YL4073 is a potent autophagy-stimulating antitumor agent in an in vivo model of Lewis lung carcinoma

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Abstract. Cancer cells activate autophagy in response to anticancer therapies. Autophagy induction is a promising therapeutic approach to treat cancer. In a previous study, YL4073 inhibited the growth of liver cancer and induced liver cancer cell apoptosis. Here, we demonstrated the anticancer activity and specific mechanisms of YL4073 in Lewis lung carcinoma LL/2 cells. Our results show that YL4073-induced autophagy was followed by apoptotic cell death. The anticancer and autophagy stimulating efficacy was confirmed by several factors, including the appearance of autophagic vacuoles, formation of acidic vesicular organelles, recruitment of microtubule-associated protein 1 light chain 3 II (LC3-II) to the autophagosomes, conversion and cleavage of LC3-I to LC3-II, upregulation of Beclin 1 expression, and formation of the Atg12-Atg5 conjugate in LL/2 cells after YL4073 treatment for 24 or 48 h. Furthermore, P53 activation and p-histone H3 phosphorylation occurred after cell exposure to YL4073 for 48 h, suggesting that cell apoptosis had occurred. Pharmacological inhibition of autophagy using 3-methyladenine increased cell apoptosis. Molecular level studies revealed that YL4073 inhibited survival signalling by blocking the activation of Akt and mTOR phosphorylation and reduced the expression of p-mTOR downstream targets for phosphorylation, including p70S6K, p-TSC, p-MAPK, and p-AMPK. This suggests that the Akt/mTOR/p70S6K and TSC/MAPK/AMPK pathways are involved in the effects of YL4073 treatment in LL/2 cells. In addition, YL4073 significantly inhibited LL/2 tumor growth and induced apoptosis in vivo. These data suggest that YL4073 has a significant anticancer effect, with a pathway-specific mechanism of autophagy both in vitro and in vivo. 

Introduction 

Lung cancer, the most common type of cancer, represents a major public health problem worldwide (1,2). Lung cancers have been ranked as the main cause of cancer-related morbidity and mortality (3-5). Unfortunately, lung cancer therapy has not been explored extensively in the field of pharmaceutics (6). Because of the poor outcomes associated with lung cancer, drugs with high efficacy are needed to treat this malignancy (7-9).

Small molecular agents have previously exhibited efficacy in anticancer therapies (10,11). YL4073 has thus far exhibited a profound effect on liver cancer (12). It is possible that YL4073 may have novel pharmacological application as a medicinal product with inhibitory activity in other cancers, such as Lewis lung cancer.

Autophagy could induce cell death and is activated in response to stress and nutrient deprivation (13). Autophagy is a complex catabolic mechanism of lysosomal degradation of proteins and other sub-cellular constituents (14-16). Several signaling pathways have been identified involved in cancer associated with the response to autophagy (17,18). Although autophagy has different roles in the modulation of cancers (19,20), it is known as a mechanism of cell death and tumor suppression (21-23).

In the present study, we identified YL4073 as a potent anticancer agent capable of inducing tumor cell autophagy. We focused our research mainly on lung carcinoma, owing to its
poor prognosis and lack of effective therapies for this tumor type in clinical settings, in an attempt to provide preclinical study profiles for clinical treatment. We have shown that YL4073, a small molecular agent, was a potent targeted autophagy-associated protein, which induced autophagy in LL/2 cells. Cell autophagy was followed by apoptosis indicating the involvement of Akt/m-TOR/p70S6K and TSC/MAPK/AMPK pathways. In addition, YL4073 significantly inhibited the growth of LL/2 tumors in vivo. Our results indicated that YL4073 has significant anticancer activity and autophagy was suggested to be involved in this activity. To the best of our knowledge, this study was the first to demonstrate that YL4073 was able to induce autophagy in cancer cells.

