An Active Endocytosis Pathway Is Required for the Cytotoxic Effects of Glycosylated Antitumor Ether Lipids

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Abstract. *Background: Glycosylated antitumor ether lipids (GAELs) kill cells by an apoptosis-independent pathway. A hallmark of this pathway is the formation of large acidic vacuoles; however, very little is known about the process. We examined the hypothesis that 1-O-hexadecyl-2-O-methyl-3-O-(2'-amino-2'-deoxy-β-D-glucopyranosyl)-sn-glycerol (Gln), a potent GAEL, diffuses across cell membranes into lysosomes, where protonation of the amine leads to its accumulation and generation of the vacuoles. Materials and Methods: N-Benzylamine analogs with similar pKa values, but with greater hydrophobicity than the parental Gln were synthesized and their activities against epithelial cancer cell lines were compared. The role of endocytosis in Gln action was investigated by inhibiting endocytosis with methyl-β-cyclodextrin (MCD), and inhibiting the maturation of the endocytic vesicles by low temperature incubation and analyzing their effects on Gln activity. Results: The N-benzylamines were either inactive or less active than Gln, indicating that activity was unrelated to diffusion or protonation. Toxicity was only observed with analogs that generated vacuoles. The incubation of cells with MCD inhibited the generation of the vacuoles and the toxic effects of Gln. The toxic effect of Gln was inhibited when cells were incubated with the drug at 20˚C, a temperature that inhibits the maturation of early endosomes. Conclusion: The results of the study show that GAELs are taken up by endocytosis and an active endocytic pathway is required for the formation of large acidic vacuoles by GAELs and manifestation of their cytotoxic effects.*

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Very few drugs are available for the treatment of advanced cancer and, furthermore, some types of cancer do not currently have any effective treatments. The commonly used chemotherapeutic drugs target cellular DNA and kill cells by inducing apoptosis (1). Cancer cells can evade cell death by several mechanisms which may be intrinsic to the cells or induced during the course of chemotherapeutic treatment (2, 3). The net effect is that cells are able to survive treatment with apoptosis-inducing compounds, leading to an inability to eradicate the tumor. There is clearly a need for novel anticancer drugs that kill cells by pathways other than apoptosis.

Antitumor ether lipids (AELs) are synthetic analogs of 2-lysophosphatidylcholine (4). After entering cells, they act on multitude targets to inhibit cell proliferation and/or induce cell death (5-9). In contrast to many of the currently used chemotherapeutic agents, AELs do not interact with DNA; therefore, they are not mutagenic. AELs with a sugar moiety in place of the phosphocholine group of the prototype AEL, 1-*O*-octadecyl-2-*O*-methyl-3-*O*-glycerophosphocholine (ET-18-OCH_3), have been developed and are collectively called glycosylated antitumor ether lipids (GAELs) (10-15). 1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-amino-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (Gln), a potent GAEL, has an amino group at the 2 position of the D-glucose moiety (11). Although the detailed mechanism of action of GAELs is not known, recent studies have shown that Gln may kill cells by an apoptosis-independent paraptosis-like cell death pathway (16) that may involve cathepsins as executors (17). Thus, GAELs may represent a class of compounds that are effective against apoptosis-resistant tumors and cancer, for which effective chemotherapeutic agents are currently unavailable. We previously found that GAEL-induced cell death was associated with the generation of large acidic vacuoles (LAVs) with lysosomal characteristics (16, 17), and postulated that Gln diffuses across the cell membrane into lysosomes, where protonation of the primary amine occurs, leading to its accumulation in that organelle (17). The mode of GAEL uptake and accumulation in lysosomes is not known. To gain insight

into the mechanism of action of GAELs, we synthesized a series of lipophilic analogs of Gln and investigated their cytotoxic effects against a panel of human cancer cell lines.

Materials and Methods

Chemical synthesis of GAELs. For general methods, see references 10, 11, and 15. Gln was synthesized as described previously (11). Benzaldehyde, *p*-cyano-, *p*-chloro-, *p*-methoxy, and *p-N,N*-dimethylaminobenzaldehyde were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The Astec diol HPLC column was purchased from Advanced Separation Technologies (Whippany, NJ, USA) and the Econosphere amino column was purchased from Alltech (Deerfield, IL, USA).

