4-O-Carboxymethyl Ascochlorin Causes ER Stress and Induced Autophagy in Human Hepatocellular Carcinoma Cells

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Background: 4-O-Carboxymethyl ascochlorin (AS-6), a PPARγ agonist, reduces serum glucose levels in a diabetic mouse model.

Results: Proteomics analysis revealed that many proteins up-regulated after AS-6 treatment were associated with ER stress response.

Conclusion: AS-6 produces an ER unfolded protein response, inducing autophagy, and activating transcription factor CHOP-dependent apoptosis.

Significance: By stimulating autophagy, AS-6 might be effective in blocking proliferation of cancer cells.

The synthetic derivative of ascochlorin, 4-O-carboxymethyl ascochlorin (AS-6) is of interest because it has been shown to induce differentiation in mouse pre-adipocytes and to ameliorate type II diabetes in a murine model. AS-6 was cytotoxic when added at micromolar concentrations to cultures of three different human cancer cell lines. We used gel electrophoresis and mass spectrometry to identify proteins with altered expression in human hepatocarcinoma cells (HepG2) after 12 h in the presence of AS-6 and found 58 proteins that were differentially expressed. Many of the proteins showed increased expression in cells treated with AS-6 are involved in protein quality control, including glucose-regulated protein 78 (GRP78/BiP), a regulator of ER stress responses, and the transcriptional regulator CHOP, which mediates ER stress-induced apoptosis.

AS-6 produces an ER unfolded protein response, inducing autophagy, and activating transcription factor CHOP-dependent apoptosis. Cells treated with AS-6 undergo an autophagic response accompanied by increased expression of beclin1, ATG5, and LC3-II and autophagosome formation marked by the appearance of large vesicles containing LC3-II. Grp78 induction was inhibited when the PPARγ antagonist, GW9662, was added together with AS-6, and autophagy and cell death were partially blocked. 3-methyl-adenine (3-MA), an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase) prevented induction of ATG5 and activation of LC3-II and blocked autophagosome formation. 3-MA also blocked induction of GRP78 and CHOP, suggesting that PI3-kinase, which is known to mediate ER stress-induced autophagy, also plays a role in initiating apoptosis in response to ER stress. Together these data establish that the cytotoxicity of AS-6 operates by a mechanism dependent on ER stress-induced autophagy and apoptosis.

Ascochlorin (ASC),2 a terpenoid antibiotic isolated from an incomplete fungus, Ascochyta viciae, was shown to have broad antiviral and antifungal activity (1). ASC inhibits invasive activity of renal carcinoma cells mediated by matrix metalloproteinase-9 (MMP-9) and thus has potential anti-tumor activity (2). ASC acts through a range of receptors and regulatory proteins exerting diverse effects on various cell types. For example, ASC suppresses oxidized low density lipoprotein (oxLDL)-induced MMP-9 expression by inhibiting the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway in human THP-1 macrophages (2, 3), suppresses the activator protein-1 (AP-1) activity of estrogen receptor-negative human breast cancer cells, partly due to induction of apoptosis (4), and promotes activation of p53 and inhibition of mitochondrial respiration (5). Recent results show that ASC inhibits mitochondrial electron transport by binding to cytochrome bc1. The x-ray crystal structure of the complex shows ASC bound at both active sites of cytochrome bc1 (6).

Synthetic derivatives of ASC display overlapping activities but also bind to specific receptors and other targets with a broad range of affinities and thus exert unique physiological effects. One synthetic derivative of ASC, 4-O-carboxymethyl ascochlorin (AS-6) is of interest because it has been shown to function as an orally active hypoglycemic agent in both obese

2 The abbreviations used are: ASC, ascochlorin; AS-6, 4-O-carboxymethyl ascochlorin; GRP78 (BiP), glucose-regulated protein 78; PDI, protein-disulfide isomerase; MMP-9, matrix metalloproteinase-9; AP-1, activator protein-1; PPARγ, peroxisome proliferator-activated receptor-γ; TNFα, tumor necrosis factor-α; NF-κB, nuclear factor-κB; VCA-1, vascular cell adhesion molecule-1; MCP-1, monocytes chemotactic protein-1; CHOP C/EBP homologous protein; CFP, cyan fluorescent protein; ATG, autophagy related; LC3-1 (ATG8), microtubule-associated protein light chain 1; GRP75 (mHSP70 and mortalin); GRP78 stabilized; PDI, phosphatidylinositol 3-kinase; EBSS, Earle's balanced salt solution; 3-MA, 3-methyl adenine; UPR, unfolded protein response; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor; PERK, pancreatic endoplasmic reticulum eIF2α kinase; Hop (stimulated phosphoprotein 1), Hsp70/Hsp90-organizing protein; VDAC, voltage-dependent anion-selective channel; apo B-100, apolipoprotein B-100; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

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Hyperinsulinemic and insulin-deficient diabetic animal models (7). AS-6 is as a potent activator of peroxisome proliferator-activated receptor-γ (PPAR-γ). AS-6 interacts directly with PPAR-γ in vitro, and treatment of the mouse pre-adipocyte cell line 3T3-L1 with AS-6 induces differentiation (8). Studies with rat vascular smooth muscle cells revealed that AS-6, as an agonist of PPARγ, inhibits tumor necrosis factor-α (TNF-α)-stimulated nuclear factor-κB (NF-κB) activity, thereby down regulating expression of several pro-inflammatory molecules, including vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), and fractalkine (CX3CL1) (9).

