The JAK2/STAT3 and mitochondrial pathways are essential for quercetin nanoliposome-induced C6 glioma cell death

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The formulation of quercetin nanoliposomes (QUE-NLs) has been shown to enhance QUE antitumor activity in C6 glioma cells. At high concentrations, QUE-NLs induce necrotic cell death. In this study, we probed the molecular mechanisms of QUE-NL-induced C6 glioma cell death and examined whether QUE-NL-induced programmed cell death involved Bcl-2 family and mitochondrial pathway through STAT3 signal transduction pathway. Downregulation of Bcl-2 and the overexpression of Bax by QUE-NL supported the involvement of Bcl-2 family proteins upstream of C6 glioma cell death. In addition, the activation of JAK2 and STAT3 were altered following exposure to QUE-NLs in C6 glioma cells, suggesting that QUE-NLs downregulated Bcl-2 mRNAs expression and enhanced the expression of mitochondrial mRNAs through STAT3-mediated signaling pathways either via direct or indirect mechanisms. There are several components such as ROS, mitochondrial, and Bcl-2 family shared by the necrotic and apoptotic pathways. Our studies indicate that the signaling cross point of the mitochondrial pathway and the JAK2/STAT3 signaling pathway in C6 glioma cell death is modulated by QUE-NLs. In conclusion, regulation of JAK2/STAT3 and ROS-mediated mitochondrial pathway agonists alone or in combination with treatment by QUE-NLs could be a more effective method of treating chemical-resistant glioma.

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Despite the recent advancements in the treatment of glioma, the disease remains incurable by standard therapies that target the apoptotic pathway.1 Mammalian cell death can be broadly sub-classified into apoptosis, autophagy, and necrosis.2 Although all three types of cell death involve a sequential mechanism of programmed cell death (PCD), the majority of conventional anti-cancer therapeutic agents through the apoptotic pathway to induce cell death.3 However, this approach has been unsuccessful for curing glioma owing to drug-resistant apoptotic machinery involving receptors and pro-apoptotic/anti-apoptotic proteins. Several components such as reactive oxygen species (ROS), mitochondrial, and B-cell lymphoma/leukemia-2 (Bcl-2) family shared by the necrotic and apoptotic pathways have been identified, indicating there are crosstalk between the different signaling pathways.4,6 Therefore, the mode of PCD can be changed from apoptosis to necrosis and vice versa, suggesting that necrosis is programmed and controllable.6 In the context of glioma, agents that initiate a non-apoptotic PCD mechanism may readily overcome the inherent deficiencies of the apoptotic machinery. The manipulation of alternative PCD pathways may represent an attractive approach to increase the overall tumor cell-killing efficiency of glioma therapies.

Necrosis often is defined as a default PCD pathway. This concept is supported by evidence that in mouse embryonic fibroblasts and in immortalized baby mouse kidney epithelial cells, overexpression of Bcl-2 (anti-apoptotic) or simaneous knockdown of the pro-apoptotic Bcl-2-associated X protein (Bax) or Bcl-2-associated killer (Bak) and depletion of Beclin 1 (autophagic) lead to necrotic cell death when cells are exposed to hypoxia or etoposide.7,8 Regarding biochemical changes, loss of mitochondrial membrane potential (Δψm) is considered a hallmark of necrotic cell death. Δψm loss has been described as a response to increased cytosolic-free calcium, anoxia, and overproduction of ROS.9 Although both apoptosis and necrosis require Δψm loss, necrotic Δψm loss is accompanied by a loss in total cellular adenosine triphosphate (ATP). In contrast, ATP is a maintained and required factor for apoptosis.10

Quercetin (QUE) is a potential chemopreventer that functions in the suppression of many tumor-related processes, including apoptosis and proliferation.