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-(*p*-methoxybenzylamino)-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol (see Figure 1): To a solution of Gln (123 mg, 0.25 mmol) and *p*-methoxybenzaldehyde (61 μl, 0.50 mmol) in 10 ml of MeOH was added $NaBH_3CN$ (32 mg, 0.51 mmol). After the mixture was stirred overnight at room temperature, the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (elution with 10:1 $CHCl_3$-MeOH), giving 136 mg (89%) of the target glucopyranose as a colorless oil: $[\alpha]^{25}_D$ −3.15˚ (*c* 3.5, $CHCl_3$/MeOH 1:1); HPLC (Alltech Econosphere NH_2 5 μm column, 4.6×250 mm) R_t 9.8 min (elution with a gradient of $CHCl_3$-MeOH; 100% $CHCl_3$ for 5 min, 0-50% MeOH for 5 min, and then 50% MeOH); ^{1}H NMR (400 MHz, $CDCl_3$ and a few drops of CD_3OD) δ 7.27 (d, 2H, *J*=7.4 Hz), 6.85 (d, 2H, *J*=7.4 Hz), 4.49 (d, 1H, *J*=7.8 Hz), 3.30-4.10 (m), 3.78 (s, 3H), 3.45 (s, 3H) 1.56 (m, 2H), 1.25 (s, 26H), 0.88 (t, 3H, *J*=6.4 Hz); ^{13}C NMR δ 159.0, 130.0, 114.0, 103.7, 79.1, 75.6, 74.5, 71.9, 71.1, 70.1, 69.0, 62.3, 62.0, 57.9, 55.2, 51.4, 31.9, 29.7, 29.6, 29.5, 29.4, 26.1, 22.7, 14.1; FAB HRMS (MH+) calcd. for $C_{34}H_{62}NO_8$ 612.4475, found 612.4494.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-*p*-chlorobenzylamino)-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol: This compound was prepared in 90% yield from Gln and *p*-chlorobenzaldehyde by the procedure described above: $[\alpha]^{25}_D$ −2.76˚ (c 4.1, $CHCl_3$/MeOH 1:1); HPLC (Astec diol 5 μm column, 4.6×250 mm) R_t 15.3 min (elution with a gradient of $CHCl_3$-MeOH; 100% $CHCl_3$ for 5 min, 0-50% MeOH for 5 min, and then 50% MeOH); ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD) δ 7.25-7.36 (m, 4H), 4.44 (d, 1H, *J*=7.8 Hz), 3.30-4.10 (m), 3.43 (s, 3H), 1.55 (m, 2H), 1.25 (s, 26H), 0.88 (t, 3H, *J*=6.4 Hz); ^{13}C NMR δ 129.9, 128.7, 79.1, 75.5, 74.8, 71.9, 71.1, 70.1, 69.0, 62.2, 62.1, 57.8, 51.4, 31.9, 29.69, 29.65, 29.58, 29.49, 29.35, 26.1, 22.7, 14.1; FAB HRMS (MH^+) calcd. for $C_{33}H_{58}NO_7Cl$ 616.3980, found 616.3978.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-benzylamino)-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol: This compound was prepared from Gln and benzaldehyde by the procedure described above: ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD) δ 7.25-7.36 (m, 5H), 4.44 (d, 1H, *J*=7.8 Hz), 3.30-4.10 (m), 3.43 (s, 3H), 1.55 (m, 2H), 1.25 (s, 26H), 0.88 (t, 3H, *J*=6.4 Hz); ^{13}C NMR δ 129.9, 128.7, 79.1, 75.5, 74.8, 71.9, 71.1, 70.1, 69.0, 62.2, 62.1, 57.8, 51.4, 31.9, 29.69, 29.65, 29.58, 29.49, 29.35, 26.1, 22.7, 14.1.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-*p-N,N*-dimethylaminobenzyl-amino)-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol: This compound was prepared from Gln and *N,N*-dimethylaminobenzaldehyde by the procedure described above: ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD) δ 7.05 (d, 2H, *J*=5.6 Hz), 6.67 (d, 2H, *J*=5.6 Hz), 4.44 (d, 1H, *J*=7.8 Hz), 3.30-4.10 (m), 3.43 (s, 3H), 2.81 (s, 6H), 1.55 (m, 2H), 1.25 (s, 26H), 0.88 (t, 3H, *J*=6.4 Hz); ^{13}C NMR δ 149.8, 129.2, 127.5 112.8, 103.7, 79.0, 75.9, 74.7, 71.7, 70.2, 70.0, 68.6, 62.0, 61.3, 57.6, 52.1, 31.7, 29.44, 29.40, 29.27, 29.25, 29.11, 25.8, 22.4, 13.8.

1-*O*-Hexadecyl-2-*O*-methyl-3-*O*-(2'-*p*-cyanobenzylamino)-2'-deoxy-β-D-glucopyranosyl)-*sn*-glycerol: This compound was prepared from Gln and *p*-cyanobenzaldehyde by the procedure described above: ^{1}H NMR ($CDCl_3$ and a few drops of CD_3OD) δ 7.63 (d, 2H, *J*=8.2 Hz). 7.49 (d, 2H, *J*=8.2 Hz), 4.35 (d, 1H, *J*=7.8 Hz), 3.30-4.10 (m), 3.43 (s, 3H), 1.55 (m, 2H), 1.25 (s, 26H), 0.88 (t, 3H, *J*=6.4 Hz); ^{13}C NMR δ 145.8, 132.1, 128.8, 118.8, 110.3, 104.2, 79.0, 75.8, 74.9, 71.7, 70.1, 69.9, 68.6, 61.8, 61.3, 57.6, 52.0, 31.7, 29.49, 29.45, 29.3, 29.28, 29.16, 25.8, 22.5, 13.9.

Cell cultures. DU145, U251, MiaPaCa2, and HeLa cell lines were obtained from frozen stocks of cell lines originally obtained from the ATCC (Manassas, VA, USA). ASK1 –/– cells were obtained from Dr H. Ichijo, University of Tokyo, Tokyo, Japan. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/0.1 mg/ml streptomycin. The growth medium for MIAPaCa-2 was supplemented with 2.5% horse serum in addition to 10% FBS.

Antiproliferation assay. The proliferation of the cancer cells was monitored by the CyQuant assay according to the instructions of the manufacturer (Invitrogen, Burlington, ON, Canada), as previously described (18). Cancer cells were dispersed into 48-well plates and incubated until they were in log phase. The medium was subsequently replaced with one containing varying concentrations of the GAELs (0-15 μM dissolved in ethanol) for 48 h. The concentration of ethanol in all wells was 0.1%. Each drug concentration was replicated 6 times and the experiments repeated twice.

Viability assay. Cells were grown in 6-well dishes or 60 mm dishes and treated with the compounds for the times indicated. The medium along with any floating cells was removed and placed into a conical tube. The cells attached to the tissue cultureware were detached by trypsinization and added to the medium in the tube. After centrifugation, the cell pellet was resuspended in phosphate buffered saline (PBS) and subjected to trypan blue dye exclusion assay using the BioRad TC10 Automated Cell Counter (BioRad, Mississauga, ON, Canada) according to the manufacturer's instructions.

