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GDC-0980-induced apoptosis is enhanced by autophagy inhibition in human pancreatic cancer cells

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ABSTRACT

Pancreatic cancer remains fatal to the fast majority of affected patients. Activation of phosphoinositide-3 kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) pathway plays an important role in pancreatic cancer progression and chemo-resistance. In the present study, we examined the activity of GDC-0980, a novel class I PI3K/mTOR kinase inhibitor, against pancreatic cancer cells *in vitro*. GDC-0980 inhibited AKT-mTOR activation and pancreatic cancer cell (PANC-1 and Capan-1 lines) survival. In both cancer cell lines, GDC-0980 simultaneously activated apoptosis and autophagy, the latter was detected by p62 degradation, Beclin-1 upregulation and light chain 3B (LC3B) conversion from a cytosolic (LC3B-I) to a membrane-bound (LC3B-II) form. Autophagy inhibitors including 3-methyladenine, hydroxychloroquine, NH_4Cl and bafilomycin A1 enhanced apoptosis and cytotoxicity by GDC-0980, such an effect was reversed by caspase inhibitors (z-VAD-FMK and z-ITED-FMK). Furthermore, knockdown of *LC3B* or *Beclin-1* through siRNA increased GDC-0980-induced anti-pancreatic cancer cell activity. Thus, inhibition of autophagy sensitizes GDC-0980-induced anti-pancreatic cancer activity, suggesting a novel therapeutic strategy for GDC-0980 sensitization.

1. Introduction

Pancreatic cancer remains fatal to the fast majority of affected patients, these patients were mostly diagnosed at the advanced stages [1–3]. The current standard treatments for pancreatic cancer include surgery (only for early stages), radiation and/or gemcitabine-based chemotherapies. However, pancreatic cancer is among the most intrinsically resistant malignancies to both radiation and chemotherapy, resulting in a median overall survival (OS) less than 7%. Hence, scientists including ours [4,5] and oncologists are searching for novel and more efficient anti-pancreatic cancer agents or adjuvants [1,6].

Phosphoinositide-3 kinase (PI3K)–AKT–mTOR pathway plays a vital role in cancer cell growth, survival and metastases [7–10]. In pancreatic cancer, this pathway could be activated by overexpression of growth factor receptors, as well as by mutation, amplification, and deletion of genes encoding components of the pathway [11–14]. For example, most pancreatic cancers have activating mutations in *KRAS* (*Kirsten rat sarcoma viral oncogene homolog*), resulting in an abnormal RAS protein that is 'locked' in the activated form [12,15,16]. This leads to aberrant activation of the RAS-mediated downstream signalings (i.e. PI3K–AKT–mTOR and Erk–MAPK) to promote cancer cell progression [12,15,16]. Activation of PI3K–AKT–mTOR pathway also mediates acquired resistance to both chemotherapies and molecule targeted agents [7–10].

Thus, therapeutic targeting of the PI3K–AKT–mTOR pathway with small molecule inhibitors may have clinical benefit for pancreatic cancer, either as single agents or used more broadly in combination with other conventional or targeted therapies. As a matter of fact, several inhibitors of this kind have now entered clinical trials [17,18]. GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor. However, it activity in pancreatic cancer is somehow not as effective

Abbreviations: 3-MA, 3-methyladenine; BafA1, bafilomycin A1; Cq, hydroxychloroquine; LC3B, light chain 3B; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide-3 kinase; SE, standard error.

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as in other cancers [19]. Here we report that GDC-0980 activates autophagy in human pancreatic cancer cells. Inhibition of autophagy by pharmacologic or genetic means drives pancreatic cancer cells into apoptosis, and significantly enhances GDC-0980 activity.

2. Materials and methods

2.1. Cells

Human pancreatic cancer cell lines (PANC-1 and Capan-1) were maintained in RPMI medium (Invitrogen, Shanghai, China), supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin/streptomycin (1:100, Invitrogen) and 4 mM L-glutamine (Sigma–Aldrich, St. Louis, MO) in a CO_2 incubator at 37 °C.