Given the diverse effects described for AS-6 in different model systems, we initiated a study to measure global changes in cellular protein levels and activities in response to AS-6 treatment to gain further insight into the metabolic and regulatory pathways affected and to provide information regarding other regulatory proteins targeted by AS-6. Using two-dimensional electrophoresis combined with MALDI-TOF-TOF mass spectrometry we separated 1500 proteins and identified 58 proteins differentially expressed in HepG2 cells following AS-6 treatment. Nearly a third of the proteins up-regulated in cells treated with AS-6 are involved in protein quality control. Immunostaining of AS-6-treated cells showed accumulation of the transcription factor, CHOP (C/EBP homologous protein), indicative of an ER unfolded protein response. Induction of ER stress proteins by AS-6 results in an autophagic response, and thus AS-6 has potential as an effective agent for blocking proliferation of cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—The human hepatocellular liver carcinoma cell line (HepG2)—was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum. The cells were maintained at 37 °C. The HepG2 cells were grown on culture plates to 60–70% confluence in complete medium containing 10% FBS for 12 h, and then changed to serum-free medium after washing twice with medium. The cells were then incubated with AS-6 at various concentrations. AS-6 is a synthetic derivative of ascorcholin described previously (10) and was kindly provided by Dr. Junji Magae, Central Research Institute of Electric Power Industry, Tokyo, JAPAN. GW9662, 3-methyl adenine, and wortmannin were purchased from Sigma-Aldrich. Troglitazone and antibodies specific for GRP78, PDI, prohibitin, Beclin 1, CHOP, p97, p-eIF2α, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for LC3 (ATG8) and ATG5 were purchased from ABGENT (Abgent Inc, CA). Lon specific antibody was prepared in this laboratory (11).

Cell Death Assay—Loss of cell viability was assessed by propidium iodide (PI) staining. Cells were harvested by centrifugation, washed three times with PBS, suspended in PBS, and then incubated for 15 min at room temperature after addition of 1 μM PI. Stained cells were counted using a flow cytometer (BD Bioscience).

Protein Extraction—HepG2 cells were washed three times with ice-cold PBS. Cells were lysed with a buffer containing 5 mM EDTA, 9.5 mM urea, 4% (v/v) CHAPS, 65 mM DTT, and protease inhibitors (Complete kit, Roche Diagnostics) for 1 h at 24 °C. Cellular debris was removed by centrifugation for 15 min at 20,000 × g at 4 °C. Protein samples were stored at −70 °C. Protein concentrations were quantified using a commercial Bradford Kit (DC reagent kit, Bio-Rad). In some cases, HepG2 cells were treated with AS-6 for 12 h. We obtained the NRK58 cell line that expresses CFP-LC3 from Jennifer Lippincott-Schwartz, NICHD, NIH, Bethesda, MD.

Two-dimensional Gel Electrophoresis—Two-dimensional electrophoresis was performed using an established procedure (12). A whole cell lysate (400 μg) was added to immobilized pH 3–10 linear gradient strip (ReadyStrip IPG strip, Bio-Rad), which was rehydrated in a Protein IEF cell. Complete sample uptake into the strip was achieved after 12 h at 20 °C. Focusing was performed with a linear voltage increase up to 250 V for 30 min, 10,000 V for 3 h, and then 10,000 V for 5.5 h. Current was limited to 50 μA per strip, and the temperature was maintained at 20 °C for all isoelectric focusing steps. For the second dimension electrophoresis, the IPG strip was incubated in equilibration buffer containing 37.5 mM Tris-HCL (pH 8.8), 6 mM urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 2% (w/v) DTT for 15 min, and then incubated for 15 min in equilibration buffer supplemented with 2.5% (w/v) iodoacetamide. The equilibrated IPG strip was transferred onto a 12% Duraaryl gel (180 × 160 × 1.5 mm) for SDS-PAGE. Gel staining was performed as described by Neuhoff et al. (13). To ensure the reproducibility of the observed changes in protein expression, experiments were performed three times with independent cell cultures treated or untreated with AS-6. For the differential analysis, statistical significance was estimated with the Student’s t test. Values of p < 0.05 were considered significant.