Effect of methyl-β-cyclodextrin (MCD) on LAV formation. ASK1 –/– cells were grown in 60-mm dishes at 37˚C in an incubator with 5% CO_2. The cells in experimental dishes were preincubated with or without 5 mM MCD for 1 h. The medium was removed and replaced with one containing 5 mM MCD plus 12 μM Gln, 12 μM Gln, or 5 mM MCD. Control cells were incubated with ethanol (0.1%). After incubation for selected periods, the presence of LAVs in the cells were observed by phase contrast microscopy using an Olympus IX-100 microscope.

Effect of incubation temperature on Gln-induced vacuole formation. ASK1 –/– cells were grown in 60-mm dishes at 37˚C in an incubator with 5% CO_2. Gln solution at the required concentration was prepared and warmed to 37˚C or cooled to 20˚C prior to addition to the cells. The cells were incubated at 37 or 20˚C in 5%

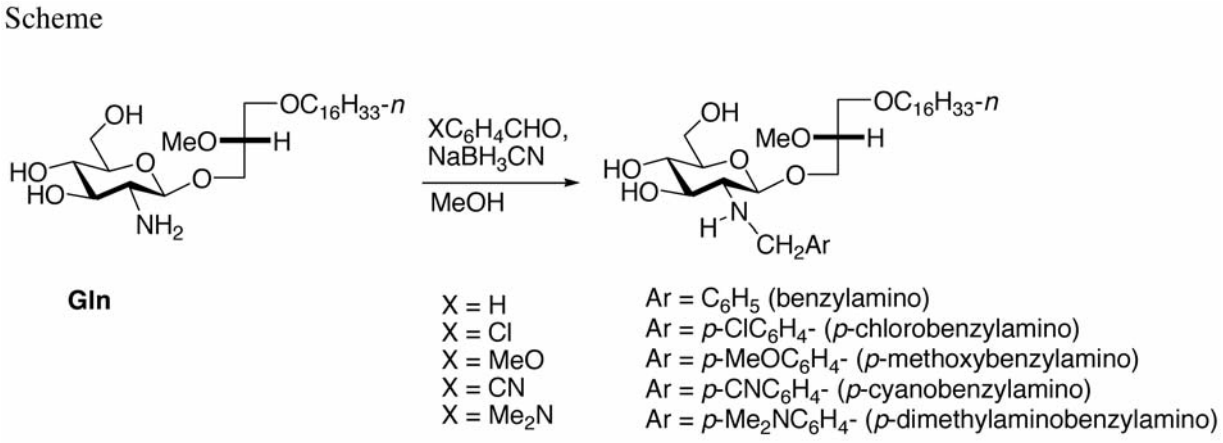

Figure 1. *Structure and synthesis of glycosylated antitumor ether lipids (GAELs).*

CO_2 for varying times and the cells were examined by phase-contrast microscopy for the appearance of LAVs using an Olympus I×70 microscope.

Results and Discussion

Gln kills cells by an apoptosis-independent mechanism that involves the generation of LAVs which we have previously characterized as having lysosomal characteristics (13, 14). While the mechanism of vacuole formation is unknown, it has been postulated that Gln enters the cell and organelles by diffusion (17). In the acidic lumen of the lysosomes, Gln is protonated and trapped, resulting in its accumulation. Accumulation of Gln in the lysosomes is postulated to lead to LAV formation (14). To test this hypothesis, several benzylamine analogs of Gln were synthesized. The synthesis of the benzylamine analogs was achieved by reaction of Gln with *p*-substituted benzaldehyde derivatives in methanol, followed by reduction of the resulting Schiff base with sodium cyanoborohydride, and purification by column chromatography on silica gel. Benzylamine analogs have similar pK_a values to the parental Gln (19) and would, therefore, be protonated in a similar fashion but the enhanced lipophilicity of benzylamines enhances their diffusion into the cells relative to Gln.

Exponentially growing cancer cell lines derived from prostate (DU145), pancreatic (MIAPaCa-2), glioblastoma (U251), and cervical cancer (HeLa) were incubated with different concentrations of GAELs for 48 h. The effects on cell proliferation were assessed with the CyQuant assay (18) while the proportion of viable cells was quantitated by the trypan blue dye exclusion assay with a TC10 Cell Counter. The effects of the benzylamine-Gln analogs were compared with the activity of the parental Gln. Gln inhibited the growth of all the cell lines with a half- maximal inhibitory concentration (IC_{50}) of about 10 μM (Figure 2A). Gln concentrations of 12 μM and higher were sufficient to kill the cell lines after 48 h incubation as assessed by the trypan blue dye exclusion assay. Viability ranged from 11-17% compared to 97-99% for the controls. Benzylamino-Gln (15 μM), on the other hand, had no effect on HeLa cells, but 15 μM inhibited the proliferation of U251 cells by about 35% and DU145 and MIAPaCa-2 cells by 50% (Figure 2B). It had no effect on the viability of any of the cells as assessed by the trypan blue dye exclusion assay. The effects of benzylamines bearing electron-withdrawing groups, *i.e.*, *p*-cyanobenzylamino-Gln and *p*-chlorobenzylamino-Gln, were similar to that of benzylamino-Gln (Figures 2C and 2D). Viability studies conducted with 15 μM, the highest concentration tested, revealed that after 48 h incubation with both analogs, viability of all the cell lines was 97-99% and identical to that of the controls incubated with the vehicle alone. As shown in Figure 2E, incubation of the cancer cells for 48 h with *p*-methoxybenzylamino-Gln, an analog with an electron-releasing group, inhibited the proliferation of DU145, HeLa, U251, and MIAPaCa-2 cells with IC_{50} values of 9-13 μM. Incubation of HeLa, DU145, and U251 with 15 μM *p*-methoxybenzylamino-Gln for 48 h gave viability values of 15%, 9% and 19%, respectively. With MIAPaCa-2 cells, incubation with 15 μM *p*-methoxybenzylamino-Gln for 48 h inhibited proliferation by 80% but had minimal effect on the viability of the cells. Viability was 90% with the compound compared to 99% for the controls. *p*-Dimethylaminobenzylamino-Gln, another analog with an electron-releasing group, affected the proliferation of DU145 and U251 cells similar to that observed with *p*-methoxybenzylamino-Gln, with an IC_{50} value of ~12 μM but had little effect on MIAPaCa-2 and HeLa cells, with IC_{50} values being greater than 15 μM (Figure 2F). At the highest concentration tested, 15 μM, proliferation of DU145