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Abbreviations: QUE-NL, quercetin nanoliposomes; PCD, programmed cell death; NAC, N-acetyl-cysteine; IL, interleukin; JAK2, Janus kinase 2; STAT3, signal transducers and activators of transcription 3; BCL-2, B-cell lymphoma/leukemia-2; Bcl-xl, B-cell lymphoma extra large; Bak, BCL-2-associated X protein; ROS, reactive oxygen species; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; PI, propidium iodide

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A study has shown the anticancer efficacy of QUE when C6 glioma cells are exposed to concentrated QUE for extended periods, and C6 glioma cells are exhibited with a reduction in glutathione content and ROS accumulation. Thus, the pro-oxidant properties of QUE could prevail over its antioxidant properties and promote cell death.\(^\text{11}\)

In this study, we explore the detailed molecular mechanisms of QUE-NL (nanoliposome)-induced glioma cell death, including the mode of cell death, the involvement of major intracellular cell death signaling cascades, and QUE-NL-induced specific cell death signal transducers. The aim of this study was to optimize QUE-NL therapy for glioma treatment and to improve preclinical outcomes. We demonstrate that NLs enhance QUE bioactivity in inhibiting tumors. QUE-NLs induce necrotic cell death in C6 glioma cells as evidenced by:

- (a) decreased Δψm;
- (b) loss of ATP; and
- (c) increased ROS production. Moreover, treatment with QUE-NLs resulted in necrotic cell death, because it did not trigger the activation of caspases from the mitochondrial pathway.\(^\text{12}\)

QUE-NL-induced necrotic cell death was partially reversible by pretreatment with AG490, a JAK2 (Janus kinase 2) specific inhibitor.\(^\text{13}\) Paradoxically, AG490 effectively enhanced the effects of QUE-NL-induced apoptosis. These data further support pre-clinical development of QUE-NLs to preferentially target alternative cell death pathways.

### Results

**Effects of QUE-NLs and AG490 on cell morphology and viability.** Exposure of C6 glioma cells to QUE-NLs resulted in necrotic morphological changes and a decrease in the percentage of viable cells (Figure 1a). These effects were dose- and time-dependent. Compared with QUE-NLs alone, the mode of PCD exhibited by C6 glioma cells was changed from necrosis to apoptosis when AG490 was administered in combination with QUE-NLs (Figure 1b). In contrast, exposure of cells to control such as blank, 0.1% dimethyl sulfoxide (DMSO), or blank NLs had no significant effects on viability (Figure 1c). Hematoxylin and eosin staining was used to detect chromatin condensation in necrotic or apoptotic cells. During a period of 12–24 h post exposure, the proportion of necrotic cells increased with an increase in the concentration of QUE-NLs from 150 to 200 μM (Figure 1d), and necrotic cell death decreased substantially when AG490 was administered in combination with QUE-NLs (Figure 1e) compared with control (Figure 1f). These results support that the JAK2/STAT3 (signal transducers and activators of transcription 3) pathway is involved in QUE-NL-induced C6 glioma cell death.

**Lactate dehydrogenase (LDH) activity-based cytotoxicity assays.** Using a LDH release assay, we identified a significant increase in the rate of LDH release as the concentration of QUE-NLs was increased (100, 150, and 200 μM; Figure 2). Moreover, we observed the cytotoxicity with increased QUE-NLs. Compared with QUE-NLs alone, the LDH release rate was markedly inhibited when AG490 was administered in combination with QUE-NLs. These results indicate that the JAK2/STAT3 pathway is related to the QUE-NL-induced cytotoxicity of C6 glioma cells (Figure 2).

### Effects of QUE-NLs or AG490 on cell death.** QUE-NLs induced significant cell apoptosis at concentrations of 50 or 100 μM when cells were exposed for 6, 12, or 24 h. In contrast, C6 glioma cells exposed to higher concentrations of QUE-NLs (150 or 200 μM) for 6, 12, or 24 h displayed significant cell death, which was mainly due to necrosis (Figures 3a and b). Under high QUE-NL conditions, the occurrence of apoptosis decreased as observed by Annexin V/propidium iodide (PI) staining. Exposure to AG490, blank, 0.1% DMSO, or blank NLs was not associated with significant necrosis (Figures 3c and d). Whereas QUE-NLs increased the percentage of necrotic cell death (Figure 3e), this process was inhibited when AG490 was administered in combination with QUE-NLs (Figure 3f).

