Research Paper

1-O-Hexadecyl-2-O-methyl-3-O-(2′-acetamido-2′-deoxy-3′-D-glucopyranosyl)-sn-glycerol (Gln) induces cell death with more autophagosomes which is autophagy-independent

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Autophagic cell death has recently received a great deal of attention. However, a dependence of this type of cell death on the actual process of autophagy has only rarely been proven. Indeed, it is important to differentiate between cell death with an accumulation of autophagosomes and cell death actually caused by excessive or inhibited autophagy. The aim of this study was to elucidate the mechanism of action involved in the cytotoxicity of 1-O-hexadecyl-2-O-methyl-3-O-(2′-acetamido-2′-deoxy-3′-D-glucopyranosyl)-sn-glycerol (Gln) and specifically the involvement of autophagy in the effects observed. Our results show that Gln induces cell death associated with large increases in autophagolysosome number and size. However the cell death is independent of autophagy and caspase activation. Instead, Gln leads to lysosomal membrane permeabilization with a resulting leakage of hydrolases into the cytosol, which are then directly involved in cell death. The increased number of autophagolysosomes, however, is just a side effect of the neutralization of the lysosomal pH by Gln.

Introduction

Cell death plays an important role during development and homeostasis in multicellular organisms. On the other hand, cell death can also be a pathological effect. Depending on the stimulus, the state of the cell and the circumstances, a cell can die in a variety of ways, some more regulated than others. Three different kinds of cell death can be differentiated morphologically: apoptosis, autophagic cell death and necrosis. These can be further characterized by certain biochemical features. Features of apoptosis include the activation of proteases of the caspase family, the condensation of chromatin and presentation of phosphatidylserine on the surface of the plasma membrane. Autophagic cell death has recently received a lot of attention, but has remained only morphologically defined. Autophagy is a degradative pathway, which involves the formation of double-membrane vesicles around portions of cytosol to form autophagosomes. These eventually fuse with lysosomes, where their contents are degraded. In autophagic cell death, an accumulation of autophagosomes can be observed; however the involvement of the degradative process in the execution of cell death has so far only been shown under really specific circumstances, e.g., in the D. melanogaster salivary gland. Indeed, autophagy is mostly considered to be a prosurvival pathway. Necrosis is morphologically characterized by cytoplasmic swelling, rupture of the plasma membrane and swelling of organelles. However, it is often used to describe cell death which does not show apoptotic or autophagic markers. Additionally, there are a variety of other possible ways for cells to die, which might combine different characteristics of the main types described above. These might often proceed in parallel with classical apoptotic pathways and may only become evident when apoptosis is blocked.

Cancer cells often show mutations in key proteins regulating apoptosis and are therefore more resistant to this form of cell death. This makes them a good model to study alternative cell death pathways, with the added benefit of the discovery of novel cancer drugs. 1-O-hexadecyl-2-O-methyl-3-O-(2′-amino-2′-deoxy-3′-D-glucopyranosyl)-sn-glycerol (Gln), has been previously shown to inhibit epithelial cancer cell growth in a variety of cell lines, with a clear cytotoxicity at higher concentrations. The targets of Gln and related drugs, which are collectively classified as glycosylated antitumor ether lipids (GAELs), are unknown. Our results show that Gln-induced cell death is associated with a large increase in autophagolysosome number and size. However the cell death is independent of autophagy and caspase activation. Instead, Gln...
leads to lysosomal membrane permeabilization with a resulting leakage of hydrolases into the cytosol, which are then directly involved in cell death. The lysosomal neutralization mediated by Gln leads to an increased number of autophagolysosomes as an epiphenomenon, as there is decreased autophagosome clearance.

**Results**

**Gln induces caspase-independent cell death.** The ether-linked glucosyl diglyceride 1-O-Hexadecyl-2-O-methyl-3-O-(2’-acetamido-2’-deoxy-3-D-glucopyranosyl)-sn-glycerol (from now on called Gln) has been previously shown to inhibit epithelial cancer cell growth in a variety of cell lines, with a clear cytotoxicity at higher concentrations. As can be observed from its structure (Fig. 1A), it contains both a long chain alkyl group (a) and primary amino group (b).

We replicated the cytotoxic effects of Gln in mouse embryonic fibroblasts (MEFs) treated with varying concentrations of the compound for only 7 hours (Fig. 1B). This effect was clearly dependent on the concentration of the compound. Cell death was assayed by trypan blue staining. Trypan blue is a dye which is excluded from live cells, but can enter into dead cells, i.e., dead cells have a specific blue color under the light microscope. However, it cannot differentiate between various forms of cell death. A similar cytotoxicity could be observed in all other cell lines (HeLa, NRK, PC12) examined (data not shown). When treated cells were stained with the nucleic acid stain 4’,6-diamidino-2-phenylindole (DAPI) and observed under the fluorescent microscope, we saw no obvious increase in the number of condensed or fragmented nuclei typically seen with apoptosis (data not shown).