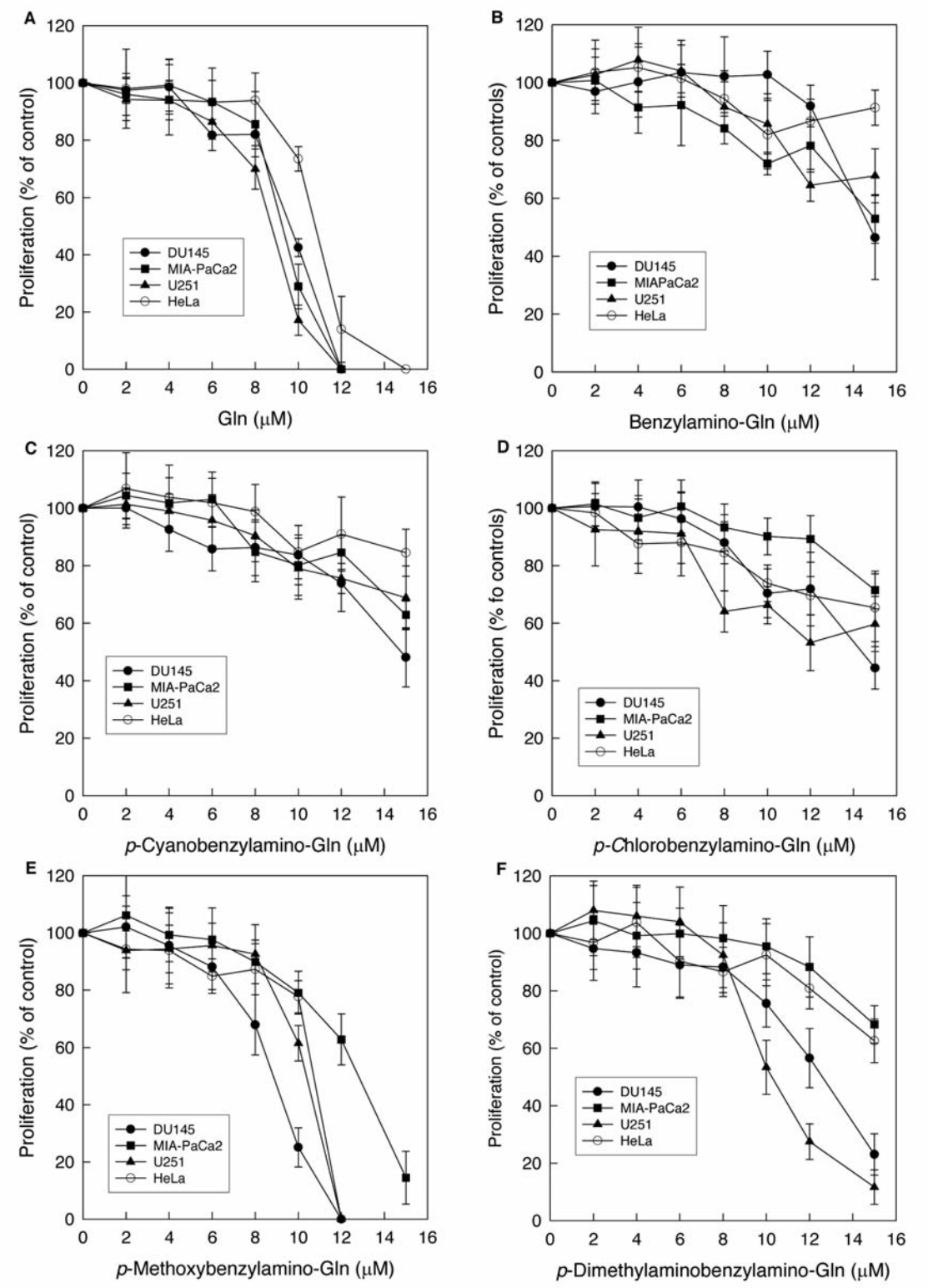

Figure 2. *Antiproliferative effects of GAELs. The effects of 0-15 μM of Gln (A), benzylamino-Gln (B), p-cyanobenzylamino-Gln (C), p-chlorobenzylamino-Gln (D), p-methoxybenzylamino-Gln (E), and p-dimethylaminobenzylamino-Gln (F) on the proliferation of DU145, MIAPaCa-2, U251, and HeLa cells were evaluated. Cells were grown in 48-well plates and incubated with different concentrations of each GAEL for 48 h. The increase in cell numbers were determined by CyQuant assay and expressed as a percentage of the increase in control wells with the vehicle (0.1% EtOH). The results are the average±SD from 12 different wells.*