Protein Identification—Gel plugs centered on the protein spots were excised from the gels, washed three times with ultrapure water, destained twice with a 1 to 1 mixture of 50 mM NH₄HCO₃ and acetonitrile, reduced with 10 mM DTT in 50 mM NH₄HCO₃, alkylated with 40 mM iodoacetamide in 50 mM NH₄HCO₃, dried twice with 100% acetonitrile, rehydrated in 50 mM NH₄HCO₃, and digested overnight at 37 °C with 50 μg/ml sequencing grade modified trypsin (Promega, Madison, WI). The peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The tryptic peptides were dissolved with 0.1% TFA in 50% acetonitrile. MS analysis was conducted with an ABI 4700 Proteomics Analyzer MALDI-TOF-TOF mass spectrometer (Applied Biosystems, Framingham, MA). Data were analyzed using GPS Explorer software (Applied Biosystems) and Mascot software (Matrix Science, London, UK) using human protein sequences in the NCBI non-redundant database as the reference set. Identification was assigned to a protein spot feature if the protein score was calculated to be greater than 65, correlating to a confidence interval of 99%.

Protein Pathway Analysis—After protein identification, the accession numbers and fold changes of the differentially expressed proteins were tabulated in Microsoft Excel and imported into IPA (Ingenuity System, Mountain View, CA).
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IPA is a software application that enables to identify the biological mechanisms, pathways, and functions matching a particular dataset of proteins. IPA is based on a database obtained by abstracting and interconnecting a large fraction of the biomedical literature according to an algorithm integrating protein functions, cellular localization, small molecules, and disease inter-relationships. The networks are displayed graphically as nodes, representing individual proteins and edges representing the biological relation between nodes. Canonical pathway analysis within IPA utilizes well characterized metabolic and cell signaling pathways, which are generated prior to data input and on which identified proteins are overlaid.

Western Blot Analysis—Total protein extracts were prepared as previously described (14). Cell lysates were prepared by suspending 3 × 10⁶ cells/60 mm-diameter dish in 30 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% (v/v) Nonidet P-40, 100 μM phenylmethylsulfonyl fluoride, 20 μM aprotinin, and 20 μM leupeptin, adjusted to pH 8.0). The cells were disrupted, and proteins were extracted at 4 °C for 30 min. The proteins were electrotransferred to PVDF membranes (Invitrogen). Detection of specific proteins was carried out with an enhanced chemiluminescence Western blotting kit following the manufacturer's (Pierce) instructions.

Quantitation of Specific mRNA—After treatment of cells with AS-6, total RNA was isolated from each preparation using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of total cellular RNA was treated for genomic DNA contamination and reverse transcribed using SA Biosciences Reverse Transcription reagents and oligo (dT) (SA Biosciences, Frederick, MD). Gene expression was analyzed using real-time PCR on an ABI7500 SDS system (Applied Biosystems, Foster City, CA). Real-time PCR was performed according to manufacturer's instructions. Then Real-time PCR, we used commercial Taq-Man CARL (Calreticulin), ALDH (Aldehyde dehydrogenase), ATF4 (activating transcription factor-4), EIF4EBP1 (eukaryotic initiation factor 4E-binding protein-1), and GAPDH probes.

siRNA-mediated ATG5 Gene Knockdown—A combination of siRNAs (total concentration 0.1 μM) against the human ATG5 (Santa Cruz Biotechnology) were transfected into 1 × 10⁶ HeLa cells using Lipofectamine 2000 (Invitrogen) for 16 h before analysis.

Fluorescence Microscopy—Cells were plated on cover slides. Following designated treatments, cells were fixed with 4% paraformaldehyde. Confocal images were acquired using a Zeiss LSM 510 system mounted on Zeiss 100M microscope (Carl Zeiss Microimage Inc. Thornwood, NY).

RESULTS

Effect of AS-6 on the Viability of HepG2 Cells—AS-6, a 2-carboxymethyl derivative of ASC (supplemental Fig. S1), has anti-biostatic activity. We tested the cytotoxic effects of AS-6 on HepG2 cells grown in serum-free medium and in medium containing 10% FBS. AS-6 was added at concentrations ranging from 5 to 25 μM for 12 h, after which dead cells were stained with PI and counted by flow cytometry. A dose-dependent increase in cell killing was observed with and without serum; the rate of killing in cultures containing FBS was consistently 80% of that of the serum-free cultures. A maximum of 16% of the cells were killed after 12 h of growth in the presence of 25 μM AS-6 in serum-free medium (Fig. 1A, left panel). After 48 h with 40 μM AS-6 growth ceased, and all cells were killed (data not shown). The right panel in Fig. 1A shows that a normal rat kidney cell line, NRK58, which was used for later experiments, was also sensitive to AS-6.

To confirm that the toxic effect of AS-6 was dependent on its interaction with PPARγ, we tested whether a PPARγ agonist, GW9662, could block killing by AS-6. Fig. 1B shows that >25% of HepG2 cells and HeLa cells were killed after 16 h of growth in the presence of AS-6 and that GW9662 almost completely prevented cell death.

Separation and Detection of Differentially Expressed Proteins from HepG2 Cells—To determine the changes in protein expression resulting from treatment of cells with AS-6, we used two-dimensional gel electrophoresis to separate total cell proteins from HepG2 cells. Each gel was loaded with 400 μg of protein, and ~1500 individual spots were resolved in this manner. In preliminary experiments we determined that on average individual spots containing 1 ng of protein were detectable using sensitive colloidal staining. We expected that many individual spots would contain more than one protein, and this was borne out by subsequent analysis by mass spectrometry. In addition, we also found that in a number of instances, the same protein was present in multiple spots most likely the products of post-translational modifications or alternative splicing at the mRNA level. Nevertheless, we estimated that we were able to resolve at least 1200 individual proteins, which provided a representative sample of the cellular proteins and allow us to identify many differentially expressed proteins.