To characterize the type of cell death further, we first assessed caspase-3 and caspase-9 activation as a diagnostic tests for caspase-mediated apoptosis. No caspase activation could be observed upon Gln treatment, while around 70% of cells were dead at this time point at the highest concentration and observed by trypan blue staining. Staurosporine, however, which was used as positive control, clearly led to caspase-3 and caspase-9 activation (Fig. 1C and Suppl. Fig. 4A). This suggested that Gln kills the cells independently of caspase-3 and caspase-9 activation. Since caspase-3 is an execution caspase that is activated by upstream caspases induced by either the intrinsic or extrinsic apoptotic pathways, these data suggested that the cell death induced by Gln is not associated with caspase activation. Indeed caspase inhibition did not influence the toxicity of Gln. The nonapoptotic nature of the cell death caused by Gln was supported by the absence of a loss of mitochondrial membrane potential or leakage of cytochrome c into the cytoplasm (Fig. 1E and F).

**Gln induces vacuolarization.** Interestingly, when observing the treated cells under the light microscope, a clear vacuolarization could be observed (Fig. 1D). Bright vacuoles started to form around the nucleus even at low Gln concentrations, which increased in size and number over time. This was clearly concentration-dependent, i.e., at higher concentrations the cells became literally filled with these structures. This was not only observed in MEFs, but also in all other cell lines examined (data not shown).

The combination of those two effects, i.e., caspase-independent cell death and perinuclear vacuolarization, suggested that the process of autophagy could be involved in the drug’s effects. One could hypothesize that the compound either induced excessive autophagy or blocked autophagosome maturation, thereby leading to the vacuolization observed and eventually cell death.

**Gln blocks autophagy.** To test this hypothesis, we first assessed the compound’s effects on autophagy by analyzing LC3-II levels. LC3 is a ubiquitin-like protein, which can be found in two forms in the cell: LC3-I is cytosolic, but can then be conjugated to phosphatidylethanolamine, yielding LC3-II, which is targeted to the autophagosome membrane. It remains on the autophagosome at least until fusion with the lysosome, making it an excellent marker for autophagosomes. LC3-positive vesicles are autophagosomes or autophagolysosomes (autophagosomes fused with lysosomes) and LC3-II levels correlate with the number of autophagosomes.

HeLa cells were therefore treated with 10, 20 or 50 μM Gln for 7, 24 or 48 hours (with replacement of the drug after 24 hours). Gln largely increased LC3-II levels, an effect which was clearly concentration dependent (Fig. 2A). However, the highest concentration used was highly cytotoxic, as all cells were rounded up and detached after treatments longer than 7 hours. Longer treatments did not seem to have any additional effects, as seen for LC3-II for the 24 hours and 48 hours time points. Gln also induced LC3-II levels in parallel with cell death in mouse embryonic fibroblasts, in a concentration-dependent manner, without affecting caspase 3 activation (Fig. 1B and C). An increase in LC3-II levels can have two possible causes—either an increased autophagosome formation or a block in autophagosome maturation. The latter could be due to a block in autophagosome-lysosome fusion or lysosomal degradation. To differentiate between those two possibilities, cells were additionally treated with a saturating concentration of bafilomycin A1, an inhibitor of the vacuolar proton pump, i.e., an inhibitor of lysosomal acidification. As this treatment blocks autophagosome maturation completely, only an inducer of autophagosome formation could increase LC3-II levels further, while an inhibitor of autophagosome maturation would have no additional effects.

Indeed, combined treatment with Gln and bafilomycin did not increase LC3-II levels above the levels due to bafilomycin alone (Fig. 2B). This suggested that Gln blocks autophagosome maturation, as opposed to increasing autophagosome synthesis. This block in maturation would consequently inhibit autophagic degradation. Interestingly, bafilomycin treatment also abolished the vacuolarization induced by Gln treatment (Fig. 2C). But note that the vacuolarization is not equivalent to autophagosome numbers. This block in maturation would consequently inhibit autophagic degradation. In order to test this hypothesis, we used a rat pheochromocytoma (PC12) cell line stably expressing a mutant huntingtin exon1 construct fused to GFP under the control of the tet-on system. Mutant huntingtin (the protein which causes Huntington’s disease) is a well-characterized autophagic substrate.