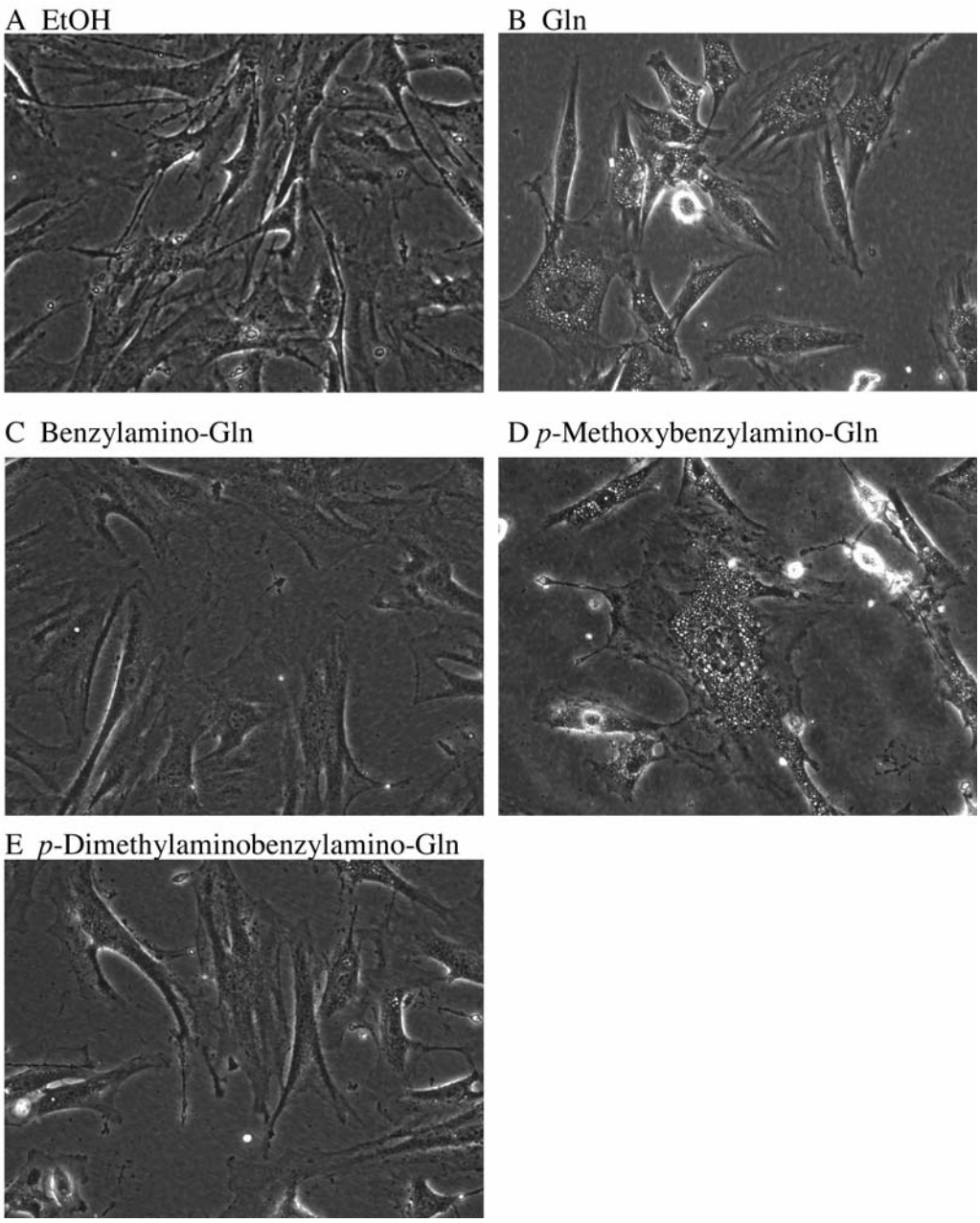

Figure 3. *Effect of GAEL analogs on vacuole formation in ASK1 –/– cells. Proliferating ASK1 –/– cells were incubated with vehicle (A) or with 10 μM each of Gln (B), benzylamino-Gln (C), p-methoxybenzylamino-Gln, (D), or p-dimethylaminobenzylamino-Gln (E). After incubation for 5 h, the cells were visualized by phase-contrast microscopy and photographed at a magnification of ×30.*

and U251 cells was inhibited by 75 and 90%, respectively. Viability studies with the trypan blue dye exclusion assay revealed that viability was 97-99% for DU145 cells and U251 cells incubated with or without 15 μM *p*-dimethylaminobenzylamino-Gln for 48 h. Thus, while the cytotoxic and antiproliferative activities of *p*-methoxybenzylamino-Gln were similar to that of Gln, *p*-dimethylaminobenzylamino-Gln was much less active.

From the above results, we conclude that GAEL activity cannot be explained simply as a function of protonation in the lysosomes as has been proposed (17). Since the pKa of benzylamine is ~9.4 (19), the extent of amino group protonation in the various Gln analogs is expected to be very similar; nevertheless, not all of the compounds are active. Indeed, a flaw in the original hypothesis may be the notion that GAELs remain in the cytosol as unprotonated molecules