Materials and methods

Materials. Dimethyl sulfoxide (DMSO), propidium iodide (PI), acridine orange, Z-VAD-FMK, and 3-methyladenine (3-MA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratory (Dojin, Japan); all chemicals employed in this study were of pure analytic and culture grade. The primary antibodies for LC-3/LC3-II, Beclin 1, Atg5/Atg12, p-mTOR, p-Akt, p-P70S6K, p-TSC, p-AMPK, p-p44/42 MAPK, P53, PTEN, and p-Histone H3 were purchased from Cell Signaling Technology (Beverly, MA, USA); horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibodies were purchased from Boster Biotechnology (Wuhan, China). For all in vitro assays, YL4073 and 3-MA were dissolved in DMSO to prepare a stock solution of 40 mM and 5 M, respectively, and stored at 4°C. The stock solutions were diluted in the relevant media to the final DMSO concentration of 0.05% v/v when used. For all in vivo studies, YL4073 was suspended in ultrapure water and Cremophor EL/ethanol (50:50, Cremophor EL, 95% ethyl alcohol; Sigma Chemical Co.) and administered at 10 ml/kg/day of body weight by intraperitoneal injection.

Cell culture. Murine Lewis lung carcinoma LL/2, mammary carcinoma cell 4T1, fibroblast NIH-3T3, and human proximal tubular cell human kidney-2 (HK-2) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in RPMI-1640 or Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Bedford, MA, USA) containing 10% heat-inactivated FBS (Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin, and streptomycin in a humid chamber at 37°C and 5% CO2.

Cell viability by CCK-8 assay. Cells were plated in 96-well plates for 24 h and cultured with YL4073 for 72 h. 10 µl CCK-8 solution was added to each well. The IC50 of YL4073 was calculated and optical density (OD) was measured at 450 nm with a Multiskan Spectrum instrument (Thermo Lab Systems, USA) after cells were incubated with CCK-8 for 2–4 h at 37°C.

EdU-DNA incorporation assay. 5-Ethynyl-2’-deoxyuridine (EdU) is a nucleoside analogue of thymidine, which can be incorporated into DNA during DNA synthesis. An EdU-DNA incorporation assay kit (RiboBio, China) was used to detect the percentage of uptake in proliferating LL/2 cells. Briefly, 3x103 LL/2 cells were seeded in 96-well plates for 24 h and treated with YL4073 for 48 h. Then, the cells were incubated with EdU for 2–3 h at 37°C, and the plates observed with an inverted fluorescent microscope (Carl Zeiss, Germany). EdU-positive cells were counted in five fields per well; the average was calculated according to the following formula: EdU (%) = (EdU-positive cells)/(Hoechst-positive cells) x 100. The experiment was done in triplicate.

Detection of acidic vesicular organelles. For detection of acidic vesicular organelles (AVO) in vitro (24), 1x103 LL/2 cells were plated in 6-well plates and treated with 20 µM YL4073 or 2 mM 3-MA for 48 h. The cells were then stained with 1 µg/ml acridine orange for 15 min, after which images were obtained under a fluorescent microscope equipped with a digital camera (25,26).

Autophagy and apoptosis analysis by flow cytometry (FCM). To further confirm autophagy of the cells, we first analyzed LL/2 cells by FCM after AVO staining. Cell culture and drug treatment were carried out as described above. After being treated with 1 µg/ml AVO for 10 min, cells were collected and immediately analyzed by FCM (Beckman Coulter, Miami, FL, USA). Furthermore, LL/2 cells were treated with YL4073 for 48 h. Cells were collected and incubated with 1 ml hypotonic fluorochrome solution containing 50 ng/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100, and were immediately analyzed by FCM. Finally, the cell-permeable caspase inhibitor Z-VAD-FMK and autophagy inhibitor 3-MA were used to study whether caspase family protein kinases and/or autophagy was involved in YL4073-induced apoptosis and autophagy. For this assay, LL/2 cells were treated with 20 µM YL4073 combined with 10 mM Z-VAD-FMK or 2 mM 3-MA, respectively. PI staining was performed and analyzed by FCM 24 and 48 h later.