2.2. Reagents and chemicals

GDC-0980 was obtained from Selleck China (Shanghai, China). Bafilomycin A1 (BafA1), 3-methyaldenine (3-MA), hydroxychloroquine (Cq), NH_4Cl, and mouse monoclonal antibody against β-actin were obtained from Sigma–Aldrich Co. (St. Louis, MO). The broad caspase inhibitor z-VAD-fmk and the caspase-8 specific inhibitor z-ITED-fmk were from Calbiochem (Darmstadt, Germany). Anti-light chain 3B (LC3B), Beclin-1 and rabbit/mouse IgG-horseradish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were from Cell Signaling Tech (Denver, MA).

2.3. Cell viability assay

After treatment, relative numbers of viable cells were estimated using CellTiter-Glo (Promega, Shanghai, China) and total luminescence was measured on a Wallac Multilabel Reader (PerkinElmer) [19]. The value of treatment group was normalized to the vehicle (0.1% DMSO)-treated control group. The concentration of drug resulting in 50% inhibition of cell viability (IC_{50}) was determined using Prism software (GraphPad).

2.4. Flow cytometry detecting cell apoptosis

Pancreatic cancer cell apoptosis was determined by the Annexin V In Situ Cell Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's instruction. Cells were also stained with propidium iodide (PI, Molecular Probes). Annexin V^+ cells were detected through a flow cytometry (BD Bioscience). Annexin V percentage was recorded to quantify apoptosis after indicated treatment.

2.5. Western blotting

Protein samples were prepared in lysis buffer [5 mM $MgCl_2$, 137 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 10 mM HEPES (pH 7.5)], normalized using nanodrop measurement (Thermo Scientific, Nanjing, China), and boiled in SDS sample buffer. Samples were then loaded onto 10% SDS–PAGE gels (14% for detection of LC3B) with electrophoretic transfer onto a polyvinylidene difluoride (PVDF) membrane, and then labeled with indicated primary and secondary antibodies. Blots were quantified using Image J software (National Institutes of Health, Baltimore, US). All experiments were repeated at least twice and similar results were obtained.

2.6. Cytotoxicity assay

The number of dead cancer cells (trypan blue positive) after indicated treatment was counted, and the percentage (%) of dead cells (cytotoxicity) was calculated by the number of the trypan blue stained cells divided by the total number of the cells.

2.7. Beclin-1 shRNA

The targeted shRNA sequences for two different sites of human Beclin-1 were 5′-CTCAGGAGAGGAGCCATTT-3′ [termed as shBeclin-1(-1)] [20] and 5′-CAGTTTGGCACAATCAATA-3′ [termed as shBeclin-1(-2)] [21]. The annealed inserts were cloned into the RNAi-Ready pSIREN-RetroQ vector (Clontech) that had been previously digested with EcoRI-BamHI. The negative control shRNA (shSC) was purchased from Kaiji Biotech (Shanghai, China). The Beclin-1 shRNA (−1/−2) or a negative control shRNA was transfected into pancreatic cancer cells twice separated by 24 h using Lipofectamine and Plus transfection reagents (Invitrogen) according the protocol. The shRNA efficiency was verified by Western blotting.

2.8. LC3B siRNA

LC3B siRNA-1 (CS-6212, cellular signaling), LC3B siRNA-2 (sc-43391, Santa Cruz Biotechnology) and LC3B siRNA-3 (LC-201, Kaiji Biotech, Shanghai, China) were purchased from commercial sources. Cells were cultured in six-well plates and transfected at 60% confluence. Transient transfection with 40 nM siRNA was performed with Lipofectamine and Plus reagents (Invitrogen), after 3 h, 1% FBS was added, and cells were left for another 36 h before they were trypsinized and used for experiments. The siRNA efficiency was again verified by Western blotting.

