Puquitinib mesylate (XC-302) induces autophagy via inhibiting the PI3K/AKT/mTOR signaling pathway in nasopharyngeal cancer cells

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Abstract. There are numerous studies that demonstrate the anti-neoplastic activity of phosphatidylinositol 3-kinase (PI3K) inhibitors and the mechanisms of inducing autophagy in cancer cells. The new anticancer drug puquitinib mesylate (XC-302) is a molecular-targeted drug, which suppresses the activity of PI3K directly. However, it remains unclear whether XC-302 can develop an antitumor effect by inducing autophagy in nasopharyngeal cancer cells. The MTT assay was used to study the anti-proliferative effects of XC-302. Subsequently, autophagy was determined by monodansylcadaverine (MDC) staining, punctate localization of green fluorescent protein (GFP)-light chain 3 (LC3), LC3 protein blotting and electron microscopy. The expression levels of beclin 1, p62, protein kinase B (AKT), phospho (p)-AKT, mechanistic target of rapamycin (mTOR) and p-mTOR in XC-302-induced autophagy were detected. Autophagy inhibition was assayed by 3-methyladenine (3-MA) or small interfering RNA (siRNA) silencing of beclin 1. XC-302 inhibited the viability of CNE-2 in a dose-dependent manner and the IC_{50} of 72 h was 5.2 μmol/l. After cells were exposed to XC-302 for 24h, MDC-labeled autophagolysosomes were evident in CNE-2 cells by fluorescence microscope. Autophagosomes and autolysosomes were identified by transmission electron microscopy. Following transfection with GFP-LC3, XC-302 induced a significant accumulation of GFP-LC3, as monitored by a confocal microscope, which was reduced by 3-MA. XC-302 induced the formation of LC3-II, increased beclin 1 levels and decreased the expression of p62. Additionally, the expression levels of p-AKT and p-mTOR were reduced with the elevation of XC-302. Knockdown of beclin 1 with siRNA or co-treatment with 3-MA enhanced significantly the survival of CNE-2 and promoted the ability of clone formation. XC-302 also induced apoptosis in CNE-2, and when autophagy was inhibited by 3-MA, the apoptosis rate was decreased. The present data provides the evidence that XC-302 can induce autophagy in CNE-2, which promotes the program of cell death and inhibits the PI3K/AKT/mTOR signaling pathway. Furthermore, XC-302 also promoted apoptosis in CNE-2 cells, which could be reduced when autophagy was suppressed, meaning that autophagy may interact with apoptosis to induce cell death.

Introduction

The aberrant activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway is an essential component of cellular processes that are critical for malignant tumor development, such as growth, proliferation and invasion (1,2). The small molecule inhibitors, including PI3K itself and associated pathway proteins, have been investigated as potential targets for new anticancer drugs (3). Puquitinib mesylate (XC-302) is a novel multiple-target agent that has been developed independently by Xinchang Pharmacy Corp. (Zhejiang Medicine Co., Ltd., Zhejiang, China). The ability to immediately suppress the activity of PI3K (subtype IA, IC_{50} against PI3K isoforms: p110\_\alpha=766.6 nM, p110\_\beta=699.4 nM, p110\_\delta=2.8 nM and p110\_\gamma=89.7 nM) in vitro has been reported in a preclinical study. It also can apparently inhibit the protein kinase B (AKT) phosphorylation mediated by epidermal growth factor receptor, the activity of receptor tyrosine kinase (kinase insert domain receptor and platelet-derived growth factor receptor \(\beta\)) and the formation of the vascular endothelial cells in the lumen. Preliminary studies have reported that XC-302 has clear antitumor efficacy in xenograft nude mice and in vitro, including cells of colon cancer, lung cancer and lymphoma (all with the IC_{50} in a range of 0.5-2.0 μM). In addition, XC-302 shows low toxicity in toxicological experiments. It is also rapid to absorb and has a high-level absolute bioavailability, which have been indicated by the preclinical pharmacokinetics tests. The phase I clinical trials also exhibit notable safety, tolerability and efficiency of XC-302 in advanced solid tumors and hematological malignancies (data are unpublished information from Xinchang Pharmacy Corp.).