Three gels per sample were processed simultaneously and analyzed with PDQUEST two-dimensional software to obtain quantitative comparisons of proteins recovered from treated and untreated cultures. The two-dimensional patterns of the
proteins recovered from HepG2 cells cultured in the presence of 10 μM AS-6 and control cells that received DMSO only are shown in supplemental Fig. S2. Most protein spots appeared in the size range from 17 to 130 kDa, with a few over 130 kDa. Fig. 2, A and B show enlarged views of gel regions containing spots that were, respectively, significantly increased and decreased in staining intensity after AS-6 treatment.

Identification of Differentially Expressed Proteins by MS—Protein spots from the two-dimensional gels were subjected to trypsin digestion and MALDI-TOF-TOF analysis. Of the 70 spots that showed significant changes, 58 provided definitive protein identifications. Several proteins were identified multiple times because they were present in more than one spot. Supplemental Table S1 lists the proteins with the greatest differential expression, which had staining intensity ratios in samples from treated and untreated cells of ≥1.5 or ≤0.6. More than 95% of the spots had sequence coverage exceeding 10% and were identified at the 95% confidence level. Identification for many proteins was further validated by agreement between the apparent molecular weights and isoelectric points estimated from position in the two-dimensional gels and the theoretical values for the identified proteins (ΔMr <20% or ΔpI <0.5) (supplemental Table S1).

The proteins were classified into categories based on function and cellular location using classifications from the SwissProt/TrEMBL protein knowledge base. The distribution of proteins among the different classes is depicted as a bar graph in Fig. 3A. The functional categories with the highest representatives were protein folding (~20%), oxidation-reduction (17%), and signal transduction (10%). The proteins assigned to the various functional groups are given in supplemental Table S1. In addition, 45% of the proteins are localized in the cytoplasm (Fig. 3B).

Protein Pathway Analysis of Differentially Expressed Proteins—To provide some insight into the cellular activities that were affected by AS-6 treatment cells, we performed a pathway analysis to place the proteins into different functional networks. As shown in Fig. 3A, 28 proteins involved in protein folding (~20%), oxidation-reduction (17%), and signal transduction (10%) were grouped together. Selected results of the IPA were validated using biochemical techniques.

Validation of Selected Proteins by Western Blot Analysis and Q-RT-PCR—To confirm the changes in protein expression after AS-6 treatment, we performed Western blot analysis to measure the levels of specific proteins and quantitative RT-PCR analysis to measure changes in the corresponding mRNAs. Because a large number of proteins showing differential expres-
We also performed quantitative RT-PCR analysis of selected other genes. Fig. 4D shows the output from analysis of mRNA levels for one protein, calreticulin, that was up-regulated, and one protein, aldehyde dehydrogenase, that was down-regulated. The increase in calreticulin mRNA (∼2.5-fold) is in good agreement with the change in protein expression estimated from Sensitive colloidal staining (1.7–3-fold). Aldehyde dehydrogenase mRNA levels dropped nearly 80% in 12 h after AS-6 addition, while a somewhat smaller change was observed for the protein, most likely reflecting the intrinsic stability for this enzyme, which apparently is turned over slowly under these conditions. In summary, the Western blot analysis and the mRNA measurements confirm that the changes observed by staining following two-dimensional gel electrophoresis reflect changes in proteins levels within the cell and should reflect regulatory mechanism triggered by direct or indirect interaction of AS-6 with one or more regulatory or signaling molecules in the cell.

AS-6 Induces ER Stress and Activates Autophagy—Induction of GRP78 is a marker for ER stress (15, 16). Accumulation of
other proteins also pointed to ER stress in AS-6 treated cells. For example, levels of the ATP-dependent protease, Lon, which is up-regulated by ER stress (17), increased in a time and dose-dependent manner following treatment of cells with AS-6 (Fig. 5, A and B). The levels of p97, which is up-regulated during stress and plays a critical role in ER-associated protein degradation, were also increased by AS-6 treatment (supplemental Fig. S4). ClpP, the proteolytic component of the ATP-dependent ClpXP protease complex is up-regulated by a mitochondria-specific unfolded protein response (UPR) requiring CHOP-activated transcription as well as activation of the JNK pathway (18). ClpP levels were increased during the first 6 h after addition of AS-6 although they began to decline at later times (Fig. 5B).