**ROS production of QUE-NLs or AG490.** To evaluate the function of ROS in C6 glioma cell death induced by QUE-NLs, cells were treated with AG490, which efficiently inhibits STAT3 in vivo and has been used widely for inhibiting JAK2.\(^\text{14,15}\) In this study, treatment efficiency was estimated by flow cytometry. ROS activity was markedly increased in C6 glioma cells exposed to QUE-NLs (50, 100, and 200 μM) reaching 90, 170, and 215%, respectively, compared with control levels of approximately 20% (Figure 4a). ROS level was 93, 190, and 249%, respectively, when C6 glioma cells were exposed to AG490 in combination with QUE-NLs (Figures 4b and c).

**QUE-NL-induced cell death involves the p53 signaling pathway.** To identify potential signaling pathways involved in QUE-NL-induced C6 glioma cell death, we measured the expression of p53 and phospho-p53 in QUE-NL-treated cells using western blot analysis.\(^\text{16}\) We detected increased p53 expression associated with exposure to QUE-NL (100–200 μM) and/or AG490, and there was no significant difference in p53 expression between the absence or presence of AG490 (Figure 5a). Compared with control, QUE-NLs (100 or 200 μM) downregulated the expression of phospho-p53. AG490 substantially inhibited the effects of QUE-NLs on p53 but had no significant effect on phospho-p53 in combination with 200 μM QUE-NLs (Figure 5b). These results suggest that QUE-NLs affect p53-mediated cell death, particularly at a high concentration of 200 μM.

**QUE-NL-induced cell death via the p53 ROS signaling pathway.** To dissect how the ROS signaling pathway might be involved in p53-mediated C6 glioma cell death following QUE-NL exposure, we measured the expression levels of p53 and phospho-p53 and the levels of ROS in cells exposed to QUE-NLs (Figure 6a). It was shown that downregulation of phospho-p53 associated with increased activity of ROS were enhanced when C6 glioma cells were exposed to QUE-NLs (Figure 6b). These results suggest that QUE-NLs affect p53-mediated cell death in association with endogenous ROS. We also investigated whether the p53-mediated ROS pathway, which is important in regulating cell apoptosis and necrosis, was involved in QUE-NL-induced necrosis. We measured phospho-p53 after cells were exposed to 200 μM QUE-NLs for 12–24 h. Compared with untreated
cells, the downregulation of phospho-p53 induced by QUE-NLs was significantly inhibited by the ROS inhibitor N-acetyl-cysteine (NAC) (Figure 6b). In contrast, NAC increased the expression of phospho-p53. Collectively, these results indicate that necrosis is induced by QUE-NLs in C6 glioma cells through p53-mediated ROS pathways.

**Relationship between STAT3 and p53-mediated ROS pathways in QUE-NL-induced cell death.** We next investigated whether QUE-NL-induced C6 glioma cell death via p53-mediated ROS pathways also involved STAT3, which is important in regulating cell apoptosis and necrosis. The level of ROS increased significantly and was associated with bright green fluorescence in C6 glioma cells induced with QUE-NLs (Figures 7a and b). The necrotic effects of QUE-NLs were significantly inhibited with AG490 pretreatment (Figure 7c). These results indicate that QUE-NL-induced C6 glioma cell death is associated with STAT3 and p53-mediated ROS pathways. We next measured STAT3 and phospho-STAT3. Necrotic cells that had been exposed to QUE-NLs (200 μM) exhibited significantly increased ROS (Figures 7d and e), with no significant effects on phospho-STAT3 (Figure 7f). However, apoptotic cells that had been exposed to QUE-NLs (100 mM) displayed...
downregulated phospho-STAT3 that was synergistically downregulated when QUE-NL-exposed cells were pretreated with AG490, a JAK2 inhibitor. These results demonstrate that necrotic C6 glioma cell death is independent of phospho-STAT3, whereas apoptotic cell death is dependent on the STAT3 pathway.