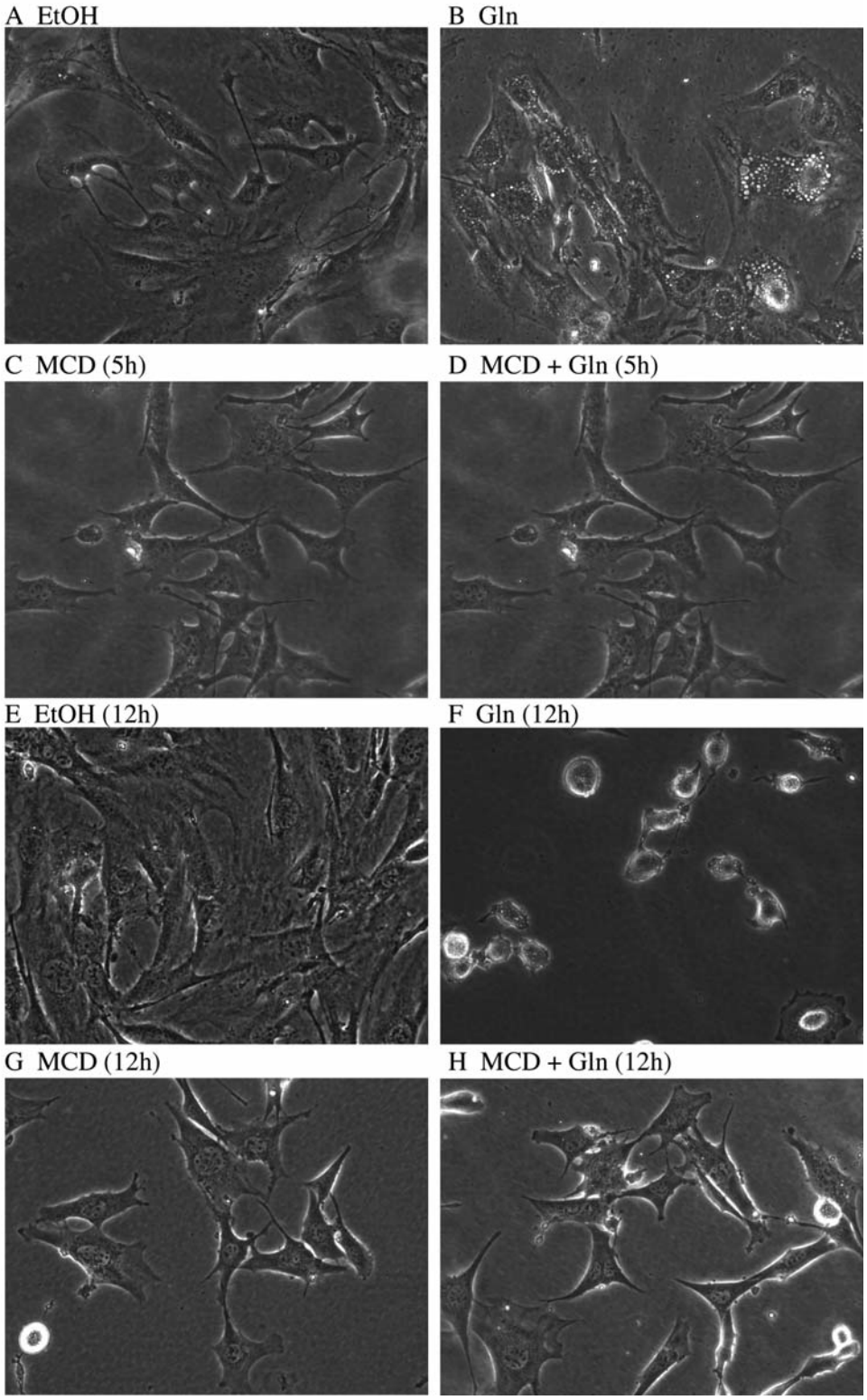

Figure 4. *Effect of methyl-β-cyclodextrin (MCD) on generation of vacuoles by Gln in ASK1 –/– cells. ASK 1 –/– cells were preincubated with EtOH (0.1%) or 5 mM MCD in growth medium for 1 h. Medium incubated with vehicle was replaced with growth medium containing vehicle (A, E) or 12 μM Gln (B, F). In cells preincubated with MCD, the medium was replaced with growth medium containing 5 mM MCD (C, G) or 5 mM MCD plus 12 μM Gln (D, H). After incubation for 5 h (A, B, C, D) or 12 h (E, F, G, H), the cells were visualized by phase-contrast microscopy and photographed. Magnification, ×30.*

and only become protonated in the low pH medium of the lysosome. Gln and its analogs that diffuse across the cell membrane will become protonated at the cytosolic pH of 7.4. The charged molecules would not diffuse into the lysosome (20). It is not known why the addition of the N-benzyl group to the amino group of Gln results in a complete loss of cytotoxic activity, but it appears to be unrelated to the basicity of the compounds.

The differential cytotoxic effects observed with the GAELs led us to test the hypothesis that GAEL cytotoxic activity requires the generation of LAVs (16). Even though LAVs are formed in all cell types we have investigated to date (16), ASK1 –/– mouse embryonic fibroblasts (MEFs) were used for these studies, as they are very sensitive to GAELs and form very prominent vacuoles (16). ASK1 –/– cells were incubated with 10 μM Gln, and with the benzylamino-Gln analogs for 5 h, the cells were examined by microscopy (Figure 3A-E). Cells that had been incubated with 10 μM Gln displayed prominent vacuoles in the cytoplasm (Figure 3B). Similar structures were observed in cells incubated with *p*-methoxybenzylamino-Gln (Figure 3D). In contrast, cells incubated with the vehicle (Figure 3A) and cells incubated with similar concentrations of the non-cytotoxic benzylamines (Figures 3C and 3E) did not have such vacuoles. Since vacuoles were only observed in cells incubated with cytotoxic GAELs, the results suggest that generation of the LAVs is intimately linked to the cytotoxicity of the compounds.