ER stress is known to trigger increased autophagy (19–21), which can rid cells of damaged proteins and organelles but can also lead to cell death (22). To investigate whether ER stress following AS-6 treatment of cells might have generated an autophagic response, we used Western blotting to probe expression of ATG5, a known marker for autophagy, and to monitor conversion of LC3-I (ATG8) to LC3-II, which is required for formation of autophagosomes (23, 24). We also measured changes in the level of beclin 1, which has been implicated specifically in ER stress-induced autophagy (25). The protein levels for both ATG5 and beclin 1 were increased by AS-6 treatment in a dose- and time-dependent manner (Fig. 5, A and B). LC3-I was converted to the phosphatidylethanolamine-conjugated form LC3-II (23, 24) in a similar dose- and time-dependent manner after AS-6 addition (Fig. 5, A and B).

**Direct Visualization of Autophagosomes by Fluorescence Microscopy**—We examined autophagosome formation after AS-6 treatment of NRK58B cells, a clonal Normal Rat Kidney cell line expressing cyan fluorescent protein (CFP) fused to LC3. Under normal growth conditions, CFP-LC3 is distributed between cytosolic and nuclear pools, with very little observed on autophagic membranes. Under starvation conditions, CFP-LC3 is converted to CFP-LC3-II, and appears in punctate structures, which serves as a marker for formation of autophagosomes. We examined the distribution of CFP-LC3 in growing cells and in cells treated with AS-6. As a positive control for autophagosome formation, we also examined cells that had been incubated for 12 h in starvation medium, EBSS. Fig. 6B shows that in untreated cells, CFP-LC3 shows a diffuse distribution throughout the cells. In contrast, AS-6 treatment for 12 h results in collection of CFP-LC3 into bright punctate structures that are indistinguishable from those formed in cells that are undergoing autophagy in response to starvation.

Formation of membrane-enclosed structures containing CFP-LC3 requires class III PI3K (26, 27) and has been shown to be inhibited by the class II PI3K inhibitor 3-MA. Addition of 3-MA abolished recruitment of CFP-LC3 into punctate structures in cells treated with AS-6 as well as positive control cells incubated in EBSS (Fig. 6B). Wortmannin had almost no discernable effect on the appearance of punctate structures in the cells treated with AS-6, although as expected it inhibited autophagosome formation in cells incubated in starvation medium. These results confirm that damage caused by AS-6 induces an autophagic response, and suggest that the pathway

**FIGURE 5.** Expression of marker proteins for autophagy and ER stress after AS-6 treatment. A, AS-6-dose dependence of expression of autophagy markers. HepG2 cells were treated for 12 h with 5, 10, or 20 μM AS-6. Cell lysates were separated by SDS-PAGE, and the levels of ATG5, LC3 and Beclin 1 were assessed by Western blotting using specific antibodies. GRP78, a marker of ER stress, and Lon protease were also probed using anti-GRP78 and anti-Lon antibodies, respectively. GRP78, a marker of ER stress, and Lon protease were also probed using anti-GRP78 and anti-Lon antibodies, respectively. β-Actin was probed as the loading control.

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A.

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FIGURE 5.
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either involves a PI3K complex that is more sensitive to 3-MA than to Wortmannin or an unknown target of 3-MA.

ER Stress Induced by AS-6 Leads to an Unfolded Protein Response (UPR)—ER stress can result in a UPR mediated by PERK-dependent phosphorylation of eIF2α, which leads to increased expression of ATF4 and its downstream target, CHOP (28, 29). CHOP promotes stress-linked apoptosis by activating transcription of Bim and other pro-apoptotic proteins (30). We probed for CHOP by immunofluorescence staining of whole cells, and we also probed in parallel for the accumulation of GRP78 in the cytosol to confirm the ER stress response. CHOP was undetectable in cells grown without AS-6 but accumulated to high levels in the nucleus of cells cultured in the presence of AS-6 (Fig. 6A, lower panels). Cytosolic GRP78 was low in untreated cells and increased after AS-6 treatment (Fig. 6A, upper panels). To ask whether the effect of AS-6 in inducing a UPR is general, we used Western blotting to measure CHOP levels in NRK58 cells and in HeLa cells with and without AS-6 treatment. CHOP was expressed in low amounts in untreated cells and was highly induced after AS-6 treatment in both cell lines (supplemental Fig. S5).

To confirm that the increase in CHOP reflected a UPR, we used Q-RT-PCR to monitor expression of ATF4, which mediates upstream signaling from PERK. ATF4 transcription was increased following treatment of HepG2 cells with AS-6 (supplemental Fig. S6). Increased activity of ATF4 should activate expression of EGFBP1 in addition to CHOP, and our Q-RT-PCR measurements showed that EGF-BP1 expression was also increased following AS-6 treatment (supplemental Fig. S6). Together these data support the conclusion AS-6 causes ER stress, inducing a UPR and activating CHOP-mediated apoptosis.

If the above hypothesis is correct, knockdown of CHOP should prevent cell death after AS-6 treatment. Fig. 7A shows that treating HeLa cells with anti-CHOP siRNA for 12 h prior to addition of AS-6 blocked expression of CHOP >90% and reduced killing from 39% to 7%.