The JAK2/STAT3 cascade positively regulates QUE-NL-induced cell death through the mitochondrial pathway. As the involvement of the JAK2/STAT3 pathway has been highlighted recently in various models of induced cell death, we next explored the involvement of the JAK2/STAT3 pathway in QUE-NL-induced glioma cell death. We measured the levels of interleukin (IL)-8 and IL-6 in C6 glioma cells after QUE-NL treatment using the enzyme-linked immunosorbent assay (ELISA). We then examined the phosphorylation of JAK2, which has been reported to correlate with cell death induction, using western blotting.12 The dynamic activation of JAK2 was observed 12–24 h after QUE-NL treatment. We therefore presumed that JAK2 was involved in QUE-NL-induced C6 glioma cell death. To test this idea, C6 glioma cells were pretreated with AG490. AG490 and QUE-NLs in combination downregulated levels of IL-8 and IL-6 in C6 glioma cells (Figure 8a). AG490 specifically downregulated the activation of JAK2 (Figure 8b). Necrotic cell death associated with high QUE-NL exposure (200 μM) did not significantly alter the downregulation of STAT3 (Figure 7f), and JAK2 was not obviously downregulated. However, exposure of C6 glioma cells to a moderate concentration of QUE-NLs (100 μM) downregulated the expression of JAK2, and pretreatment with AG490 synergistically affected this downregulation. Collectively, these data suggest that the kinase activity of JAK2 and STAT3 is essential for glioma cell death.

On the basis of these results, we further examined the contact and relationship of the JAK2/STAT3 pathway with the mitochondrial pathway in the context of QUE-NLs-induced cell death. Considering a mechanism of caspase activation, the mitochondria are critical for relaying caspase cascade-transduction of PCD by diverse cell death stimuli through the mitochondrial pathway.17,18 Therefore, we monitored Bax and Bcl-2 activation. Bax self-oligomerizes, and Bcl-2 forms a pore-forming oligomer in the mitochondrial outer membrane.19 In response to QUE-NLs, we detected Bax in the mitochondria, and self-oligomerization of Bcl-2 and Bax was confirmed (Figure 8c).

Exposure to QUE-NLs (100 or 200 μM) affected caspase-3 activity and cytochrome c protein levels (Figures 8d and e). QUE-NLs had no effect on the activity of caspase-8 and -9 in necrotic cells; these results are in agreement with other reports.20 QUE-NL exposure enhanced the protein levels of cytochrome c in C6 glioma cells (Figure 8d) and enhanced the release of cytochrome c from mitochondria. Caspase-3 activity was inhibited significantly when QUE-NLs were administered in combination with AG490. These results demonstrate that QUE-NL-induced cell death is independent of caspase-8 and -9, whereas apoptotic cell death is dependent on caspase-3 when QUE-NLs and AG490 are administered in combination. Thus, Bcl-2 and Bax are essential for QUE-NL-induced glioma cell death, and caspase-3, excluding caspase-8 and -9, are activated downstream of mitochondrial pro-apoptotic Bcl-2 family protein activation.

Discussion

Although the clinical efficacy of QUE therapy has been established,21,22 the detailed molecular effects of QUE on glioma cells remain unclear. Several studies have reported that certain types of cell death share apoptotic and necrotic features; this phenomenon has been deemed ‘necroptosis’.6,23 Nanoliposomes may enhance the solubility of QUE and thereby enhance its bioactivity in inhibiting tumors. The observed anti-cancer effects of QUE administered to C6 glioma cells at high concentrations and for an extended duration may be associated with the accumulation of ROS. Thus the pro-oxidant feature of QUE could prevail over its antioxidant feature and result in cell death.

QUE-NLs induced necrotic morphological changes in cells and decreased cell viability in a dose- and time-dependent manner. Several common points in the necrotic and apoptotic pathways exist, suggesting crosstalk between the different pathways. During conventional chemotherapy, tumor cells...
typically are observed to undergo apoptosis. Histological analysis of human tumor specimens indicates necrotic changes as a result of high-dose chemical agents. To our knowledge, this is the first study to elucidate the molecular mechanisms of QUE-NL-induced glioma cell death, including the type of cell death and the molecular induction

