificity of action of GW4869 on N-SMase activity both in vitro and in vivo. Finally, the use of this novel N-SMase inhibitor provided strong evidence implicating activation of N-SMase in the regulation of a number of biological processes induced by TNF.

During a high throughput screen with a preparation of de-lipidated rat brain N-SMase, we successfully identified
GW4869, a molecule that exhibited significant and specific inhibitory activity. The compound is a noncompetitive inhibitor of the enzyme with an IC50 of 1 \( \mu M \). GW4869 showed no or minor inhibitory activity versus other hydrolytic enzymes, such as bacterial phosphatidylcholine-PLC and bovine protein phosphatase 2A, and it showed significantly higher activity versus the rat brain enzyme compared with the human lyso-PAF PLC (Table I). Importantly, GW6948 showed no inhibition of the human acid SMase.

The inhibitory activity of GW4869 against N-SMase observed in vitro (Figs. 2–4 and Table I) was also confirmed at a cellular level. GW4869 prevented SM hydrolysis induced by TNF treatment and partially inhibited ceramide accumulation (Fig. 5). These effects have been observed using concentrations ranging from 5 to 20 \( \mu M \). The requirement for higher concentrations for cellular action could be due to limitation in permeability through the plasma membrane.

Specificity of action of GW4869 to the N-SMase was also confirmed in vivo by multiple lines of evidence. 1) GW4869 only targeted ceramide formation from the N-SMase pathway. In MCF7 cells, TNF treatment activates at least two major routes for ceramide generation, the N-SMase and the de novo biosynthetic pathways (38). GW4869 completely inhibited ceramide accumulation only at an early phase of incubation (12–18 h), when at the same time it also inhibited SM hydrolysis (Fig. 5). At a later time, corresponding to the activation of the de novo pathway, the inhibitor no longer prevented the rise of ceramide levels. When the effects of the inhibitor were directly tested on this later ceramide-generating pathway, no effects were observed on de novo incorporation of palmitate into ceramide (Fig. 7). Since the de novo pathway requires many enzymes including serine palmitoyl transferase and ceramide synthase, these results demonstrate that GW6948 does not affect these enzymes. It is also interesting to note that, in addition to demonstrating specificity of action of GW4869 on N-SMase-generated ceramide, these results also indicate that the two pathways of ceramide generation are independent. 2) GW4869 did not inhibit the drop in glutathione induced by TNF treatment, which has been recently shown to precede the activation of N-SMase in response to TNF (13, 49–51). These results also add to the cellular specificity of action of GW4869 (Fig. 6). 3) GW4869 did not inhibit ceramide-mediated cell death. This indicates that indeed the target of GW4869 is localized upstream of ceramide generation and that the N-SMase inhibitor does not affect directly any of the targets downstream of ceramide action (such as caspases, cytochrome c, nucleases, etc.). 4) GW4869 did not affect other molecular events triggered by TNF that are known to be independent of ceramide accumulation. Thus, the lack of effects on NF-κB demonstrates that GW4869 does not interfere with initial events in TNF action (including interaction with the TNF receptors, activation of Nik, Ikk, or the proteosome). Additionally, GW4869 did not
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 affect TNF-induced prostaglandin production in both A549 lung cancer cells and L929 murine fibroblasts, thus allowing a possible effect of the compound on either cPLA₂ or cyclooxygenase.

2 The use of GW4869 as a specific inhibitor generated specific insight into the molecular mechanisms that are dependent on activation of N-SMase. Thus, inhibition of N-SMase not only blocked PARP cleavage (target for caspase-3) but also prevented the activation of other effector caspases such as caspase 6 and caspase 9. Processing of caspase 9 occurs by auto-cleavage as the result of the formation of the so-called “apoptosome,” a complex constituted by cytochrome c released from mitochondria, apoptotic protease-activating factor-1, and procaspase 9 (52, 53). Therefore, the possibility that activation of N-SMase leads to the induction of effector caspases by targeting specific mitochondrial events was verified and confirmed, since inhibition of N-SMase partially, but significantly, prevented the release of cytochrome c from the mitochondria (Fig. 13). The observation that the activation of N-SMase promotes cell death through mitochondrial dysfunction is in agreement with results obtained in MCF7 overexpressing the Bcl2 protein. Indeed, the overexpression of Bcl2 has been shown to almost completely prevent TNF-induced cell death (27) and PARP proteolysis (13), only moderately blocking ceramide formation (13), and only moderately blocking ceramide formation (13). In addition, these results are in agreement with some studies that previously reported the ability of short chain ceramide analogs to induce cytochrome c release in both isolated mitochondria (54) and intact cells (55, 56).

Although these results start to shed light on the possible biological targets of ceramide generated through the N-SMase pathway, they also raise a number of important questions that require further evaluation. First, how does the ceramide from the N-SMase signal to mitochondria? It has been recently demonstrated that targeting of an N-SMase to mitochondria induced accumulation of ceramide that was sufficient to induce mitochondrial dysfunction and signal cell death (57). Therefore, one possible scenario implicates the activation by TNF of an N-SMase isoform, which resides in mitochondria or in close proximity. Second, what are the direct effectors of the mitochondrial dysfunction induced by accumulation of ceramide? Recent studies suggest a few candidates. For example, it has been shown that ceramide induces conformational changes of the Bax protein and synergizes with Bax, altering mitochondrial functions (58). On the other hand, ceramide was found to specifically activate a mitochondrial protein phosphatase 2A, which rapidly and completely dephosphorylates Bcl-2, leading to cell death (59).

It is also important to note that inhibition of N-SMase did not completely prevent either the formation of ceramide (Fig. 5B) or some of the biological effects induced by TNF in MCF7, such as cytochrome c release (Fig. 13A) and nuclear condensation (Fig. 9B). Interestingly, the predominant peripheral condensation of the chromatin observed in MCF7 treated with TNF in the presence of the N-SMase inhibitor (Fig. 11H) closely resembles the pattern of nuclear changes induced by activation of the apoptosis-inducing factor-mediated pathway (60). These results suggest that this specific process, which is known to be independent of caspases, may also be independent of N-SMase.

In conclusion, by using a newly characterized inhibitor of N-SMase, we show for the first time that activation of the N-SMase leads to mitochondrial dysfunction and that it is required for the full development of the cytotoxic program induced by TNF.

B. Pettus and Y. A. Hannun, unpublished observations.
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