2.9. Statistical analyses

Data were analyzed with SPSS 13.0 software using one-way analyses of variance (ANOVA). Differences between the control and treated samples were analyzed by using individual contrasts when the factor consisted of more than two levels. P values of <0.05 were considered statistically significant.

3. Results

3.1. GDC-0980 inhibits AKT-S6K1 phosphorylation and pancreatic cancer cell survival

We first tested the effect of GDC-0980 on survival of human pancreatic cancer cells (PANC-1 and Capan-1 lines). The viability assay results in Fig. 1A demonstrated that GDC-0980 (0.1–10 μM) dose-dependently decreased the survival of both cells. GDC-0980 was more effective in PANC-1 cells (IC_{50} = 0.88 μM), and was less effective in Capan-1 cells (IC_{50} = 5.85 μM). GDC-0980 is a potent small-molecule inhibitor of class I PI3K and mTOR kinase. Thus we examined its role on AKT–mTOR activation. Western blotting results in Fig. 1B showed clearly that GDC-0980 (1 μM) dramatically inhibited phosphorylation of AKT (Ser-473 and Thr-308) and S6K1 (Thr-389) in both PANC-1 cells and Capan-1 cells. Together, these results demonstrate that GDC-0980 inhibits AKT-S6K1 phosphorylation and pancreatic cancer cell survival.

3.2. GDC-0980 activates autophagy and apoptosis in pancreatic cancer cells

Above studies show that GDC-0980 inhibits pancreatic cancer cell survival. The effect of GDC-0980 on cell apoptosis and autophagy was then examined. In both PANC-1 cells and Capan-1 cells, GDC-0980 (1–10 μM) induced autophagy activation, which was detected by LC-I to LC-II conversion, p62 degradation and

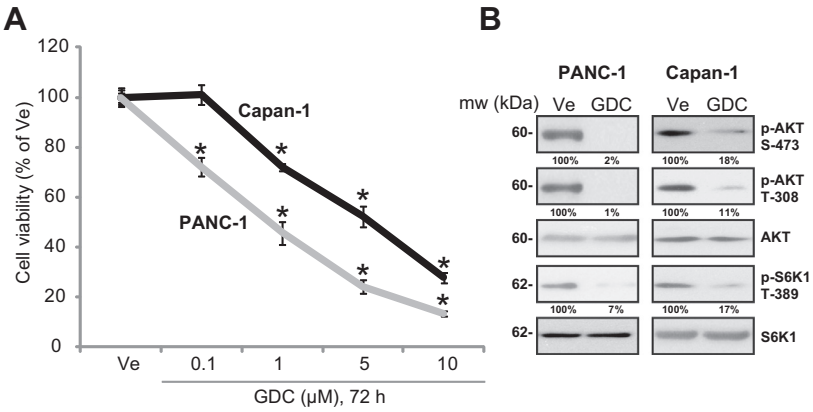

Fig. 1. GDC-0980 inhibits AKT-S6K1 phosphorylation and pancreatic cancer cell survival. Viability of PANC-1 cells and Capan-1 cells after indicated GDC-0980 treatment (GDC, 0.1–10 μM, 72 h) was tested (A). PANC-1 cells and Capan-1 cells were treated with GDC-0980 (1 μM) for 24 h, expressions of indicated proteins were tested by Western blotting (B). Experiments in this figure were repeated three–four times with similar results obtained. Data were presented as mean ± SE. "Ve" means vehicle control (0.1% DMSO). "mw" stands for molecular weight. Phosphorylation of AKT and S6K1 was quantified and normalized to "Ve" group. $^{*}p < 0.05$ vs. "Ve" group.

Beclin-1 up-regulation (Fig. 2A and B). Meanwhile, GDC-0980 also activated cell apoptosis, as the level of cleaved-PARP (Fig. 2A and B) and the percentage of Annexin V positive cancer cells (Fig. 2C and D) went up after GDC-0980 stimulation. These results show that GDC-0980, similar to many other anti-cancer agents [22–25], activates autophagy and apoptosis simultaneously in cultured pancreatic cancer cells.