Mutant huntingtin exon 1 constructs form aggregates when expressed in cells, and the number of cells with aggregates correlates with the expression levels of the construct. The expression of the transgene was induced for 8 hours with doxycycline, then the cells were incubated in doxycycline-free medium for 24 hours and finally treated with 15 μM Gln or 400 nM bafilomycin for the
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Figure 1. For figure legend, see page 838.
Figure 1 (See previous page). 1-O-Hexadecyl-2-O-methyl-3-O-[2’-acetamido-2’-deoxy-3-D-gluco-pyranosyl]-sn-glycerol (Gln) induces caspase-independent cell death and vacuolarization. (A) Structure of Et-18-OCH<sub>3</sub> and Gln. Gln has a long aliphatic sidechain (a) and a primary amine (b). (B) Gln induces cell death in a concentration-dependent manner. Wild-type MEFs were treated for 7 h with the concentrations of Gln indicated, the cells were collected and stained with trypan blue. The percentage of trypan blue positive cells was then determined using a haemocytometer. Cumulative data from 3 independent experiments are reported; statistical analysis was performed by Student’s t-Test: p < 0.05: *; not significant: n.s. (C) No caspase-3 activation caused by Gln-induced cell death. Cells were treated for 7 hours with 3 μM staurosporine as positive control and cell death was determined as in (B). wt = wild type MEFs, KO = Atg5-deficient autophagy incompetent MEFs. Low exp. means short time of X-ray film exposure to the blot, while high exp. means longer time of X-ray film exposure to the blot. (D) Gln induces vacuolarization. Wt MEFs were treated with 30 μM Gln for 4 h. Scale bars represent 30 μm. (E) Gln does not induce mitochondrial membrane permeabilization. HeLa cells were treated for 8 hours with 30 μM Gln or with 3 μM staurosporine (STS) as positive control and subjected to cell fractionation. Both the membrane and cytosolic fractions were analyzed by western blot with the indicated antibodies. Cumulative data from 3 independent experiments are reported; the p values for the densitometric analysis were determined by factorial ANOVA test, where the control condition was set to 100: p < 0.05: *; not significant: n.s. (F) Gln does not affect mitochondrial membrane potential. HeLa cells were either left untreated or incubated for 8 hours with 30 μM Gln and then stained with JC-1 reagent as detailed in the material and methods section. When there is a loss of mitochondrial membrane potential, this results in a loss of the red fluorescence, plotted on the Y axis. This is obvious when one looks at the data from the standard positive control, the mitochondrial uncoupling agent CCCP, used for 5 minutes at 50 μM final concentration in control cells. However, we did not observe any reduction in the mean changes in 2 triplicate experiments using Gln. wt = wild type MEFs, KO = Atg5-deficient autophagy incompetent MEFs. Low exp. means short time of X-ray film exposure to the blot, while high exp. means longer time of X-ray film exposure to the blot.

last 24 hours. This regime assesses the effects of drugs on the ability of cells to clear the aggregate-prone protein, as it tests the effects of the drug after the synthesis of the transgene is “switched off.” Gln led to a clear increase in the number of cells with aggregates, albeit not as strong as the one observed upon bafilomycin treatment (Fig. 3A). To corroborate this, we have shown that Gln increases the accumulation of huntingtin exon 1 aggregates in HeLa cells (Fig. 3D–E), a phenomenon we also see with a range of other autophagy inhibitors. Likewise, Gln causes the accumulation of the endogenous autophagy substrate, p62 (Fig. 3B-C). Thus, Gln blocks autophagosome maturation, and this would slow clearance of autophagic substrates such as mutant huntingtin.

To further investigate the mechanisms of this block of autophagosome maturation, NRK cells were transiently transfected with an RFP-GFP-LC3 expression vector. Due to the vastly different pK<sub>a</sub> of the two fluorescent proteins (pK<sub>a</sub> (GFP) around 6, pK<sub>a</sub> (RFP) <4.5), this construct can be used as a probe for autophagosome maturation. At physiological pH, i.e., in newly formed autophagosomes, both proteins are stable, leading to both red and green fluorescence. Upon acidification, i.e., fusion with the lysosome, green fluorescence is rapidly lost due to the high pK<sub>a</sub> of GFP and only red fluorescence remains (Fig. 4A). Treatment with 15 μM Gln for 24 hours led to a decrease in the proportion of vesicles that were...
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Gln treatment, which clearly decreased the percentage of double-positive vesicles (Suppl. Fig. 1B), showing that it decreases autophagosome-lysosome fusion at this time point, Gln treatment led to an increase in double-positive vesicles, i.e., autophago/lysosomes (Fig. 4C and D). Moreover, those autophago/lysosomes were clearly increased in size, suggesting that they are the vacuoles visible under the light microscope. This hypothesis was further confirmed by staining NRK cells treated with increasing concentrations of Gln with an antibody against lgp120. Thereby, a clear increase in the size of lgp120-positive vesicles, i.e., autophago/lysosomes could be observed (Fig. 4E). These data suggest that only red (i.e., acidified), similar to bafilomycin treatment (Suppl. Fig. 1A), but in contrast to rapamycin treatment, a well-known inducer of autophagy (Fig. 4B and D). These data were confirmed in HeLa cells stably expressing RFP-GFP-LC3 constructs (Suppl. Fig. 3). As mentioned above, this could be due to a decrease in fusion or a decrease in acidification. To differentiate between those two possibilities, cells were cotransfected with mCherry-LC3 mCherry is a novel red fluorescent protein and lgp120-GFP in a parallel experiment. Lgp120 is the rat homologue of LAMP-1, a transmembrane glycoprotein, which is mainly localized to the limiting membranes of lysosomes and late endosomes. Unlike bafilomycin treatment, which clearly decreased the percentage of double-positive vesicles (Suppl. Fig. 1B), showing that it decreases autophagosome-lysosome fusion at this time point, Gln treatment led to an increase in double-positive vesicles, i.e., autophago/lysosomes (Fig. 4C and D). Moreover, those autophago/lysosomes were clearly increased in size, suggesting that they are the vacuoles visible under the light microscope. This hypothesis was further confirmed by staining NRK cells treated with increasing concentrations of Gln with an antibody against lgp120. Thereby, a clear increase in the size of lgp120-positive vesicles, i.e., autophago/lysosomes could be observed (Fig. 4E). These data suggest that