Figure 3 QUE-NL-induced apoptosis and necrosis in C6 glioma cells. C6 glioma cells were treated with the indicated concentrations of QUE-NLs in the absence or presence of AG490 for 12–24 h. (a) Dose-dependent apoptosis and necrosis in C6 glioma cells exposed to QUE-NLs. Cells were stained with Annexin V-FITC and analyzed by flow cytometry. (b) Dose-dependent apoptosis and necrosis in C6 glioma cells exposed to QUE-NLs and/or AG490. Cells were stained with Annexin V-FITC and analyzed by flow cytometry. (c) Apoptosis and necrosis of C6 glioma cells exposed to QUE-NLs and treated with AG490 for 24 h. Cells were stained with Annexin V-FITC and analyzed by flow cytometry. (d) Control cells, including blank, DMSO, and blank NL. (e) Time- and dose-dependent apoptosis and necrosis of C6 glioma cells exposed to QUE-NLs in the presence or absence of AG490. Representative measurements of at least three independent experiments are shown. (f) Cell death was measured as the percentage of PI-positive cells using flow cytometry. *\(P<0.01\) versus blank NL. Cell death values (apoptosis and necrosis) are reported as the mean ± S.D. of three separate experiments. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) versus control cells.
mechanisms. The role of p53 in tumor cell growth arrest/death is generally recognized, and the effect of p53 in the context of QUE-NLs treatment has been demonstrated.26,27 However, chemical-resistant gliomas have been reported to harbor mutations in the p53 gene.28 Therefore, we used a p53-mutated glioma cell line in this study to investigate the efficacy of QUE-NL treatment to specifically kill p53-mutated tumor cells. In addition, the activation of specific caspase cascades following cell stress is poorly understood. Regarding conventional chemical therapy, the involvement of the intrinsic pathway, the extrinsic pathway, or both have been reported.29 In contrast, induction of the apoptotic pathway by QUE specifically via intrinsic caspase-3 activation in p53 wild-type/mutant cells has been reported.30

AG490, administered alone or in combination with the Chk1 inhibitor UCN-01, exerted antagonist effects on cell proliferation and viability and dramatically enhanced the response to UCN-01 in p53-mutated or -deleted glioma cells. AG490 enhanced UCN-01-induced cytotoxicity by suppressing BAD phosphorylation in p53-defective cell lines that appeared to protect against UCN-01-induced cytotoxicity.31 Because QUE-NLs and JAK/STAT pathway inhibitors such as AG490 interfere with survival signaling by different mechanisms, we reasoned that these agents might cooperate to block tumor cell proliferation and induce apoptosis. The identification of the kinases responsible for STAT3 phosphorylation via AG490 may clarify the molecular mechanism associated with QUE-NL-induced glioma cell death.

The prosurvival role of JAK2/STAT3 in cell death proceeds via the downstream transcription of anti-apoptotic genes and the downregulation of pro-apoptotic genes. However, the pro-apoptotic action of STAT3 has also been reported in several systems.32–34 Among the pro-apoptotic actions of STAT3, the function of JAK2/STAT3 pathway has been well studied, and the role of p53/ROS-mediated pathway in cell death is explained by p53-mediated regulation of ROS activation.35–37 In the present model, ROS production (Figure 6a) and the effects of pro-oxidants in QUE-NL-induced glioma cell death are confirmed (Figure 6b). Thus, p53-induced ROS-dependent necrotic cell death is associated with high-dose QUE-NLs.

Figure 4 QUE-NL-induced apoptosis and necrosis of C6 glioma cells involves ROS accumulation. C6 glioma cells were treated with the indicated concentrations of QUE-NLs for 24 h in the presence or absence of AG490. Cells then were loaded with DCFH-DA for 30 min. (a) ROS generation in C6 glioma cells treated with QUE-NLs alone as estimated by flow cytometry. (b) ROS generation in C6 glioma cells treated with QUE-NLs and AG490 as estimated by flow cytometry. (c) ROS measurement in C6 glioma cells treated with QUE-NLs in the presence or absence of AG490 using flow cytometry. *P < 0.05 versus blank NL.
In this study, the recovered activation of STAT3 observed 12–24 h after QUE-NL treatment was considered to have an essential role in QUE-NL-induced glioma cell death (Figure 7a). A previous report indicated a time-dependent increase in STAT3 activity from baseline corresponding to its cell death-inducing ability.\(^3\)\(^8\) The present data suggest that suppression of the JAK2/STAT3 pathway by AG490 did not prevent cell death completely. Thus, at least in the present model, a JAK2/STAT3-independent pathway could contribute to QUE-NL-induced glioma cell death. Considering the mechanism of QUE-NL-induced cell death, the involvement of p53-induced ROS-mediated extrinsic cell death signals, especially those related to ROS-mediated cell death, have been demonstrated previously.\(^3\)\(^9\) Thus, we speculate that the JAK2/STAT3 pathway has an important association with ROS/p53-mediated cell death and the extrinsic pathway of apoptosis in the present system. We report that the ROS-mediated signal is activated in C6 glioma cells exposed to QUE-NLs and is regulated by a STAT3-independent mechanism. However, an antagonistic-STAT3 inhibitor failed to prevent QUE-NL-induced cell death. Therefore, in the present system, the QUE-NL-induced ROS-mediated extrinsic pathway of apoptosis is not essential for cell death induction.