It has been postulated that the primary mode of Gln entry into cells is by diffusion (17), but there is no experimental evidence in support of this. Furthermore, the observation that lipophilic benzylamino-Gln analogs did not generate LAVs and were not cytotoxic suggests that uptake by diffusion may be insufficient to initiate the processes culminating in cell death. This led us to investigate if Gln is taken up by endocytosis. Therefore, ASK1 –/– cells were incubated with 12 μM Gln in the presence or absence of the endocytosis inhibitor, MCD (21), and the appearance of the cytoplasmic vesicles was monitored. No vacuoles were present in control cells incubated for 5 h with EtOH, the vehicle (Figure 4A), or with 5 mM MCD alone (Figure 4C). However, 5 h after incubation with Gln, cytoplasmic vacuoles are clearly visible in the cells (Figure 4B). In cells incubated with 12 μM Gln and MCD, such vacuoles are not present (Figure 4D). After 12 h incubation with 12 μM Gln, the cells were all rounded but vacuoles were still visible in some cells (Figure 4F). Analysis of the floating and adherent cells by trypan blue exclusion assay showed viability of 13%, while control cells with the vehicle gave a value of 97%. Thus most of the cells were dead following 12 h incubation with 12 μM Gln. In contrast, vacuoles were still not observed in the cells incubated with Gln and MCD and few rounded up cells were evident (Figure 4H) relative to the controls with MCD alone (Figure 4G). Trypan blue dye exclusion assay showed that viability was 93% compared with 96% for control cells with MCD alone. The results demonstrate that MCD inhibits the formation of vacuoles and prevents Gln-induced cell death. Since MCD inhibits both caveolar-mediated and raft-mediated endocytosis (21), these observations indicate that Gln uptake takes place *via* endocytosis and the vacuoles observed may be formed by Gln-induced perturbation of the endocytic process. Future studies will investigate if uptake is by raft-mediated or caveolin-mediated endocytosis.

In the endocytic pathway, early endosomes undergo fission and fusion events to mature into late endosomes which also undergo changes to form lysosomes (22). If Gln enters the cells *via* endocytosis, as our results indicate, then it should first be associated with early endosomes in cells and would subsequently be found in late endosomes/lysosomes. Even though LAVs have been reported to be derived from lysosomes (17), the results reported above suggest that LAVs could be derived from any one or all of the endocytic vesicles. The demonstration that some LAVs may be derived from early endosomes would support such a scenario.

To investigate whether LAVs may be derived from early endosomes, our approach was to inhibit the formation of late endosomes or lysosomes. Incubation of cells at or below 26˚C permits endocytic traffic to proceed from early endosomes to the endosomal carrier vesicles but not into late endosomes/lysosomes (23-26). Therefore, ASK 1 –/– MEFs were incubated with and without 12 μM Gln at 37 or 20˚C for different periods (5 and 24 h), and the appearance of vacuoles was observed by light microscopy. Viability analyses were carried out by the trypan blue dye exclusion assay. Five hours after incubation with 12 μM Gln, vacuoles were observed in cells incubated at 37˚C (Figure 5B) and 20˚C (Figure 5D) but there were more vacuoles in the cells incubated at 37˚C than those incubated at 20˚C. At 24 h after incubation with 12 μM Gln, only rounded up dead cells (confirmed by trypan blue analysis) were observed in the dishes incubated at 37˚C (Figure 5F), whereas the cells in dishes incubated with Gln at 20˚C were still viable (96%) and contained vacuoles (Figure 5H). When cells were incubated with Gln for 4 h at 37˚C and subsequently cooled to 20˚C and maintained at 20˚C for 20 h, vacuoles were still present and the cells were viable (Figure 5I). The results indicate that LAVs can be derived from early endosomal vesicles since at temperatures that inhibit maturation of early endosomes into late endosomes and lysosomes, LAVs are formed. Furthermore, active endocytosis is required for manifestation of the cytotoxicity of Gln since inhibiting late endosome/lysosomal formation by incubating the cells at 20˚C curtailed Gln-induced cell death even though LAVs derived from early endosomes were present.

The need for an active endocytic pathway for manifestation of Gln activity is also supported by the results of the MCD study (Figure 4). The inability of the early endosome-derived