3.3. Autophagy inhibitors enhance GDC-0980-induced pancreatic cancer cell apoptosis

Since both apoptosis and autophagy were activated by GDC-0980, we thus aimed to increase GDC-0980 activity in pancreatic cancer cells through regulating autophagy. 3-methyaldenine (3-MA), an autophagy inhibitor which disrupts autophagosome formation, significantly increased GDC-0980 (1 μM)-induced Capan-1 cell viability decrease (Fig. 3A) and cell death (Fig. 3B). Such effects by 3-MA were largely inhibited by the caspase-8 inhibitor z-ITED-fmk and the broad-spectrum caspase inhibitor z-VAD-fmk (Fig. 3A and B). Meanwhile, in Capan-1 cells, 3-MA enhanced GDC-0980-induced apoptosis, which was again blocked by z-ITED-fmk and z-VAD-fmk (Fig. 3C). Note that 3-MA alone also inhibited Capan-1 cell survival, while inducing cytotoxicity and apoptosis (Fig. 3A–C). These results suggest that 3-MA increases GDC-0980-induced anti-Capan-1 cell activity through promoting cell apoptosis. Three other autophagic inhibitors including hydroxychloroquine (Cq), NH_4Cl and bafilomycin A1 (BafA1) similarly enhanced GDC-0980-induced viability reduction in Capan-1 cells (Fig. 3D). The GDC-0980 sensitization by 3-MA was also observed in PANC-1 cells, as 3-MA increased GDC-0980-induced PANC-1 cell survival loss (Fig. 3E) and apoptosis (Fig. 3F), which were again reversed by the two caspase inhibitors (Fig. 3E and F). Together, these results show that autophagy inhibitors significantly increase GDC-0980-induced apoptosis in pancreatic cancer cells.

3.4. Beclin-1 or LC3B knockdown sensitizes pancreatic cells to GDC-0980

Pharmacological inhibition of autophagy might be affected by off-targeted side effects of the inhibitors. We therefore utilized siRNAs to knock down *key* genes implicated in the autophagic pathway [26]. The essential *LC3B* was first targeted. We utilized three non-overlapping siRNAs to down-regulate LC3B expression. Western blotting results in Fig. 4A demonstrated that these siRNAs all showed high efficiency in decreasing LC3B expression. Significantly, GDC-0980-induced cell viability reduction and apoptosis were enhanced in *LC3B-depleted* Capan-1 cells (Fig. 4B and C). Meanwhile, knocking-down of Beclin-1 through two different shRNAs (Fig. 4D) also enhanced the activity of GDC-0980 in Capan-1 cells (Fig. 4E and F). Higher level of cytotoxicity and apoptosis by GDC-0980 were