Inhibition of STAT3 expression by RNA interference in glioblastoma U251 cells via a lentivirus-based shRNA vector significantly and efficiently suppressed STAT3 expression and activation of U251 cells.\(^4\)\(^0\) Knockdown of STAT3 expression suppressed the growth of U251 cells and induced their apoptosis by downregulating Bcl-2. It indicated that there were probable additional signaling pathways associated with the STAT3 pathway.

Our studies indicate that STAT3 acts as an essential mediator of Bcl-2 family proteins and mitochondrial activity.
through ROS-dependent and ROS-independent mechanisms (Figure 9). Various oncogenic signals can trigger the constitutive activation of STAT3,41 either directly or indirectly. When STAT3 is activated, it migrates into the nucleus and constitutive activation of STAT3,41 either directly or indirectly. Effective Bcl-2-specific antagonists or inhibitors of IAP family proteins18,19 that abrogate caspase activation downstream of the mitochondria have been developed. QUE-NL exposure alone or in combination with these inhibitors (Figure 9) could be an effective method of treating chemical-resistant gliomas.

Inhibition of STAT3 activity sensitizes cells to the effects of several anti-cancer drugs.44–46 Our findings suggest that the general inhibition of protein synthesis may reduce STAT3 activity, thereby increasing the cytotoxic effects of anti-cancer drugs. The present study suggests a novel mechanism involved in the downregulation of phospho-STAT3 levels. These findings might help inform new anti-cancer strategies. Effective Bcl-2-specific antagonists or inhibitors of IAP family proteins18,19 that abrogate caspase activation downstream of the mitochondria have been developed. QUE-NL exposure alone or in combination with these inhibitors (Figure 9) could be an effective method of treating chemical-resistant gliomas.

Figure 7 Changes in ROS and STAT3 signaling. (a and b) ROS fluorescence was visualized using a fluorescence microscope. (c) Cells exposed to QUE-NL were pretreated with AG490 to inhibit the necrotic function of QUE-NLs. Cell death was measured as the percentage of PI-positive cells using flow cytometry. *P<0.05, **P<0.01 versus control. (d and e) QUE-NLs induced a significant increase in ROS generation, and the level of ROS was enhanced with AG490 pretreatment, as evaluated using flow cytometry. Representative measurements of at least three independent experiments are shown. Values represent the mean ± S.D. of three separate experiments. *P<0.05, **P<0.01 versus control cells. (f) QUE-NL-induced decreases in phospho-p53 and phospho-STAT3 were inhibited with AG490 pretreatment. Alterations in phospho-p53, phospho-STAT3, and actin were analyzed by western blotting. *P<0.05, **P<0.01 versus control cells.
expression of Bcl-2, upregulated Bax protein expression, and promoted C6 glioma cell apoptosis or necrosis via the mitochondrial pathway. Conversely, a lower concentration of QUE-NLs (100 μM) regulated C6 glioma cell apoptosis by adjusting the JAK2/STAT3 signal transduction pathway and associated signaling molecules (IL-8, IL-6) and proteins.
In summary, this study provides rational evidence for further preclinical development of QUE-NLs that preferentially target alternative cell death pathways. The application of QUE-NLs to glioma treatment may result in improved preclinical outcomes.