Figure 3. Gln decreases autophagic clearance. (A) Gln increases mutant huntingtin aggregation in PC12 74.10 cells. Expression of huntingtin-Q74-GFP was induced for 8 h with doxycycline. Then cells were washed and incubated in doxycycline-free medium for 48 h. In the last 24 h, cells were treated with 15 μM Gln or Bafilomycin. The percentage of cells with aggregates was assessed as described previously. Values represent means with SDs. Statistical analysis by Student’s t-test: *p < 0.05, **p < 0.005. (B) HeLa cells were treated for 24 hours with 15 μM Gln and the effect on autophagy was monitored by analysing the levels of LC3-II and of the autophagy substrate p62. (C) The graphs show cumulative data from three independent experiments performed as reported in (B); the p values for the densitometric analysis were determined by factorial ANOVA test, where the control condition was set to 100: *p < 0.05; not significant: n.s. (D) HeLa cells seeded on glass coverslips were transfected with 1.5 μg of the EGFP-HDQ74 expression vector. 24 h after transfection, cells were incubated with the indicated drugs for 24 h, and finally fixed and analyzed under a fluorescence microscope. The p values for assessing EGFP-HDQ74 aggregation were determined using Student’s t-test (n = 3; *p < 0.05, **p < 0.005). (E) Representative pictures of the experiment performed in (D). The arrowheads indicate the presence of the EGFP-HDQ74 intracellular aggregates.
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| Figure 4. Gln decreases maturation of autophagosomes and increases LC3/lgp120 colocalization. (A) Rationale of the RFP-GFP-LC3 assay. Under physiological pH LC3+ vesicles fluoresce emit both green and red fluorescence. Upon fusion with lysosomes and concomitant acidification, GFP fluorescence is rapidly lost, resulting in vesicles with only RFP fluorescence. (B) Gln decreases maturation of autophagosomes. NRK cells were transiently transfected with RFP-GFP-LC3, then treated with 15 μM Gln for 24 hours. (C) Gln increases mCherry-LC3 lgp120-GFP colocalization and increases lysosomal size. NRK cells were transiently transfected, then treated with 15 μM Gln for 24 hours. (D) Quantitative analysis of experiments performed in (B and C). As controls, cells were treated with 0.2 μg/ml Rapamycin or 400 nM Bafilomycin for 24 hours. (mean with standard deviation, 20 cells each, tested against control). Statistical analysis by Student’s t-test: **p < 0.005. Scale bar represents 10 μm. (E) Gln-induced vacuoles are of lysosomal origin. NRK cells were treated for 4 h with 7.5, 15 or 30 μM Gln and then stained for lgp120. Scale bars represent 20 μM. (F) Gln treatment decreases acidification at low concentrations, but leads to the formation of large acidic vesicles at high concentrations. Wild-type MEFs were preloaded with 2.5 μg/ml acridine orange for 15 min and then treated for 4 h with 7.5 μM Gln, or with 400 nM bafilomycin as positive control. Images were then taken of living cells.
Gln treatment does not inhibit autophagosome-lysosome fusion, but rather increases lysosomal pH. As a result, lysosomal degradation of LC3 is reduced, leading to its accumulation upon Gln treatment. This effect on acidification could be explained by Gln being trapped in acidic lysosomes after protonation of the primary amine (Fig. 1A, b), leading to its accumulation in the lysosomes and a decrease in lysosomal pH, a mechanism involved in the function of a subset of other compounds referred to as lysosomotropic agents. To further investigate this effect on acidification, wild-type MEFs were stained with acridine orange for 15 min and then treated for 4 hours with 7.5 μM Gln or with 400 nM bafilomycin as a control. For this aim, acridine orange dye represents a lysosomotropic dye, i.e., upon protonation it is retained in acidic organelles (e.g., lysosomes) and emits fluorescence at around 650 nm (red). Cells treated with Gln showed a clear decrease in the size and intensity of acidic (red) vesicles, suggesting decreased acidification (Fig. 4F). Interestingly, an appearance of green vesicles could also be observed, which could possibly be explained by a complete loss of acidification, resulting in loss of protonation and therefore green fluorescence of the accumulated acidine orange. As expected, bafilomycin treatment clearly led to a complete loss of acidic vesicles (Fig. 4F). It has also been suggested that vacuolarization by lysosomotropic agents can be reduced by addition of the sugar mannitol to the culture medium, by increasing cytosolic osmolarity. However, this seems to be highly cell-type dependent, as we observed an increase in LC3-II levels and vacuolarization when adding the similar sugar sorbitol at the same concentration to NRK cells (data not shown). This might be explained by endocytosis of the sugar, which has also been previously observed.