Western blot analysis. Standard western blot analysis was performed to identify the possible mechanism of YL4073. Briefly, LL/2 cells were treated by YL4073 for 48 h and cell proteins were extracted. Western blotting was further examined via electrophoretic transfer of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), separation of proteins on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubation with primary and secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences Corp., Piscataway, NJ, USA).

Pharmacokinetics analysis of YL4073 in SD rats. SD male rats (weight, 180–200 g) were obtained from the Beijing Animal Center (Beijing, China) received an intraperitoneal injection with a single dose of 30 mg/kg YL4073 and the pharmacokinetic profiles were determined by high-pressure liquid chromatography (HPLC; Waters, USA). Briefly, the blood of SD rats (n=3) was collected in heparinized tubes at designated time-points after drug administration and centrifuged immediately to separate plasma from blood cells. Samples (90 µl) were mixed with 100 µl of acetonitrile containing 10 µl internal standards (5 µg/ml) and centrifuged at 6,000 x g for 10 min.
Then, 100 µl supernatant was evaporated and re-dissolved in a solution of 50 µl acetonitrile: water 50:50 v/v containing 10% formic acid and subjected to HPLC. Pharmacokinetic parameters were analyzed using a Pharmacokinetic software of Drug and Statistics (DAS, edited by Mathematical Pharmacology Professional Committee of China, version 2.1.1). All animal experiment protocols were conducted in full compliance with our universities for the Care and Use of Laboratory Animals and Experimental Animal Ethics Committee.

Table I. The effects of YL4073 on tumor cell viability.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>IC50 (µM)</th>
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<tbody>
<tr>
<td>LL/2</td>
<td>Murine Lewis lung carcinoma cell line</td>
<td>5.29</td>
</tr>
<tr>
<td>4T1</td>
<td>Mouse mammary carcinoma cell line</td>
<td>9.33</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>Mouse fibroblasts cell line</td>
<td>&gt;40</td>
</tr>
<tr>
<td>HK-2</td>
<td>Human proximal tubular cell line</td>
<td>&gt;40</td>
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CCK-8 assay was employed to detect the cell viability. Each cell line was treated with various concentrations of YL4073 for 72 h, respectively. Data are expressed as the mean from three experiments.

Pharmacodynamic analysis of YL4073 in vivo. Female C57BL/6 mice (6-8 week-old) were obtained from the Beijing Animal Center (Beijing, China) and used in the present study. Each mouse received a single injection of harvested LL/2 cells on the flank in the axillary region for the subcutaneous mouse tumor model (27). The tumors were allowed to grow for ten days, and the animals were subsequently sorted into groups of ten for treatment. Tumor volume was measured in two dimensions with vernier calipers and calculated using the following formula: Tumor volume (cm3) = (length x width x height) / 2 (28).

TUNEL assay in vivo. TUNEL [terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling] assay was performed to examine the apoptosis induction effect of YL4073 on LL/2 cells in vivo. LL/2 tumor sections from vehicle and YL4073-treated mice were subjected to TUNEL assay according to the manufacturer's instruction. TUNEL-positive cells were counted under a microscope in three equal-sized fields per slide for quantitative analysis of apoptotic cells. The percentage of apoptotic cells was evaluated as follows: Tumor apoptotic index (%) = apoptotic cells / total cells x 100 (12).