Materials and Methods

Reagents and antibodies. AG490 and NAC were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PI, DCFH-DA, DiOC6(3), and Indo-1 AM were purchased from Molecular Probes (Invitrogen, Eugene, OR, USA). Caspase-3, 8, and 9 ELISA kit was obtained from BestBio Co. (Shanghai, China). Roswell Park Memorial Institute 1640 medium (RPMI 1640), penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and L-glutamine were purchased from Gibco-BRL (Carlsbad, CA, USA). The following were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): p53 antibody, phospho-p53 antibody, STAT3 antibody, phospho-STAT3 antibody, total-JAK2 antibody, phospho-JAK2 antibody, Bax polyclonal antibody, Bcl-2 antibody, and horseradish peroxidase-conjugated anti-goat IgG secondary antibody. Cytochrome c antibody, caspase-3 antibody, caspase-8 antibody, and caspase-9 antibody were purchased from Cell Signaling Technology (San Jose, CA, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

Preparation of QUE-NLs. QUE-NLs were prepared according to an established method involving emulsion evaporation and low-temperature curing. The aqueous phase was prepared by dissolving poloxamer-188, PEG2000-DPSE, and Tween 80 (1:1:1, w/w/v) in pure water, and the solution was maintained in a water bath at 75 °C. The oil phase was made of glyceryl behenate (ATO), soy lecithin, and cholesterol (1:2:1, w/w/w). Glyceryl behenate (ATO) and cholesterol were melted in a water bath at 80 °C. QUE 1% (w/v) and soy lecithin were then dissolved in the ethanol-acetone mixed solvent (1:1, v/v) to obtain a weight ratio of lecithin to QUE of 10:1. QUE and soy lecithin were then dissolved in the oil phase, and the oil phase was injected into the aqueous phase through plastic needle tubing (internal diameter 0.45 μm, injection rate 2 μl/min) under mechanical agitation at 1000 r.p.m. After stirring for 2 h, the resulting liposome suspension was cured at −4 °C. The suspension was passed through a filter membrane (0.2 μm) to remove the nonincorporated drug, and the prepared QUE-NL system was recovered. Liposomes were also prepared without QUE in the manner described above. Before experimental use, the prepared QUE-NLs were well distributed in RPMI 1640 medium containing 10% (v/v) heat-activated FBS using ultrasound treatment to obtain a suspension of QUE-NLs.

Characteristics of QUE-NLs. To observe the particle size and morphology of QUE-NLs (Figure 1), the sample was dispersed in deionized water, and the particles were evaluated using a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan).

Cell culture and treatment. C6 glioma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C with 5% CO2 in a humidified atmosphere. C6 glioma cells then were pretreated with the indicated amounts of QUE-NLs for no more than 24 h. C6 glioma cells were then mixed in combination with AG490, taking 0.1% DMSO or blank NLs as control.

Evaluation of morphological changes by electron microscopy. Various concentrations (50, 100, 150, and 200 μg/ml) of QUE-NLs, and 0.1% DMSO or blank NLs were added to C6 glioma cells, and the cells were cultured for 12–24 h. Cells were analyzed morphologically by fluorescence microscope (Olympus, Tokyo, Japan).

LDH activity-based cytotoxicity assays. C6 glioma cells (1 × 106 cells/well) were cultured in six-well plates overnight and were then treated with various concentrations (50, 100, 150, and 200 μg/ml) of QUE-NLs for 6, 12, 24, 36, or 48 h. The release of LDH into the culture medium (extracellular LDH (LDHe)) was used as an index of cell injury. Adherent and viable cells were lysed in 0.1% NP-40 for 15 min to measure the release of LDH (intracellular LDH (LDHi)). The percentage of release rate was calculated as follows:

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\text{Percentage of release rate of LDH} = \frac{\text{LDHe/(LDHe + LDHi)}}{100}\%.
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Cell death and apoptosis assays. Cells were incubated with various concentrations (50, 100, 150, and 200 μg/ml) of QUE-NLs for 6, 12, 24, 36, or 48 h. Cells were then trypsinized and harvested by centrifugation before incubation with Annexin V and PI for 15 min at room temperature. The rates of apoptosis and necrosis were analyzed by flow cytometry using an Annexin V-FITC/PI kit (BestBio Co.). Cells were washed with an Annexin-V-binding buffer and incubated with Annexin V-FITC/PI. Cells were then analyzed by fluorescence microscopy (Olympus). For quantification of apoptosis and necrosis, cells were trypsinized and harvested by centrifugation before incubation with Annexin V and PI for 15 min at room temperature. Apoptotic and necrotic cells were observed after staining with annexin V-FITC and PI; the apoptotic and necrotic cells expressed as percentages