Gln induces lysosomal membrane permeabilization. Since Gln seemed to behave like a lysosomotropic agent, we decided to evaluate whether it also induced lysosomal membrane permeabilization, as has been already described for other compounds of this class. To test for a potential leakage of lysosomes with a highly sensitive and quantitative assay, the activity of lysosomal cysteine cathepsins was assessed in cytosolic preparations from wild-type MEFs treated with various concentrations of Gln. For this aim, cytosolic proteins were extracted from the cells with a buffer containing 50 μg/ml of the glycosidic detergent digitonin, a concentration which we had determined to be high enough for the extraction of cytosolic proteins, while still leaving lysosomal membranes intact in this cell line (see Suppl. Fig. 2A). Cathepsin activity was then assessed by a kinetic assay measuring the generation of fluorescence from the synthetic substrate Z-Phe-Arg-AMC. LDH activity was used as a control for equal cytosolic protein extraction. Values represent means with SDs of 3 independent experiments of 6 samples for each treatment. Statistical analysis was performed by Student’s t-test: not significant: n.s.; **p < 0.005. (B) HeLa cells were incubated for 8 hours with 30 μM Gln and subjected to cell fractionation. Both the membrane and cytosolic fractions were analysed by western blot with the indicated anti-cathepsin antibodies. The western blots shown are representative of experiments performed in triplicate; pre-: cathepsin B precursor, m: mature cathepsin B.

These data were confirmed by subcellular fractionation experiments showing that Gln induced leakage of cathepsins B, D and L from the lysosomes to the cytoplasm (Fig. 5B).

We did not see Bid cleavage resulting from Gln treatment (Suppl. Fig. 4B and C). While Bid may be cleaved by cathepsins, the absence of cleaved Bid may be difficult to interpret, as the levels of cleaved Bid will depend both on the amounts of cathepsin
cleavage, the amount of time the cells live, and the effects of other proteases on the turnover of the Bid fragments. Nevertheless, our failure to see Bid cleavage is compatible with the absence of a loss of mitochondrial membrane permeabilization and caspase activation.

Gln induces cell death and vacuolization independently of autophagy. Up to this point, our experiments had shown that Gln induces caspase-independent cell death, lysosomal membrane permeabilization, vacuolization and blocks autophagy. However, it had remained unclear whether autophagy was actually directly involved in these effects or just a secondary effect itself. To investigate the role of autophagy, we used MEFs generated from an Atg5−/− mouse (Atg5 KO), with matched wild-type MEFs (Atg5 wt). Atg5 is a protein which is essential for autophagosome formation, i.e., its loss leads to a complete loss of autophagosomes.18 To test for the involvement of autophagy in cell death, we treated Atg5 wt or KO MEFs with different concentrations of Gln for 7 hours, collected them and stained a small aliquot with trypan blue (see earlier). As described earlier (Fig. 1B), there was a clear concentration-dependent increase in the percentage of cell death assayed by this method (Fig. 6A). However, no significant differences between Atg5 wt and KO MEFs could be observed, suggesting that autophagy was not a major factor in the cell death observed. The remainder of the cells was then collected for western blotting. Neither cell line showed any caspase-3 activation (Fig. 1B). Interestingly, similar degrees of vacuolization could be observed in both cell lines (Fig. 6B), suggesting that the accumulation of undegraded autophagic substrates is not necessary for this effect. These results suggest that the block in autophagy is just a secondary effect of Gln treatment, without any major functional importance for the other effects observed.

Gln-induced cell death depends on cathepsin activity. Since Gln treatment leads to lysosomal membrane permeabilization, we wanted to test whether leakage of lysosomal hydrolases into the cytosol, most importantly cathepsins, were actively involved in Gln-induced cell death. When cells were treated with the aspartic cathepsin inhibitor pepstatin A, a significant rescue from Gln-induced cell death was observed (Fig. 7A and B). We also tested if E-64, a cysteine cathepsin inhibitor, reduced cell death caused by Gln. While E64 did not reduce cell death caused by Gln, the data may be difficult to interpret, as E64 induced some cell death on its own, possibly as a consequence of blocking autophagy (Fig. 7D). Similarly, bafilomycin A1 did not reduce cell death caused by Gln, but was toxic on its own probably due to its autophagy-inhibitory properties and their consequences (Fig. 7C).27 Therefore, Gln seems to induce cell death by a mechanism dependent on cathepsin activity, but independent of autophagy, caspase activation and chromatin condensation.

Discussion

Gln causes cell death and increased numbers of autophagolysosomes in a range of cell types (including mouse embryonic fibroblasts and HeLa cells),26 thus its effects may be fairly generic. Sammader et al.26 interpreted their data showing increased numbers of autophagosomes caused by Gln to be due to increased synthesis. Here we have shown with a range of assays, including of LC3-II synthesis studies (in the presence of bafilomycin A1), studies with GFP-RFP-LC3, and assays of both endogenous and exogenous autophagy substrate accumulation/degradation, that Gln primarily acts to decrease autophagic flux by impairing lysosomal acidification. However, our data showing that Gln-induced cell death is independent of autophagy and caspases are compatible with those of Sammader et al.26 Here, we have advanced the understanding of the cell death mechanism by revealing that this is due to leakage of cathepsins from lysosomes. Thus, this cell death is independent of autophagy and caspases, but appears to be due to leakage of cathepsins from lysosomes. We propose the following model for Gln’s mode of action (Fig. 8): Gln can diffuse freely through the plasma membrane and into the lysosomes. There, its
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Vacuoles and toxicity induced by Gln also occur in Atg5 null cells, suggesting that the primary toxic process is autophagy independent. Indeed, this situation has some similarities to what was previously noted by Overmeyer et al. and represents a form of cell death mediated by lysosomes.