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Epidemiology studies show that nasopharyngeal carcinoma (NPC) is a type of familiar head and neck cancer in Southern China and Southeast Asia (4). Currently, irradiation alone or chemotherapy combined with radiotherapy is the major treatment on the basis of the disease stage (5). Due to the marked breakthroughs in diagnosis and treatment (such as intensity-modulated radiation therapy), the majority of early-stage patients could be cured (6). Even in patients with locally advanced stage disease, the 5-year local control rate is more than 90% (7). However, there remain a portion of locally advanced and numerous metastasis NPC patients who fail to achieve long-term disease control and succumb from the disease when it begins to progress out of control. One of the reasons for the poor prognosis may be treatment resistant (8). Therefore, evolving novel antitumor drugs research and development is a notable prospect for oncotherapy. Preclinical studies demonstrated that the PI3K/mchanistic target of rapamycin (mTOR) signaling pathway is commonly activated in numerous cancers and inhibition of this pathway could increase radiosensitivity, including NPC (9,10), suggesting that the PI3K/mTOR pathway may be a promising target in NPC (11). A previous study has shown that BEZ235, a dual PI3K/mTOR inhibitor, has a therapeutic potential and can reverse resistance to cisplatin in NPC (10). Another study reported that GSK2126458 and PKI-587, which are also dual PI3K/mTOR inhibitors, suppress tumor progression and increase radiosensitivity in NPC (9).

The feasibility of medicines in anticancer treatments is assessed by their capacity to induce cell death (12). Three major modalities of cell death have been defined, including apoptosis (type I), autophagy (type II), which has been regarded as ‘programmed cell death’, and necrosis (type III), which has been considered as accidental and unregulated (13). There are limited studies that have observed that autophagy can be induced by drugs in NPC, including drugs targeted for the PI3K/AKT/mTOR pathway (14,15). As a consequence, XC-302 may not exhibit an antitumor effect by inducing autophagy in NPC cells. In addition, we hypothesize that the present study could provide an initial basis of XC-302 for subsequent clinical application.

Materials and methods

Cell cultures and reagents. The human NPC cell line, CNE-2 (poorly differentiated), was supplied from State Key Laboratory of Oncology (Sun-Yat-sen University Cancer Center, Guangzhou, China). The cell lines were maintained in RPMI-1640 with 10% fetal bovine serum (FBS). Cells in the logarithmic phase were used in the experiments. The puquitinib mesylate (XC-302; chemical structure shown in Fig. 1) was obtained from Xinchang Pharmacy Corp. and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a storage concentration of 40 mM.

Cell proliferation assay. In brief, tumor cells (3x10^5/100 µl/well) were plated in 96-well plates and incubated for 24 h. Culture medium containing gradient concentrations of XC-302 (16-0.125 µM) were added to each well, and 0.1% DMSO was set as the negative control. MTT (5.0 mg/mL; 20 µl) was added after the cells were incubated for 68 h, followed by the addition of 150 µl DMSO into each well to dissolve the dark blue crystals. The absorbance (A) was measured at wavelengths of 570 and 630 nm, respectively (Model-550; Bio-Rad, Hercules, CA, USA). Finally, the cell viability value was calculated using the following formula: (A_sample - A_blank)/(A_control - A_blank) x 100%. All the experiments were executed independently at least three times in triplicate.

Fig. 1. The structural formula of XC-302. C_{15}H_{16}N_{2}CH_{2}SO_{4}H + 2H_{2}O; Molecular weight: 545.59.

Monodansylcadaverine (MDC) staining. The MDC assay was used to label preliminary autophagosomes (16). Cells grown on 6-well plates were treated with XC-302 (0.5-8 µM) for 24 h, incubated with 0.05 mM MDC for 30 min at 37°C and washed with phosphate-buffered saline (PBS). Fluorescence images were captured by an Olympus IX71 fluorescence microscope (Olympus, Center Valley, PA, USA).

Transmission electron microscopy (TEM). The presence of autophagosomes in TEM is the standard for detecting autophagy. Cells grown on 6-well plates were treated with XC-302 (4 µM) and 24 h later were trypsinized and washed with PBS prior to fixing in fixative buffer. Subsequently, cells were collected by centrifugation at 500 x g for 10 min, suspended and incubated for 2 h in 2.5% glutaraldehyde. Following fixation, cell samples were treated with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated through a graded series of acetone and embedded in resin. Finally, the samples were sliced into 65-nm sections, which were processed for TEM (Hitachi electron microscope H-600; Hitachi, Ltd., Toyko, Japan).