Immunohistochemical analysis in vivo. Immunohistochemical analysis was used to detect the autophagy induction effect of

Figure 1. YL4073 inhibited cellular proliferation and induced apoptosis in vitro. (A) The EdU-DNA incorporation assay using YL4073-treated LL/2 cells. Staining with Hoechst 333258 and EdU in LL/2 cells treated with YL4073 for 48 h (x100 magnification). (B) The EdU-positive cells were analysed using Image analyzing software. Data were expressed as mean ± SD; n=3; *P<0.05; **P<0.001. (C) YL4073 induced LL/2 cell apoptosis (concentration-dependent manner). Cells were incubated with YL4073 for 48 h, PI-stained, and analyzed by FCM. Data are representative of three parallel experiments.
YL4073 on LL/2 cells in vivo. LL/2 tumor sections from vehicle and YL4073-treated mice were incubated with primary LC3-II antibody and the corresponding second antibody according to the manufacturer’s instruction. Representative images were then observed under an inverted microscope with a camera system, in three equal-sized fields per slide (Carl Zeiss) (12).

Statistical analysis. The data were analyzed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and presented as mean ± SD/SEM. Kaplan-Meier curves were used to analyze survival and tested with a log-rank test.

Results

YL4073 inhibited proliferation of murine cancer cells in vitro. CCK-8 assay was used to measure the effect of YL4073 on cell viability. Our results showed that YL4073 decreased the viability of 4T1 and LL/2 cell lines with IC_{50} values of 9.33 and 5.29 µM after treatment for 72 h, respectively (Table I). LL/2 cells were used to further study the mechanism of YL4073 in Lewis lung carcinoma. The IC_{50} for NIH-3T3 and HK-2 cell lines was >40 µM, higher than that for the cancer cell line.

Anti-proliferation effects of YL4073 in LL/2 cells in vitro. The EdU-DNA incorporation assay validated the anti-proliferation ability of YL4073. As shown in Fig. 1B and C, the percentage of EdU-positive cells was 51.05% after 5 µM YL4073 compared with that after vehicle treatment, whereas the percentage of EdU-positive cells decreased to 16.18% and 5.67% when cells were treated with 10 and 20 µM YL4073, respectively.

Effects of YL4073 on LL/2 cell apoptosis in vitro. Morphological changes of LL/2 cells (stained with PI) were assessed after treatment with YL4073. As shown in Fig. 1, the percentage of sub-G1 cells in the YL4073-treated group increased in a concentration-dependent manner. After cells were treated with 5 µM YL4073, the apoptosis rate was 43.20%; the rate of apoptosis increased to 64.80% and 76.60% after cells were treated with 10 and 20 µM YL4073 for 48 h, respectively.

Effect of caspase on YL4073-induced apoptosis. The effect of autophagy inhibitor 3-MA on YL4073-induced apoptosis was assayed by FCM. The rate of apoptosis after treatment with 20 µM YL4073 plus 2 mM 3-MA decreased from 34.7 to 23.6% and from 71.3% to 52.3% after 24 and 48 h treatment, respectively, compared with that after YL4073 treatment alone (Fig. 2). Furthermore, similar results were observed in caspase inhibitor Z-VAD-FMK-treated LL/2 cells (data not shown). These results suggest that YL4073-induced apoptosis in LL/2 cells is associated with autophagy, which may be one of mechanisms of the anticancer effect of YL4073.