3-MA Inhibits Both Autophagy and the UPR in Cells Exposed to AS-6—The phosphatidylinositol 3-kinase (PI3K) inhibitor 3-MA inhibits autophagy (31). In cells treated with ER inhibitors such as thapsigargin and tunicamycin, 3-MA blocks

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**FIGURE 6. Effects of inhibitors on ER stress signaling and autophagosome formation induced by AS-6.** A, autophagosome formation following AS-6 treatment. NRK58 cells were cultured for 12 h in DMEM medium in the presence of 20 μM AS-6 or DMSO. Autophagosome formation was detected by the appearance of dense clusters of CFP-LC3, which accumulate in the membrane of autophagosomes. As a positive control, NRK58 were also suspended in starvation medium (EBSS) to induce autophagosome formation. As further confirmation of autophagosome formation, the autophagy inhibitors, 3-MA (5 mM) and Wortmannin (100 nM), were added to separate cultures to block the accumulation of CFP-LC3. CFP fluorescence was imaged using a Zeiss LSM 5100 microscope. B, GRP78 and CHOP expression levels in intact HepG2 cells cultured in the presence of AS-6. HepG2 cells were cultured for 12 h with 20 μM AS-6 or with DMSO. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with an anti-GRP78 antibody. Images were obtained using a Zeiss LSM 5100 microscope.

**FIGURE 7. The response to AS-6 involves proteins that regulate both autophagy and UPR.** A, parallel cultures of HeLa cells were treated with 50 nM anti-CHOP siRNA or control siRNA for 12 h after which AS-6 or DMSO was added for an additional 16 h. Cell viability was measured by PI staining, and harvested cells were subjected to SDS-PAGE and Western blotting with anti-CHOP antibody. B, knockdown of ATG5 blocks autophagy and attenuates the induction of GRP78 and CHOP after AS-6 treatment. Western blotting was used to measure the increased expression of stress response proteins after AS-6 treatment of cells with normal or reduced levels of ATG5. HepG2 cells were treated with control siRNA or anti-ATG5 siRNA, and 20 μM AS-6 or DMSO was added to duplicate cultures for 12 h. Proteins were solubilized in SDS sample buffer and separated by SDS-PAGE. The levels of ATG5, GRP78, CHOP, LC3, and β-actin (as the loading control) were measured using monoclonal antibodies specific for each protein. C, HepG2 cells were treated for 12 h with different concentrations of AS-6. To evaluate the role of class III PI3 kinase in the response to AS-6, 5 mM 3MA was added to a duplicate culture that had been treated with 20 μM AS-6. After SDS-PAGE and blotting, GRP78, phosphorylated-eIF2α, and LC3-II were detected with monoclonal antibodies specific for each protein. The anti-LC3 antibody used in this experiment had poor reactivity with unprocessed LC3 but detected the processed form (LC3-II). β-Actin served as the loading control. D, three cultures of HepG2 cells were treated with 20 μM AS-6 for 12 h. Two of the cultures were treated with either 5 mM 3-MA or 100 μM GW9662, an antagonist of PPARγ.
autophagosome formation by inhibiting the beclin1–PI3K complex that is needed to initiate the pathway for activation of LC3-II (25). We found that 3-MA blocked the increase in cytosolic GRP78 in cells treated with AS-6 (Fig. 7B and supplemental Fig. S5). Wortmannin (WM), which inhibits autophagy induced by nutrient deprivation but was less inhibitory of UPR-dependent autophagy (25), had much less effect on the up-regulation of GRP78 expression in response to AS-6 treatment (Fig. 7B and supplemental Fig. S5). Because the inhibitory effects of 3-MA and wortmannin on induction of the autophagic markers differed, we sought to block the autophagic pathway by using siRNA against ATG5, which is a critical factor in the activation pathway for LC3-II during the autophagic response. Pretreatment with ATG5 siRNA blocked conversion of LC3-I to LC3-II in cells treated with AS-6 (Fig. 7B). Knockdown of ATG5 also attenuated the induction of GRP78 (Fig. 7B). These results support the conclusion that cells exposed to AS-6 experience ER stress that leads to induction of autophagy and suggest that mobilization of the autophagic machinery might generate a feedback signal needed for full induction of GRP78.

Surprisingly, 3-MA completely blocked the accumulation of CHOP in the nucleus of cells treated with AS-6 (Fig. 6A) and also blocked the increased expression of CHOP in NRK58 and HeLa cells treated with AS-6 (supplemental Fig. S5). This result cannot be explained solely by the block in autophagy, because ATG5 knockdown blocked autophagy but had only a slight effect on CHOP induction (Fig. 7B). Our results suggest that PI3K might also play a role in upstream signaling for CHOP-mediated apoptosis. Wortmannin, which was a weak inhibitor of autophagy under these conditions, also had no effect on induction of CHOP in AS-6 treated cells (Fig. 6A and supplemental Fig. S5). To confirm that the effects of 3-MA were exerted at an early step in the UPR leading to activation of CHOP, we used phosphospecific antibodies to examine the effect of 3-MA on phosphorylation of eIF2α, which is phosphorylated by PERK in response to a UPR. Fig. 7C shows that eIF2α was phosphorylated following treatment of HepG2 cells with AS-6 and that addition of 3-MA resulted in a decrease of 60–80% in the accumulation of phosphorylated eIF2α, confirming that activation of PERK under these conditions is dependent on PI3K activity and is needed for CHOP induction and CHOP-mediated apoptosis. We conclude that AS-6 causes an ER stress-induced UPR that operates through a pathway in which PI3K plays a role and that the UPR might be enhanced by but is not dependent on the autophagic response.