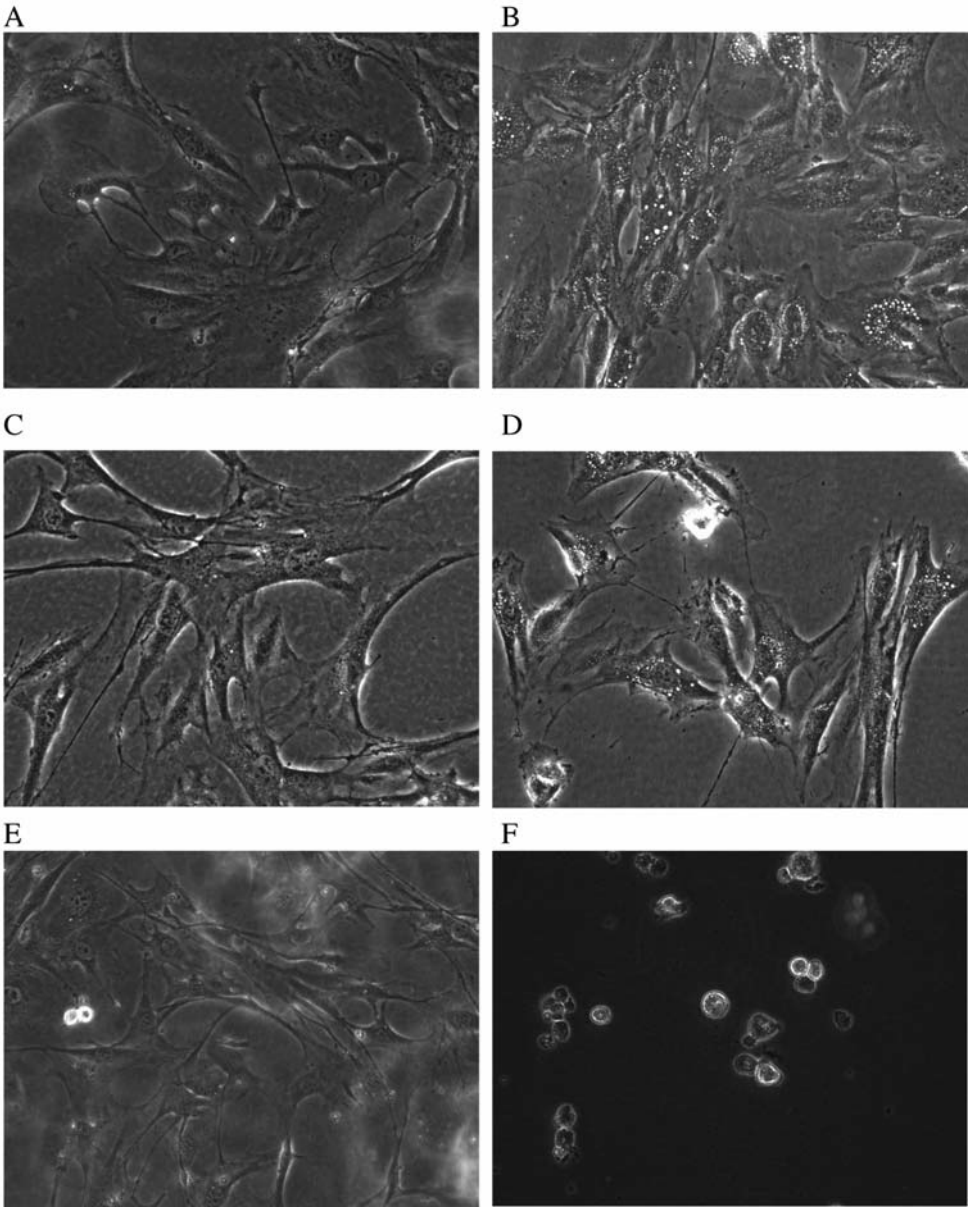

Figure 5. *continued*

vacuoles to induce cell death may be due to the absence of acidic hydrolases such as cathepsins in the early endosome, as these components are added at the late endosomal or lysosomal stages in the maturation process of endocytic vesicles (27).

In summary, the results of our studies have provided insight into the mechanism of action of GAELs. While the amphiphilic nature of GAELs should enable these compounds to readily enter cells by diffusion, our results suggest that diffusion of GAELs into cells does not lead directly to toxicity. Rather, uptake by endocytosis and an active endocytosis pathway are required for the formation of the LAVs that lead to cell death. The generation of LAVs from endocytic vesicles and their association with cell death is novel and has not been described as far as we are aware. Thus, the cellular localization of the compound appears to be critical in determining the cytotoxic activity of Gln and its analogs.

Acknowledgements

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Figure 5. *continued*

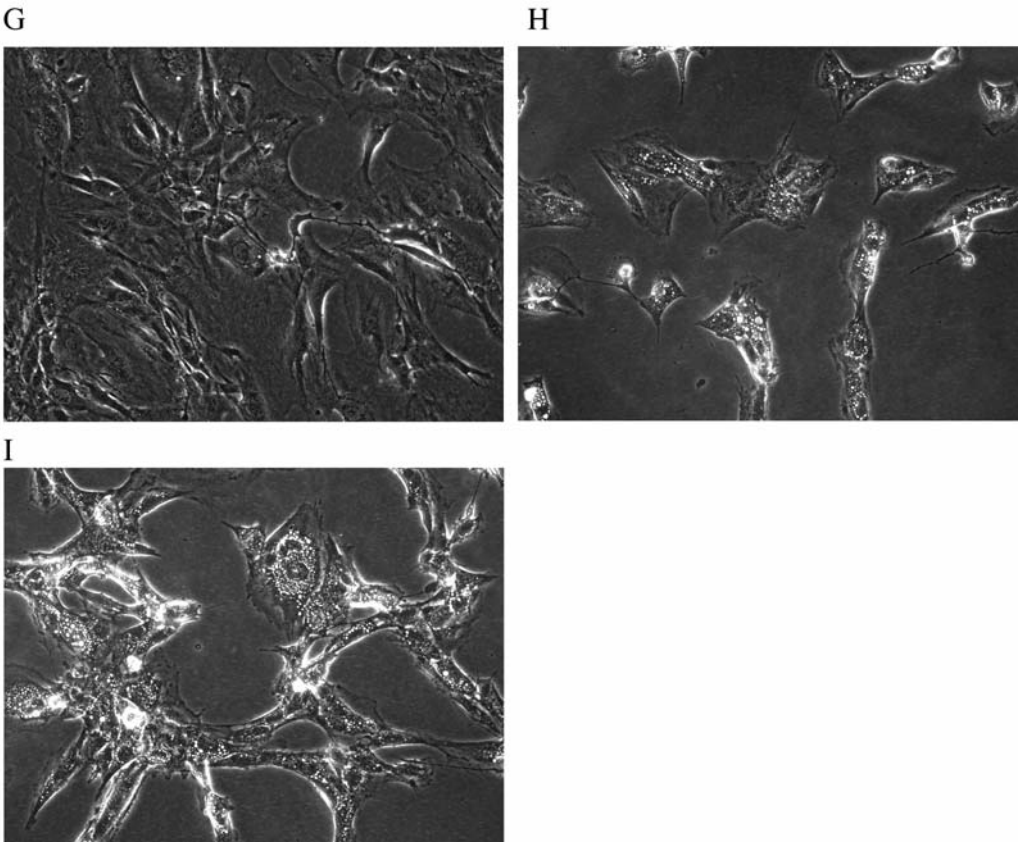

Figure 5. *Effect of temperature on LAV formation and cell death in ASK1 –/– cells. ASK1 –/– cells were incubated for 5 h at 37˚C in the absence (A) or presence of 12 μM Gln (B) or at 20˚C for the same period in the absence (C) or presence of 12 μM Gln (D). The cells were visualized by phase-contrast microscopy at a magnification of ×30. Cells incubated for 24 h at 37˚C in the absence (E) or presence of 12 μM Gln (F) or at 20˚C in the absence (G) or presence of 12 μM Gln (H) are shown at a magnification of ×20. Cells incubated at 37˚C with 12 μM Gln for 4 h and subsequently transferred to 20˚C for 24 h are shown in panel I.*

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