Induction of autophagy in LL/2 cells examined with AVO assay. Cell death by autophagy has been proposed recently (29,30). Autophagy is characterized by AVO formation, which was detected and measured by staining with acridine orange (31,32). As shown in Fig. 3A, staining LL/2 cells with acridine orange showed the accumulation of AVO in the cell cytoplasm after exposure to 20 µM YL4073. This was inhibited by addition of 2 mM 3-MA, which inhibited autophagosome sequestration (33,34). FCM analysis was used to quantify the YL4073-induced increase in the fractional volume and acidity of AVO. As shown in Fig. 3B, 20 µM YL4073 increased the intensity of fluorescence in LL/2 cells to 85.99% compared with vehicle, indicating that 2 mM 3-MA suppressed the development of AVO in LL/2 cells. YL4073 significantly affected development of AVO in LL/2 cells compared with vehicle (P<0.01), and the inhibitory effect of 3-MA on development of AVO was also significant (P<0.05).
YL4073 induces autophagosome formation in LL/2 cells. Western blotting was performed to monitor the alteration of YL4073-mediated autophagy (34). As shown in Fig. 4A, LC3-II protein was detectable in LL/2 cells following YL4073 treatment for 48 h. Activation of a series of autophagy proteins was a critical step for autophagosome formation. To determine whether the key autophagy-related proteins were synergistically activated in response to YL4073-induced autophagy, the expression of Beclin 1 and the key regulator of autophagy, Atg12-Atg5 conjugate, were assessed in LL/2 cells. As shown in Fig. 4A, YL4073 induced a marked accumulation of Beclin 1 and intracellular Atg12-Atg5 complex after 48 h compared with vehicle; moreover, p-histone H3 was significantly inhibited by YL4073, while the expression of P53 was
YL4073 inhibits Akt/mTOR/p70S6K and activates TSC/MAPK/AMPK pathway in LL/2 cells. Western blot analysis was also used to evaluate the effect of YL4073 on the Akt/mTOR/p70S6K pathway, because it is the main pathway that down-regulates autophagy (35). Treatment with YL4073 decreased phosphorylated Akt (p-Akt) effectively for 48 h in LL/2 cells; p-mTOR activity was also affected (Fig. 4C). These results suggest that the upstream pathway of Akt was influenced by YL4073 treatment. Furthermore, YL4073 decreased p-p70S6K and p-TSC gradually in LL/2 cells after 48 h. Because the AMPK/MAPK pathway upregulated autophagy in starved cancer cells (36), we examined the effects of YL4073 treatment on this pathway. As shown in Fig. 4D, YL4073 decreased p-AMPK and p-p44/42MAPK for 48 h in LL/2 cells. Collectively, these results indicated that YL4073 could inhibit both the Akt/mTOR/p70S6K pathway and the AMPK/MAPK pathway, and both changes potentially mediated YL4073-induced autophagy.

Pharmacokinetic and pharmacodynamic profile of YL4073. YL4073 was used as an anticancer therapeutic agent model in a pharmacokinetics study. We determined certain pharmacokinetic parameters of YL4073 in blood serum of SD rats by HPLC. A concentration versus time curve was measured in serum at each time-point. Our results shown that the plasma concentration of YL4073 at 5 min was 5.53 µg/ml after treatment with a 30 mg/kg dose. Pharmacokinetic modelling suggested that the plasma concentration-time profile can be described using a two-compartment model with an estimated $t_{1/2}$ of 4.4 h and urinary clearance representing 5.1% of systemic clearance, with substantial variation in the plasma concentration. The AUC was 11.9 mg/l per hour. We used AUC to estimate the efficacy of YL4073; it was also used to predict toxicity. The results indicated that YL4073 had high efficacy and low toxicity.

We evaluated whether YL4073 inhibited the growth of LL/2 subcutaneous tumors by inducing apoptosis and
autophagy in vivo. C57BL/6 mice were inoculated subcutaneously with LL/2 cells, and after ten days, tumor volume reached about 50 to 70 mm³. Intraperitoneal injections of 60 mg/kg/day YL4073, 30 mg/kg/day YL4073, vehicle, and N.S. were administered to different groups. Tumor growth was observed for 14 days after the initiation of treatment. On day 14, there was 29.1% and 52.6% tumor growth inhibition in 60 mg/kg/day and 30 mg/kg/day YL4073 groups compared with vehicle (P<0.05; Fig. 5A). In addition, immunohistochemical analysis and TUNEL assay was used to examine whether YL4073 induced autophagy and apoptosis in vivo. As shown in Fig. 5B-D, the expression levels of LC3-II increased, and the number of apoptotic cells in YL4073-treated groups was more than that in the vehicle group, after treatment with YL4073 in vivo. The results were consistent with the results of WB in vitro. These results suggest that YL4073 inhibited LL/2 tumor growth in vivo by inducing autophagy and apoptosis.