It has been previously suggested that some lysosomotropic amines can act as detergents, once they reach a high enough concentration in the lysosomes. This could indeed be the case for Gln, as it has a long aliphatic chain and also shows a threshold response for lysosomal membrane permeabilization, i.e., increasing the concentration above a certain level does not increase cathepsin release any further. This detergent effect might be further exacerbated by the accumulation of undegraded autophagocytosed material.

Importantly, cysteine cathepsins have been shown to be stable and active outside of their optimal (lysosomal) pH, suggesting a high degradative potential in the cytosol. Thus cathepsins can wreak havoc if let loose in the cytoplasm even when the pH is suboptimal. The concentrations of the Gln necessary for the effects to appear varied for different cell lines, a phenomenon which might be due to cell type specific differences or possibly due to slightly defective acidification, a change which can occur upon transformation of cells. Interestingly, transformation of cells seems to lead to increased susceptibility to lysosomal cell death, suggesting a possible application of lysosomotropic agents to induce cell death specifically in cancer cells.

In conclusion, Gln provides a clear example of cell death with increased autophagosomes where cell death is autophagy independent and where the increased numbers of autophagosomes are due to an impaired removal of these vesicles.

Figure 7. Gln-induced cell death is mediated by cathepsins. (A) Gln-induced cell death is significantly reduced by treatment with the aspartic cathepsin inhibitor pepstatin A. Cells were treated for 7 h with 30 μM Gln or 30 μM Gln and 10 μg/ml pepstatin A, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (B) Gln-induced cell death is reduced by pepstatin A. HeLa cells were treated for 7 h with 30 μM Gln, 10 μg/ml pepstatin A or in combination, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (C) Gln-induced cell death is not reduced by Bafilomycin A1. HeLa cells were treated for 7 h with 30 μM Gln, 400 nM Baf A1 or in combination, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (D) Gln-induced cell death is not reduced by the cysteine protease inhibitor E-64. HeLa cells were treated for 7 h with 10 mM E-64, 30 μM Gln or both, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (E) Gln-induced cell death is not reduced by the cysteine protease inhibitor E-64. HeLa cells were treated for 7 h with 10 mM E-64, 30 μM Gln or both, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (F) Gln-induced cell death is not reduced by the cysteine protease inhibitor E-64. HeLa cells were treated for 7 h with 10 mM E-64, 30 μM Gln or both, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (G) Gln-induced cell death is not reduced by the cysteine protease inhibitor E-64. HeLa cells were treated for 7 h with 10 mM E-64, 30 μM Gln or both, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001. (H) Gln-induced cell death is not reduced by the cysteine protease inhibitor E-64. HeLa cells were treated for 7 h with 10 mM E-64, 30 μM Gln or both, collected and stained with Trypan blue. Percentage of dead cells was then determined as reported in (A). Statistical analysis was performed by unconditional logistical regression analysis: **p < 0.0001.
Materials and Methods

Constructs. We are grateful to T. Yoshimori for mRFP-GFP-LC3 and P. Luzio for GFP-lyp120. The EGFP-HDQ74 has been extensively characterized. The FLAG-tagged human Bid construct has been already described. The cloning of mCherry-hLC3B has previously been described.

Antibodies. Rabbit anti-actin (cat. n° A2066), rabbit anti-FLAG epitope (cat. n° F7425), and mouse monoclonal anti-cathepsin D (cat. n° C0715), and L (cat. n° C2970), antibodies were from Sigma; mouse monoclonal anti-cathepsin B (cat. n° ab33538), was from Abcam; mouse monoclonal anti-complex-I (cat. n° A21344), was from Invitrogen; rabbit polyclonal anti-caspase-3 (cat. n° 9665), mouse anti-caspase-9 (cat. n° 9508), rabbit anti-Bid (cat. n° 2002) and anti-cytochrome c (cat. n° 4280), were from Cell Signaling; rabbit anti-LC3 from Novus Biological (cat. n° NB100-2220), mouse monoclonal anti-p62 (cat. n° 610832), was from BD Biosciences; mouse anti-rat lgp120 a kind gift from P. Luzio, anti-mouse Alexa 488 (cat. n° A-11001) from Invitrogen, and anti-mouse/rabbit HRP-linked antibodies (cat. n° NA-931-1; NA-934-1), were from GE Healthcare.

Chemicals. The following chemicals were used (concentrations, solvents used and catalog number are reported in brackets): Acridine orange (2.5 μg/ml, H2O, cat n° A-3568, Invitrogen), bafilomycin A1 (400 nM, DMSO, cat n° 19-148, Millipore), 4',6-diamidino-2-phenylindole (3 μg/ml, cat n° D-9564, Sigma), digitonin (cat n° 300410, Calbiochem), doxycycline (1 μg/ml, DMSO, cat n° D9891, Sigma), E-64 (10 μM, DMSO, cat n° E3132, Sigma), beta-NADH (0.5 mM, cat n° N6660, Sigma), pepstatin A (10 μg/ml, 10% acetic acid in MeOH, cat n° P5318, Sigma), rapamycin (0.2 μg/ml, DMSO, cat n° R0325, Sigma), staurosporine (3 μM, DMSO, cat n° S4400, Sigma), trypan blue solution (cat n° T8154, Sigma), Z-Phe-Arg-7-amido-4-methylcoumarine (Z-Phe-Arg-AMC, 20 μM, cat n° 03-32-1501, Merck). All other standard laboratory chemicals were purchased from Sigma, unless otherwise indicated.