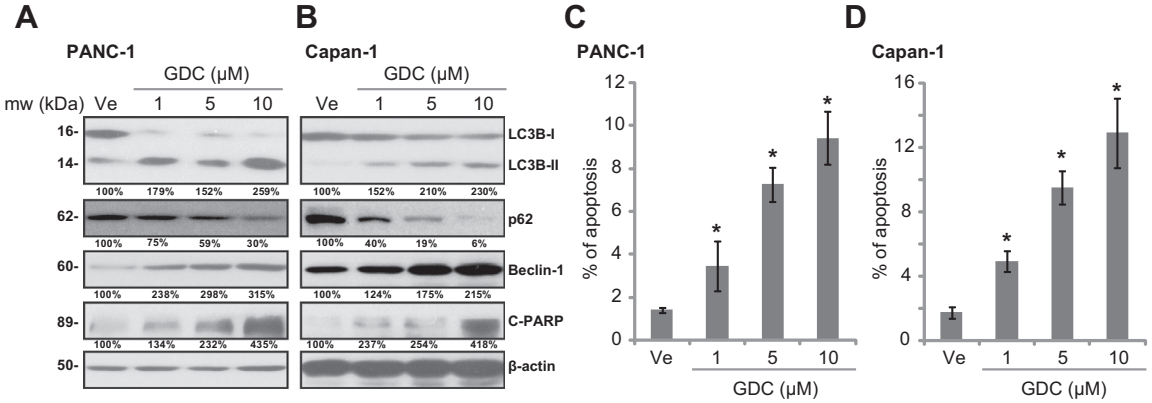

Fig. 2. GDC-0980 activates autophagy and apoptosis in pancreatic cancer cells. PANC-1 cells or Capan-1 cells were stimulated with indicated concentration of GDC-0980 (GDC, 1–10 μM), after 24 h, expressions of LC3B, p62, Beclin-1, Cleaved-PARP (c-PARP) and β-actin (loading) were tested by Western blotting (A and B), Annexin V FACS assay was also performed after 36 h stimulation (C and D). Experiments in this figure were repeated three times, and similar results obtained. Expressions of LC3B-II, p62, Beclin-1 and C-PARP were quantified, and normalized to the "Ve" group. Data were presented as mean ± standard error (SE). "Ve" means vehicle control (0.1% DMSO). $^{*}p < 0.05$ vs. "Ve" group.