Transfection of green fluorescent protein (GFP)-light chain 3 (LC3) expression vector. The GFP-LC3 expression vector was generously provided by Professor X.F. Zhu (State Key Laboratory of Oncology). Following transfection of the GFP-LC3 expression vector with Lipofectamine 2000 (Invitrogen) for 24 h, following the manufacturer’s protocol, cells were divided into groups based as follows: i) Negative control with 0.1% DMSO (4 µM); ii) XC-302 (4 and 8 µM); iii) 3-MA (5 mM); and iv) 3-MA (5 mM) + XC-302 (4 and 8 µM). Following the indicated treatment, the localization of GFP was directly observed with a laser scanning confocal microscope.

Western blot analysis. Following treatment of various concentrations of XC-302 for different times, the total protein from the CNE-2 cells was obtained by lysing in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membranes were subsequently blocked with skimmed milk (5%) and incubated overnight at 4°C with the following antibodies: Total AKT (1:1,000, #9272, rabbit anti-human), mTOR, phospho-AKT (S473; 1:2,000, #9271, rabbit anti-human), phospho-mTOR (1:1,000, #5536, #9231, rabbit anti-human).
rabbit anti-human), LC3 (1:1,000, #4599, rabbit anti-human), beclin 1 (1:1,000, #3495, rabbit anti-human), p62 (1:1,000, #8025, rabbit anti-human) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:3,000, #5174, rabbit anti-human), which were purchased from Cell Signaling Technology. The samples were incubated with a secondary antibody (1:4,000, #9916, anti-rabbit IgG; CST) for 1 h at room temperature and the labeled proteins were detected using chemiluminescence reagent and automatic X-ray film. Equal loading GAPDH was assessed as the internal control for western blot analysis.

Downregulation of gene expression by small interfering RNA (siRNA). The specific siRNA oligomers against beclin 1 (forward, 5'-CAG TTT GGC ACA ATC AAT Att-3') and si-control (forward, 5'-UUC UCC GAA CGU GUC ACG Utt-3') were purchased from the Gene Technology Co. (Shanghai, China). The CNE-2 cells were transfected with si-beclin 1 and Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After incubation for 12 h, the transient transfected CNE-2 were used for the following experiments.

Colony formation assay. The cells were grown for 24 h and subsequently treated for 6 h as follows: Groups without intervention of siRNA, i) negative control; ii) XC-302 (4 µM); iii) 3-MA (5 mM); and iv) 3-MA (5 mM) + XC-302 (4 µM); and groups with silencing of siRNA, i) si-control; ii) si-control cells with XC-302 (4 µM); iii) si-beclin 1; and iv) si-beclin 1 cells with XC-302 (4 µM). The cells were trypsinized and subsequently seeded in 6-well culture plates (500/well) for 2 weeks. The cells were washed and fixed with ice-cold methanol for 10 min, followed by the addition of 0.5% crystal violet solution for 10 min to observe the colony formation.

Annexin V-FITC/propidium iodide (PI) double staining. Briefly, the CNE-2 cells were treated with different concentrations of XC-302 (4 and 8 µM) and/or 5 mM 3-methyladenine (3-MA, M9281; Sigma-Aldrich) for 24 h. Following collection, the cells were stained with Annexin V-FITC and PI. The apoptotic cells were estimated using a Beckman-Gallios flow cytometer (Beckman-Gallios, Miami, FL, USA).

Statistical analysis. Whole data are exhibited as mean ± standard error of the mean of more than two independent experiments in each group. The variances between each group were calculated by Student’s t-test and P<0.05 was considered to indicate a statistically significant difference.