Cell culture. HeLa, NRK and Atg5 wild-type/knockout mouse embryonic fibroblasts (a kind gift from N. Mizushima) were grown in DMEM (cat n° D6546, Sigma), supplemented with 10% FBS (cat n° F7524, Sigma), 100 U/ml penicillin/streptomycin (cat n° P0781, Sigma), 2 mM l-glutamine (cat n° G7513, Sigma). HeLa cells stably expressing mRFPGFP-LC3 reporter were grown in the same media used above, supplemented with 600 μg/ml G418 (cat n° 11811-031, GIBCO-BRL). PC12 cells stably maintained at 75 μg/ml hygromycin B (cat n° CB-0579, Calbiochem) in DMEM with 10% horse serum (cat n° H1270, Sigma), 5% FBS, 100 U/ml penicillin/streptomycin, 2 mM l-glutamine, and 100 μg/ml G418 at 37°C, 10% CO2.

Transfection. Cells were transfected 24 hours after seeding with 1.5 μg DNA and 4.5 μl Lipofectamine 2000 (cat n° P/N 52887, Invitrogen) per well for 4 hours in Optimem (cat n° 31985, GIBCO-BRL). They were then washed once in full medium and cultured in full medium for the times indicated.

Acridine orange staining and live imaging analysis. Wild-type MEFs were seeded on 42 mm glass cover slips (cat n° 0727.016, PeCon, GmbH, Germany) at a density of approximately 3 x 10^5 cells per cover slip. The next day, they were incubated for 15 minutes with 2.5 μg/ml acridine orange (Invitrogen) in full medium at 37°C, washed twice in medium, treated with drugs for the time mentioned, and finally mounted in a POC chamber (PeCon GmbH). The imaging was performed on a Zeiss Axiovert 200M microscope with a LSM 510 confocal attachment using an 63X 1.4 NA Plan Apochromat oil-immersion lens, fitted with an X-L3 incubator (PeCon GmbH) heated to 37°C. The argon laser line at 488 nm was used for excitation, while two separate emission bands (505–570 nm and 615–754 nm) were simultaneously collected.

Cell death assay. For each sample, we seeded 3 wells at 1.75 x 10^5 Atg5 wt, Atg5 KO MEFs, or Hela cells per well. The next day,
cells were treated with the compounds as described in the main text and then collected by scraping, combining the three wells into one tube. The cells were spun down and resuspended in 1 ml of full medium. Cell viability was then assayed by trypan blue dye exclusion, i.e., the percentage of dead cells was determined by counting the number of blue cells in a haemocytometer. The remainder of the cells was then collected and processed for western blotting to assay for caspase-3 activation.

**Immunocytochemistry.** For cells transfected with fluorescent proteins, cells were fixed 15 minutes in 4% paraformaldehyde and then mounted in Prolong Gold antifade solution (cat n° P36931, Invitrogen) containing DAPI (3 μg/ml, Sigma). The lg120 staining was performed as previously described.5

**Colocalization analysis.** Cover slips were blinded and 20 cells per condition were imaged on a Zeiss Axiovert 200M microscope with a LSM 510 confocal attachment using an x63 1.4 NA Plan Apochromat oil-immersion lens. Laser lines at 488 nm (lg120-GFP, RFP-GFP-LC3), 543 nm (mCherry-LC3, RFP-GFP-LC3). These cells were then analyzed in Zeiss LSM Image Browser 3.5 as follows: First, after switching off all other channels, all mCherry-LC3 positive vesicles were counted and marked. Then, the GFP-channel was switched back on and the number of colorized vesicles was counted. From these values the fraction of double-labeled vesicles was determined, i.e., the percentage of LC3-positive vesicles labeled with another/both other markers was calculated. RFP-GFP-LC3 transfected cells were imaged and analyzed in the same way.

**Assessment of autophagy by automated cellomics microscope.** Counting of autophagosomes and autolysosomes was performed on a Thermo Scientific Cellomics ArrayScan HCS reader (20x objective) using the Spot detector V3 Cellomics Bioapplication.25 For identification of cells, nuclei were stained with DAPI and detected on the primary channel by a Hoechst-associated filter dependent upon size, shape and intensity thresholding. GFP and RFP vesicles were identified on separate secondary channels using FITC- and Texas Red-associated filters, respectively, dependent upon size, shape and intensity thresholding. The mean number of vesicles per cell (object) was calculated by the ArrayScan software as the total number of vesicles per field divided by the total number of objects per field. 1,000 cells were counted per coverslip, and the analysis was done on triplicate samples at least three times.