AS-6 Acts Through PPARγ to Induce Autophagy and Apoptosis—AS-6 binds to PPAR-γ and acts as an agonist, inducing differentiation of mouse pre-adipocytes (8) and inhibiting TNFα-stimulated inflammatory responses in rat muscle (9). We asked whether induction of autophagy and apoptosis by AS-6 is dependent on its interaction with PPARγ by treating cells with GW9662, a PPARγ antagonist (32). Fig. 7D shows that the presence of GW9662 during treatment of cells with AS-6 attenuated the induction of GRP78 and blocked the appearance of activated LC3-II to a similar extent as seen with the PI3K inhibitor 3-MA. We conclude that the effects of AS-6 are mediated at least in part through activation of PPARγ. Interestingly, the induction of apoptosis by PPARγ agonists, such as the thiazolidinediones, troglitazone, and ciglitazone, is only partially blocked by antagonists, suggesting that they induce apoptosis by PPARγ-independent mechanisms. Because GW9662 addition produced only a partial reduction in CHOP induction by AS-6 (supplemental Fig. S7), our data also leave open the possibility that AS-6 might target other molecules in addition to PPARγ, and that activation of the cell death pathways stems from these other interactions. The identities of the other target(s) of AS-6 remain to be determined as is its influence on the PI3K signaling pathway.

DISCUSSION

In this study we analyzed changes in the proteome of HepG2 cells after growth in the presence of AS-6, a synthetic derivative of ascochlorin. Ascochlorin and related natural and synthetic compounds have potent anti-fungal activities and are pharmacologically active in animal models of human diseases (1–7). AS-6 itself has been shown to be an agonist of PPARγ, blocking activation of NF-κB and thereby suppressing inflammatory responses (8). AS-6 also acts through PPARγ in a manner similar to currently used drugs such as pioglitazone (32) to ameliorate type II diabetes by reducing serum glucose levels. We note that in our experiments the protein with the highest overexpression after AS-6 treatment was guanine nucleotide-binding protein-β (RACK1). RACK1 is down-regulated in a diabetes mouse model (34), and our finding suggests that the efficacy of AS-6 in ameliorating diabetes might stem in part from the increased expression of RACK1. In addition, Rab GDP dissociation inhibitor-β (GDIβ), which is up-regulated in diabetic mice (35), was reduced after AS-6 treatment, which would also contribute to the effect of AS-6 in stimulating glucose uptake.

We observed that AS-6 kills at least three different human cancer cell lines and performed proteomics analysis followed by Western blot analyses of specific proteins to identify the major regulatory proteins affected by AS-6 and to define pathways involved in cell death. We found 58 proteins that were differentially expressed after AS-6 treatment (supplemental Fig. S2 and Table S1). Pathway analysis of the data suggested that AS-6 induces ER stress, up-regulates proteins involved in glucose uptake, and down-regulates pro-inflammatory proteins. The PPARγ antagonist, GW9662, protected both HepG2 and HeLa cells from killing by AS-6 (Fig. 1B) and had moderate to strong effects in blocking induction of specific proteins required for autophagic and apoptotic cell death (Fig. 7D). While GRP78 induction and activation of LC3-II were reduced, CHOP induction was reduced about 50% (supplemental Fig. S7), suggesting that AS-6 induces autophagy through its interaction with PPARγ but might induce apoptosis through an additional unidentified target. Our results are in line with studies showing that other PPARγ ligands, such as 15-deoxy-Δ12,14-prostaglandin J2 (36), induce ER stress. Most of proteins up-regulated after AS-6 addition were molecular chaperones and enzymes associated with protein folding and ER stress. Among these proteins was GRP78, which increased 3.5-fold after 12 h in AS-6-treated cells. GRP78 is an ER chaperone and a master regulator of ER stress responses, including the unfolded protein response (UPR) (25). GRP78 constitutively blocks response regulators, inositol-requiring enzyme-1 (IRE1), activating tran-
AS-6 Causes ER Stress and Induces Autophagy in HepG2 Cells

**FIGURE 8.** Schematic model for ER stress-induced autophagy in hepatocellular liver carcinoma cells following AS-6 treatment. AS-6 leads to up-regulation of CHOP and induced expression of the ER stress-related proteins, GRP78, PDI, p97, and Lon protease. ER stress decreases the amount of Grp78 available to inhibit the sensor proteins, PERK and IRE1, thus activating PI3K to phosphorylate beclin 1 and initiating an autophagic response. ER stress accompanied by an unfolded protein response also activates autophagosomal formation via a pathway dependent on eIF2α and the c-Jun kinase. Both 3-MA and wortmannin inhibit PI3K and block beclin 1 activation (25) but only 3-MA blocks up-regulation of CHOP during AS-6-mediated ER stress, further evidence that the ER stress is accompanied by an unfolded protein response. Because 3-MA also prevented up-regulation of Grp78, it must inhibit the stress response at a stage upstream of the sensor kinases. Our results suggest that AS-6, a putative agonist of PPARγ, dysregulates protein homeostasis generating ER stress and triggering autophagy.