Results

XC-302 inhibits the proliferation of CNE-2 cells by inducing autophagy and apoptosis. To assess the effects of XC-302 on NPC cell proliferation in vitro, CNE-2 cells were treated with XC-302 at different concentrations for 72 h and subsequently
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Evaluated by the MTT assays. Exposure to XC-302 showed a dose-dependent inhibition of cell viability. MDC is known as a specific marker of autophagic vacuoles (Fig. 2A). XC-302-treated CNE-2 cells resulted in fluorescence intensity enhanced with the increasing dose of the drug (Fig. 2B). In addition to autophagy, XC-302 treatment also induced apoptosis in the CNE-2 cells. The Annexin V-FITC/PI double staining showed that cells treated with XC-302 increased the percentage of apoptosis up to 14.2% (Fig. 2C). The TEM was adopted to further examine the autophagosomes of XC-302-treated cells. Compared with the control cells, XC-302 treatment not only resulted in a rapid increase of autophagosomes (thick arrow), but also induced the formation of autolysosomes (thin arrow) in a portion of CNE-2 cells (Fig. 2D).

**XC-302 inhibits the activation of the PI3K/AKT pathway during the process of autophagy in CNE-2 cells.** Microtubule-associated protein 1 LC3 is a specific marker for autophagy. It is converted from the inactive form, LC3-I, to a cleaved form (LC3-II) during autophagy, and the LC3-II protein has been frequently used for autophagy detection. In the present study, the changes of the markers in autophagy were analyzed by western blotting analysis. The LC3-II protein levels were markedly increased by XC-302 treatment. The beclin 1 level was augmented and as a degradation product, the expression of p62 was decreased (Fig. 3A and B).

Subsequently, whether the downstream targets of PI3K could be inhibited by XC-302 was examined in the CNE-2 cell line. As shown in Fig. 3C, following exposure to XC-302 for 24 h, although the levels of AKT and mTOR did not exhibit significant changes, the phosphorylation levels of AKT and mTOR protein were attenuated when treated by XC-302 in a dose-dependent manner.
Autophagy inhibitor 3-MA reduces cell death induced by XC-302 in the CNE-2 cells. 3-MA is a known specific inhibitor of autophagy, which is frequently used to detect the role of autophagy under various treatments. Thus, the effects of 3-MA was investigated on the apoptosis, autophagy, proliferation and clone formation of XC-302-treated CNE-2 cells. The apoptosis rate of cells were markedly decreased in cells co-treated with various concentrations of XC-302 and 3-MA, compared with those treated with XC-302 alone (P<0.05) (Fig. 4A and B). To detect the formation of autophagosomes specifically, cells were transfected with the GFP-LC3 plasmid transiently. Following treatment with XC-302, cells showed increased LC3 punctures compared with the control in a dose-dependent manner (Fig. 4C), visualized by fluorescence microscopy. In addition, there was a significant degradation of green fluorescence in XC-302 (8 µM) and 3-MA co-treated cells. These results corroborate the observation that XC-302 induced autophagy, as well as 3-MA decreased the accumulation of autophagosomes, in XC-302-exposed CNE-2 cells. The proliferation of the CNE-2 cells was inhibited by XC-302 and could be reversed by exposure to 3-MA after 24 or 48 h, as revealed by the MTT assay (P<0.05) (Fig. 5A). Subsequently, clone formation assays were used to further ensure that 3-MA reduced the sensitivity of the cells to XC-302. The group combining 5 mM 3-MA with 4 µM XC-302 formed more colonies compared with the individual use of XC-302 (P<0.05) (Fig. 5B and C). These results clearly reveal that autophagy contributes to XC-302-induced cell death in CNE-2 cells.

Knockdown of beclin 1 accelerates proliferation and clone formation of CNE-2 cell line treated by XC-302. Beclin 1 is a critical regulator of autophagy, which can specifically suppress the autophagy pathway by knockdown of it. The beclin 1 gene was further knocked down using siRNA and the efficiency of the knockdown was confirmed by western blotting (Fig. 6A). Using the MTT assay, similar to the effect of 3-MA, the knockdown of beclin 1 resulted in a higher vital rate following exposure to XC-302 (P<0.05) (Fig. 6B). Additionally, clone formation is markedly increased in beclin 1 knockdown cells compared with the scramble control cells following the treatment of XC-302 (Fig. 6C and D). Taken together, these results revealed that the inhibition of autophagy by beclin 1 knockdown promoted proliferation and clone formation of CNE-2 cells that were exposed to XC-302.
Discussion

The PI3K/AKT/mTOR signaling pathway has a crucial role in the tumorigenesis of human cancers, including NPC (17,18), which is extremely attractive for targeted, molecular drug therapies. The NPC cell lines and tissues also overexpressed phosphorylated AKT (19-21). The two well-studied PI3K inhibitors are wortmannin and LY294002 (22). However, due to toxic responses, and poor pharmacological properties, their application has been primarily restricted. As a novel multiple target point inhibitor, including PI3K, which was developed independently, XC-302 exhibited promising inhibition of NPC cell proliferation, and not only induced apoptotic cell death but also autophagy in the present study.