**Huntingtin exon1 aggregation experiment.** PC12 74.10 cells were induced with 1 μg/ml doxycycline (Sigma) for 8 hours, then grown in doxycycline-free medium for 24 hours and then finally treated with Gln or bafilomycin for 24 hours. After fixation/ mounting (see above) cells were analyzed under the fluorescence microscope. The total proportion of GFP expressing cells with inclusions was determined by counting a minimum of 200 cells (cell number was determined by counting DAPI stained nuclei). HeLa cells seeded on glass coverslips were transfected with 1.5 μg of the EGFP-HDQ74. 24 hours after transfection, cells were incubated for 24 h with 15–30 μM Gln or 400 nM Baf A1 and finally fixed and analyzed under fluorescence microscope. For fluorescence microscopy, images were acquired with Nikon Digital Camera DXM1200 attached to Nikon Eclipse E600 fluorescence microscope (plan- apo 60x/1.4 oil immersion lens at room temperature) using Nikon ACT-1 v2.12 acquisition software (Nikon, Inc.). The p values for assessing EGFP-HDQ74 aggregation were determined using Student’s t-test.

**Cell fractionation assay.** 5–6 x 10⁶ HeLa cells were treated for 8 h with 30 μM Gln or 3 μM staurosporine to induce cell death; cells were washed with cold PBS and harvested by scraping. The cell pellet was resuspended in extraction buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, passed through a 22-gauge needle, kept on ice for 45 min and centrifuged (30 min, 14,000 rpm at 4°C). The supernatants (cytosolic fractions) were collected and the membrane fractions were washed twice in the same buffer an then resuspended and solubilized in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue).

**Measurement of the mitochondrial membrane potential.** Mitochondrial membrane potential was determined by the JC-1 assay, according to the manufacturer’s instructions (cat n° M34152, Invitrogen). HeLa cells were treated for 8 h with 30 μM Gln, harvested, resuspended in culture medium at approximately 1 x 10⁶ cells/ml and then incubated with 2 μM JC-1 for 15 minutes at 37°C 5% CO₂. To confirm that JC-1 response was sensitive to changes in membrane potential, control cells were pre-incubated for 5 minutes with 50 μM CCCP before the JC-1 staining. Cells were then centrifuged and washed twice with PBS. After washing, cells were resuspended in PBS and analyzed by flow cytometry using 488 nm excitation with 530/30 nm and 585/42 nm bandpass emission filters on a FACSCalibur apparatus (Becton Dickinson).

**Digitonin extraction.** For each sample, we seeded 3 wells at 175,000 wt MEFs per well. The next day, cells were treated with the compounds as described in the main text and then collected, combining the three wells into one tube. They were then extracted with 300 μl 50 μg/ml digitonin (cat n° 300410, Calbiochem) in extraction buffer (250 mM sucrose, 20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA), a concentration that had previously been determined to be optimal (concentrations ranging from 1–200 μg/ml were tried and 50 μg/ml showed good cytosolic extraction, while lysosomal leakage was still minimal), for 10 minutes on ice with repeated vortexing. The samples were then spun down (90 seconds, 13,000 rpm, 4°C) and the supernatant quickly transferred to a new tube.

**LDH assay.** To measure extraction of cytosolic protein, the enzymatic activity of lactate dehydrogenase (LDH) was assayed. 50 μl of sample was pipetted into a 96-well plate and diluted 1:2 by adding 50 μl of extraction buffer. In parallel, the assay buffer was prepared (200 mM TrisHCl pH 7.5, 10 mM sodium pyruvate, 0.5 mM β-NADH). Immediately before starting measurements, 100 μl of assay buffer were added to each well of the plate and the oxidation of β-NADH by LDH was determined by measuring the absorption at 340 nm at room temperature on a plate reader (Molecular Devices Optimax tunable microplate reader). Measurements were repeated every minute for 10 minutes. Initial velocities were determined from the linear part of the resulting absorption decay curve.
**Cathepsin assay.** To assay cathepsin activity, the synthetic substrate Z-Phe-Arg-7-amino-4-methylcoumarine was used, which can be cleaved by all lysosomal cysteine cathepsins, generating fluorescence. To do that, a 40 μl volume of the digitonin-extracted samples were added to a 96-well plate, diluted with 50 μl phosphate buffer and incubated at 37°C for 15 minutes. During the incubation, the stock of substrate (10 mM in DMSO) was diluted to 400 μM in DMSO and then diluted 1:1 in phosphate buffer. 10 μl of this working substrate solution were then added to the wells. The kinetics of generation of the free fluorescent group were then measured at 37°C in a fluorescence plate reader (Tecan; 20 minutes, 1 measurement/minute, excitation 360 nm, emission 465 nm). Initial velocities were determined from the linear part of the resulting curve.

**Western blotting.** Cell pellets were lysed on ice in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) for 30 min in the presence of protease inhibitors (cat n° 14696200, Roche Diagnostics). Samples were subjected to SDS-PAGE, and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, cat n° IPVH00010, GE Healthcare). Blots were first probed with primary antibodies (see above). Then, they were probed with the appropriate anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase secondary antibodies and probed with the appropriate anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase secondary antibodies and visualized using an enhanced chemiluminescence detection kit (cat n° RPN2106V 1/2, GE Healthcare).

**Statistical analysis.** Densitometric analysis on the immunoblots was done by Image J software. The p values for the densitometric analysis were determined by factorial ANOVA test using STATVIEW v4.53 (Abacus Concepts), where the control condition was set to 100 or by Student’s t-test. Odds ratios were using STATVIEW v4.53 (Abacus Concepts), where the control condition was set to 100 or by Student’s t-test. Odds ratios were determined from the linear part of the resulting curve. Statistical analysis.

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