- **PI3K**
- **Beclin 1**
- **ATG5**
- **LC3-II**
- **JNK**
- **Grp78**
- **CHOP**

**Autophagosome formation**

**Autophagy**

demonstrated formation of autophagosomes by the detection of fluorescent vacuoles containing CFP-LC3, a fusion of CFP and LC (41), which localizes to the membrane of autophagosomes (Fig. 6B). The autophagic response induced by AS-6 treatment was blocked by 3-MA, a class III PI3K inhibitor, which blocks formation of the beclin-PI3K complex preventing autophagy induced by ER stress brought on by drugs such as tunicamycin or thapsigargin (25). Knockdown of ATG5, a critical autophagic protein needed to target LC3-II to autophagosome precursors, also blocked autophagy induced by AS-6, confirming that the autophagic pathway is the same as that activated by impaired ER function.

The accumulation of CHOP in the nucleus of cells treated with AS-6 (Fig. 6B) provides direct evidence for an ER UPR response in these cells. ER stress accompanied by a UPR triggers the onset of apoptosis via CHOP induction and activation. CHOP promotes synthesis of Bim and leads to down-regulation of anti-apoptotic proteins such as Bcl-2 (28). In our experiments, the increase in Lon protease is also consistent with UPR in the ER (17), while the induction of the proteolytic component of human ClpXP, ClpP, points to a mitochondrial UPR as well (18). Up-regulation of GRP78 in the presence of AS-6 was also inhibited by 3-MA, which implies that PI3K activity is needed to transduce ER stress signaling through IRE1 or ATF6, and that blocking PI3K activity prevents induction of GRP78. Further support for this proposal comes from the finding that 3-MA also blocked induction of the transcription factor CHOP, which is induced in a PERK-dependent manner following ER stress. It is possible that PI3K interacts either directly or functionally with these sensor kinases or acts on another enzyme that modulates their downstream effects. Entingh et al. reported that both wortmannin and LY294002, inhibitors of PI3K, prevent induction of CHOP in response to amino acid starvation (42) and proposed that PI3K acted through an mTOR-mediated pathway to induce CHOP. Interestingly, LY294002 also blocked CHOP induction in response to oxidative stress but not in response to sodium arsenite or the calcium ionophore, A23197. In AS-6-treated cells wortmannin was not able to block up-regulation of either Grp78 or CHOP in cells treated with AS-6. Our results combined with data from other studies suggest that the extent to which specific PI3K-dependent pathways are impaired might depend on the degree of PI3K inhibition or on some other aspect of the inhibition, such as temporal or spatial distribution of PI3K.

Wortmannin was shown to inhibit autophagosomal formation via the beclin-PI3K pathway in response to nutrient starvation or ER stress but is less effective in blocking autophagy induced by the ER UPR and mediated through eIF2α down-regulation and the JNK activation (25). JNK activation is also important for CHOP expression. Our data suggest that ER stress produced by AS-6 mimics the UPR and implies that the target of AS-6 might be directly involved in a primary process needed for protein homeostasis. In our experiments, cells grown in complete medium were less sensitive to AS-6 (data not shown), and it was necessary to remove fetal calf serum to sensitize the cells to AS-6. It is likely that the combination of growth in serum-free medium and AS-6 treatment amplified our proteomics analysis directly indentified several proteins increased in AS-6-treated cells that have been associated with both autophagic and apoptotic cell death pathways. Among these proteins were endophilin 1 (Bif-1), which interacts with beclin through the PI3K/UVrag (UV resistance-associated gene protein) complex to stimulate autophagy (38), and two proapoptotic kinases, MAPK-3 and Mst1 (Stk23) (39, 40). We confirmed induction of autophagy by Western blotting, which showed that the essential autophagic proteins, beclin1, ATG5, and LC3-II, were all increased in cells treated with AS-6, and...
the ER stress and associated autophagy and resulting in a UPR that initiated apoptosis.

This is the first report of a proteomic analysis of the effects of AS-6 treatment on cultured human cells. AS-6 treatment results in ER stress and autophagy. We propose that AS-6 acts through PPARγ to attenuate NF-κB activity and that impairment of NF-κB-responsive pathways allows damage to accumulate inducing ER stress. It is possible that AS-6, like ASC and ascofaraceous, also targets other pathways adding to the stress response. Further investigation of the mechanism by which ER stress and autophagy are induced following AS-6 treatment should lead to the identification of other direct targets of AS-6 and provide a better understanding of its effects on normal and diseased cells as well as its potential therapeutic utility.

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4-O-Carboxymethyl Ascochlorin Causes ER Stress and Induced Autophagy in Human Hepatocellular Carcinoma Cells
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