As well as apoptosis, autophagy is a type of programmed cell death that is mediated by activation of the autophagy-related gene proteins. When autophagy is induced, the characteristic structure change is the formation of acidic vesicular organelles with double-membrane structures engulfing bulk cytoplasmic organelles. Numerous antitumor agents have been reported to induce autophagy (23). The results of the present study showed the autophagosome accumulations in the CNE-2 cells and MDC-positive stain following exposure to XC-302. LC3-II, beclin 1 and p62 are the reliable biomarkers of autophagy (24). As shown by western blotting analysis, XC-302 augmented LC3-II and beclin 1 levels, and downregulated p62 in the CNE-2 cells. Currently, increasing studies have suggested that the PI3K/AKT pathway has a vital role in the regulation of autophagy (25-27). The consequences of protein expression are the hypothesis that the inhibition of the PI3K/AKT/mTOR pathway is involved in XC-302 induced autophagy. In the present study, XC-302 caused autophagy in the CNE-2 cell line, which was activated when PI3K/AKT/mTOR was inhibited. In particular, 3-MA suppressed the XC-302-induced accumulation of autophagosomes. As opposed to triggering the type I PI3K by XC-302, 3-MA inhibits autophagy in mammalian cells through inhibiting class III PI3K (28,29), which may explain the diverse effects of XC-302 and 3-MA in autophagy. XC-302 treatment also triggered apoptosis, as evidenced by Annexin V-PI double staining.

However, whether autophagy represents a survival mechanism or contributes to cell death remains controversial (30). The present study has defined the role of XC-302-mediated autophagy in CNE-2 cell functions. Inhibition of autophagy by 3-MA could markedly suppress XC-302-induced cell death. Consistent with this, the present findings were further strengthened by transduction of specific target siRNAs to block autophagy, and knockdown of beclin 1 in combination with XC-302 markedly accelerated proliferation and clone formation of the CNE-2 cell line compared to using XC-302 alone. Taken together, these results propose that autophagy provided a survival disadvantage for these NPC cells.

Notably, the promoted effect of apoptosis due to XC-302 treatment was reversed when autophagy was suppressed by 3-MA in the CNE-2 cells. In NPC, the associations of apoptosis with autophagy in response to PI3Ks inhibition remain unknown. The present results demonstrate that autophagy, as well as apoptosis, participate commonly in the death of CNE-2 cells induced by XC-302, and furthermore, autophagy may serve as a significant role in the process of apoptotic cell death. The mechanisms connecting autophagy with apoptosis are not entirely clear, and studies have generated inconsistent results. However, B-cell lymphoma-2 (Bcl-2) has been defined as a central regulator of autophagic and apoptotic signaling pathways (31-34). Beclin 1 was discovered by a yeast two-hybrid system as a binding partner of Bcl-2 (Bcl-2-beclin 1 complex) (33). Recent discoveries have suggested that certain apoptotic proteins modulate autophagy (35). In the present study, the phenomenon of mitochondrial swelling was observed when cells were exposed to XC-302. Tolkovsky et al (36) considered that the cytochrome c released by injured mitochondria could activate autophagy and apoptosis when the permeability of mitochondria was altered, which prompted the membrane potential. Therefore, it is possible that the mitochondria switches the cellular program from one to another.

In conclusion, to the best of our knowledge, this is the first study to report that XC-302 may induce autophagy, which promotes the program of CNE-2 cell death probably by inhibition of the PI3K/AKT/mTOR signaling pathway. Thus, XC-302 may be a potential novel candidate for treating human NPC, and enhancing autophagy is a new way to strengthen the effects of antitumor therapies. Furthermore, XC-302 also promoted apoptosis in CNE-2 cells, which could be reversed when autophagy was suppressed. While autophagy has been served as a possible therapeutic target in NPC treated with XC-302, the exact effects and association between autophagy and apoptosis remain unclear. More studies are required in the future to elucidate the associations.

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