Figure 9 QUE-NLs induced cell death in C6 glioma cells interfere directly or indirectly with the JAK2/STAT3 and mitochondrial signaling pathways.
of the total cells were identified by brightly stained condensed chromatin and fragmented nuclei. Annexin V binds to necrotic and apoptotic cells in which phosphatidylserine is exposed on the cell surface. The percentage of necrotic cells was measured as the percentage of PI-positive cells using flow cytometry.\(^{49}\) ROS measurement. C6 glioma cells were incubated with various concentrations (50, 100, 150, and 200 \(\mu\)M) of QUE-NLs or QUE for 12–24 h. The activity of caspase-3, -8, and -9 was measured using caspase-3, -8, and -9 ELISA kit (10 \(\mu\)M).^{51}

Caspase-3, -8, and -9 activity assays. Cells were treated with QUE-NLs (150 or 200 \(\mu\)M) or DMSO for 12–24 h. The activity of caspase-3, -8, and -9 was measured using caspase-3, -8, and -9 ELISA kit (10 \(\mu\)M).^{51}

Western blot detection of cytochrome c and caspase-3, 8, and -9. Whole-cell lysates were obtained as described previously.\(^{19}\) Cell fractionation was performed as described previously with minor modifications. In brief, pelleted cells were permeabilized for 1 min in isotonic buffer containing 0.03% digitonin for 5 min on ice and then centrifuged at 15 000 r.p.m. for 10 min. The supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were collected, and the pellet was further lysed to yield the final mitochondrial lysate. For western blotting, the protein concentration of the lysates was determined using a BCA protein assay kit in accordance with the manufacturer’s instructions (Thermo Fisher Scientific). Total proteins were resolved by denaturing 8–12% SDS-polyacrylamide gel electrophoresis and were electro-transferred by semi-dry blotting (Bio-Rad Laboratories, Shanghai, China) onto a nitrocellulose membrane. Membranes were incubated with antibodies to caspase-3, -8, -9, and \(\beta\)-actin (Santa Cruz Biotechnology).

Western blot detection of JAK2/STAT3. For protein analysis, C6 glioma cells were harvested 12–24 h following the treatments described in the section ‘Cell culture and treatment’, washed with cold PBS, and incubated in ice-cold RIPA buffer. Cell lysates were sonicated for 30 s on ice and then were lysed at 4 °C for 60 min. Cell lysates were then centrifuged for 30 min at 12 000 \(\times\) g and 4 °C. Protein concentrations were measured from the supernatants using BCA. Total proteins were resolved by denaturing 8–12% SDS-polyacrylamide gel electrophoresis and were electro-transferred by semi-dry blotting (Bio-Rad Laboratories) onto a nitrocellulose membrane. Membranes were incubated with antibodies to JAK2, phospho-JAK2, STAT3, phospho-STAT3, and \(\beta\)-actin (Santa Cruz Biotechnology).

Western blot detection of Bcl-2 and Bax oligomers. In vitro cross-linking of associated Bax monomers was performed according to the method reported previously with some modifications. Briefly, cells were collected by centrifugation at 15 000 r.p.m. for 5 min, and the supernatants were subjected to western blotting using polyclonal antibodies to Bax and Bcl-2 (Santa Cruz Biotechnology) as reported previously.\(^{19}\)

Statistical analysis. Data were represented as mean ± S.D. and were analyzed by two-tailed Student’s t-tests using Statistical Program for Social Sciences 13.0 software (SPSS, Shanghai, China). Significance was assessed at \(P<0.05\). The intensity of the bands on the membrane were analyzed using the Bio-Rad image analysis system with Image-Pro software analysis (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Conflict of Interest

The authors declare no conflict of interest.

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