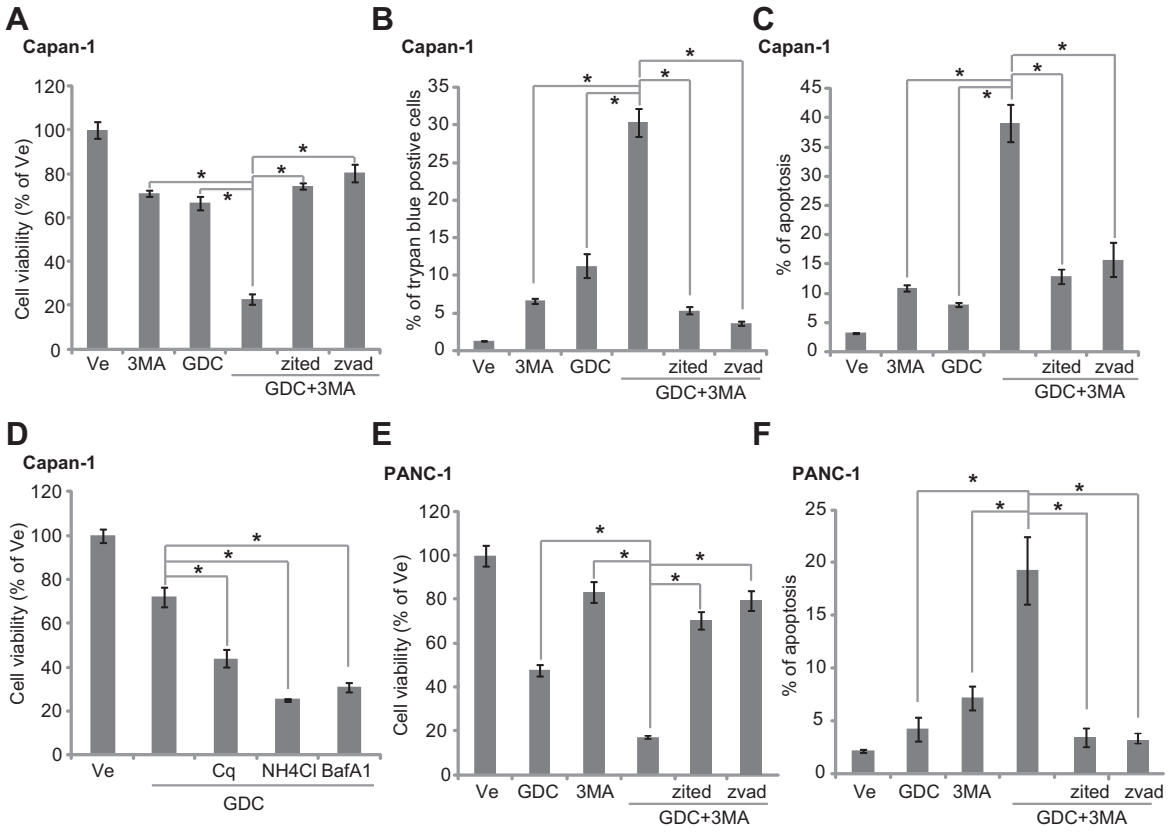

Fig. 3. Autophagy inhibitors enhance GDC-0980-induced pancreatic cancer cell apoptosis. Capan-1 cells or PANC-1 cells were treated with GDC-0980 (GDC, 1 μM), 3-methyladenine (3-MA, 1 mM) or combination (GDC+3MA), pre-treated with z-ITED-fmk (zited, 25 μM) or z-VAD-fmk (zvad, 25 μM), cells were cultured, cell survival (72 h, A and E), cell death (72 h, B) and cell apoptosis (36 h, C and F) were tested by viability assay, trypan blue staining assay and Annexin V FACS assay, respectively. Capan-1 cells were treated with GDC-0980 (1 μM), or plus hydroxychloroquine (Cq, 5 μM), NH_4Cl (2.5 mM) and Bafilomycin A1 (BafA1, 100 nM), cell viability was tested (D). Experiments in this figure were repeated three–four times with similar results obtained. "Ve" means vehicle control (0.1% DMSO). Data were presented as mean ± SE. $^*p < 0.05$.