**Discussion**

Although notable progress has been made in the management of advanced lung cancer, many challenges remain (37). Chemotherapy has been the primary treatment for patients with advanced lung cancers (38). However, recent studies suggest no significant improvement in survival rate in these patients (39). There is an urgent, unanswered medical necessity for the development of rational and effective therapies to treat advanced malignant lung cancers (40). In this study, we demonstrated that small molecular compound YL4073 inhibited autophagy and apoptosis in murine Lewis lung cancer LL/2 cells both in vitro and in vivo. YL4073 has significant anti-proliferation activities against a panel of murine cancer cell lines, including 4T1 cells and LL/2 cells; LL/2 cells were most sensitive to YL4073 treatment. Furthermore, we focused on the effect of YL4073 on LL/2 cells to study the relationship between the autophagy and apoptosis pathways involved. The anti-proliferation effect of YL4073 has yet to be studied in a wider variety of cancer cells. Susceptibility to apoptosis of tumor cells was an important determinant of effective therapy.

Based on our results, we consider that YL4073 inhibits the growth of tumors by inducing autophagy. Our results indicated YL4073 induced LL/2 cell autophagy in a concentration-dependent manner. We demonstrated that YL4073 promoted an autophagic process in LL/2 cells. Our data show that YL4073 treatment resulted in the appearance of a series of autophagic markers, including development of double-membrane autophagic vacuoles and AVO augmented conversion of LC3-I to LC3-II (41). Furthermore, YL4073 treatment caused the activation of a group of autophagy-related proteins, including Beclin 1 and Atg12-Atg5 complex, both of which are essential for autophagosome formation (42).

Inhibition of the Akt/mTOR/p70S6K and the TSC/MAPK/AMPK signalling pathways may contribute to the autophagy of tumors (43). The Akt-mTOR signalling pathway is an important downregulator of autophagy (42). The study also employed EdU-DNA incorporation assays to show that YL4073 could inhibit the proliferation of tumor cells; p-Akt and p-mTOR were also inhibited in the presence of YL4073, suggesting that inhibition of the Akt/mTOR/p70S6K signalling pathway contributed to YL4073-induced autophagy in LL/2 cells. In addition, the phosphorylation of TSC, MAPK and AMPK were inhibited by YL4073 treatment. These findings suggest that inhibition of these two signalling pathways is a potential therapeutic approach for the treatment of cancer. YL4073 effectively inhibited tumor growth in an established LL/2 lung cancer model, and induced autophagy in vivo. In addition, there were no obviously pathological changes in major organs, including the heart, liver, spleen, lung, kidney and brain, according to H&E stain (data not shown).

We also attempted to identify the primary target of YL4073, since it is critical to further study. We have carried out some limited research to this end; a bioinformatics-based ‘reverse docking’ method with computer-aided drug designed has been applied, which predicted that the possible primary targets might be CXCR3, glutathione S-transferase, chymotrypsin, and penicillopepsin. Furthermore, the results of the kinase inhibitory activity experiment showed that YL4073 did not block kinase activity of MEK, or Raf (12). Therefore, the primary molecular target of YL4073 is not yet identified. Our aim for further study is to use new approaches to identify the primary target, such as quantitative chemical proteomics, which has been used as an effective method to detect the primary molecular target of some agents (44).

In conclusion, we demonstrated that YL4073 has anti-cancer activity and that it induced autophagy and apoptosis both in vitro and in vivo. The anticancer mechanisms involved LC3, Beclin 1, and Atg5/Atg12 complexes, inhibition of the Akt/mTOR/p70S6K pathway, and activation of the AMPK/MAPK pathway in LL/2 cells. These results indicate that YL4073 warrants further in-depth study and may be a promising agent for future lung cancer therapy.

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