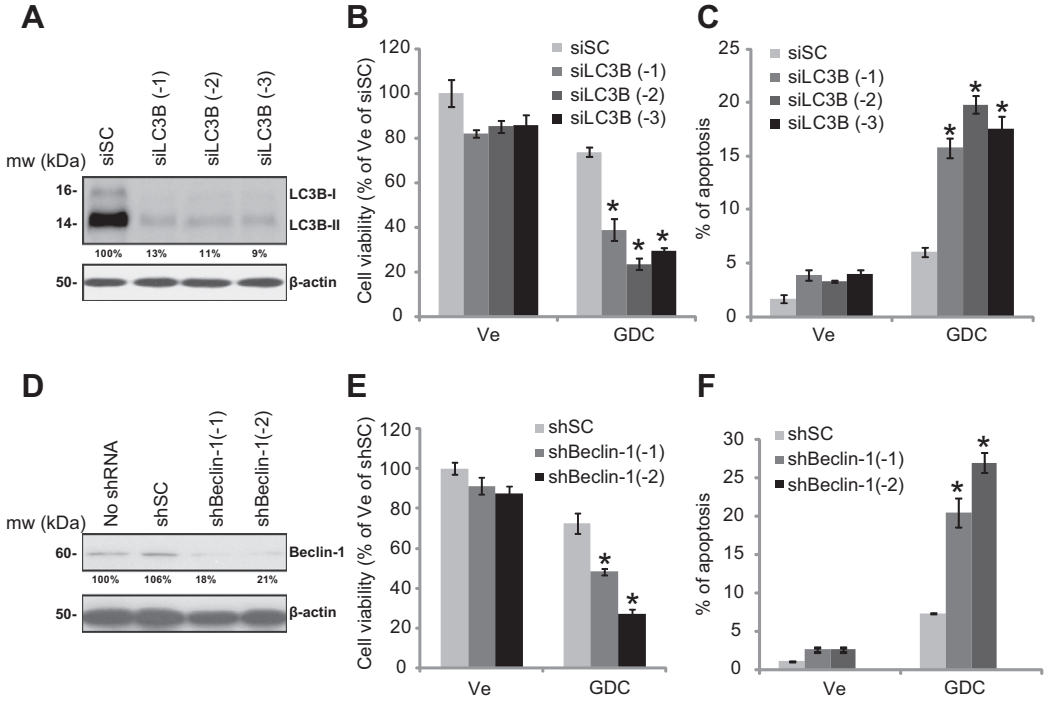

Fig. 4. Beclin-1 or LC3B knockdown sensitizes pancreatic cells to GDC-0980. Expressions of targeted protein (LC3B or Beclin-1) and β-actin (loading) in Capan-1 cell lines transfected with indicated siRNA (for LC3B) (40 nM) or shRNA (for Beclin-1) (0.5 μg/ml) were tested (A and D). Above Capan-1 cell lines were treated with GDC-0980 (1 μM), cell survival (72 h, B and E) and apoptosis (36 h, C and F) were tested by viability assay and Annexin V FACS assay, respectively. Experiments in this figure were repeated three times with similar results obtained. Data were presented as mean ± SE. Beclin-1 and LC3B relative intensity was quantified. $^*p < 0.05$ vs. scramble control siRNA/shRNA group.

achieved in Beclin-1-depleted Capan-1 cells vs. control cells (Fig. 4E and F). Note that Beclin-1 or LC3B knockdown also enhanced GDC-0980-induced death and apoptosis of PANC-1 cells (Data not shown). Together, these data suggest that Beclin-1 or LC3B knockdown increases sensitivity of GDC-0980 in pancreatic cancer cells.

4. Discussions

Autophagy represents an adaptive strategy through which cells clear damaged proteins or organelles through lysosomal degradation, thus surviving from many stresses [27–29]. At the same time, autophagy also plays a vital role in the survival of cells resistant to apoptosis when they are deprived of extracellular nutrients or growth factors [30]. In cancer cells, autophagy is recognized as a pro-survival and chemo-resistance factor, probably due to its anti-apoptosis ability [26,31,32]. This present study was performed because of numerous reports of autophagy observed in established cancer cell lines following anticancer therapies [22–24]. We found that autophagy was activated by GDC-0980 in cultured pancreatic cancer cells, which was reflected by Beclin-1 upregulation, LC3B I-II conversion and p62 degradation. These results are somehow not surprising, since AKT-mTOR inactivation was a known trigger of cell autophagy, and GDC-0980, the PI3K/mTOR inhibitor, blocked AKT-S6K1 phosphorylation in pancreatic cancer cells [33].

Many groups have shown that inhibitors of autophagy given in combination with anti-cancer agents may enhance chemo-sensitization in human cancer cells [30,34]. In consistent with these findings, we found that inhibition of autophagy utilizing pharmacological inhibitors could significantly enhance GDC-0980-induced apoptosis in pancreatic cancer cells. Hydroxychloroquine (CQ) is a lysosomotropic drug that raises intra-lysosomal pH and impairs autophagic protein degradation [35,36]. NH_4Cl and Bafilomycin A1 worked the similar way as CQ to disrupt lysosomal degradation [27]. 3-MA suppresses the formation of autophagosomes to inhibit autophagy [37]. We found that all these inhibitors enhanced GDC-0980 activity, suggesting that autophagy exerts pro-survival activity against GDC-0980, and autophagy inhibitors may be an important adjunct to enhance the efficacy of GDC-0980 against pancreatic cancer cells.

LC3B is one of the key factors in autophagosome formation and autophagy initiation. LC3B is cleaved and conjugated to phosphatidylethanolamine to become LC3B-II, which forms pre-autophagosomal puncta structure [38]. Beclin-1 was another important regulator for autophagy initiation [39]. Here we found that inhibition of the early stages of autophagy, using 3-MA or more specifically siRNA knockdown of Beclin-1 or LC3B, sensitized pancreatic cells to GDC-0980, once again confirming that inhibition of autophagy, either at early or late stages, could increase GDC-0980-induced apoptosis in human pancreatic cancer cells.

In summary, our novel findings demonstrate that GDC-0980 can induce apoptosis and autophagy in human pancreatic cancer cells. Inhibition of autophagy by pharmacologic or genetic means drives pancreatic cancer cells into apoptosis, and to enhance GDC-0980 activity. Since, an emerging theory for cancer therapy is to induce apoptotic cell death, autophagy inhibition may represent a novel therapeutic strategy to sensitize GDC-0980 in pancreatic cancers.

Conflict of interests

The authors have declared that no conflict of interest exists.

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