Depletion of 4E-BP1 and regulation of autophagy lead to YXM110-induced anticancer effects

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Natural products have always been a profuse database for developing new chemotherapeutics. YXM110 is a new synthesized phenanthroquinolinizidines that exhibits excellent anticancer activity in numerous cancer cells. In this study, we examined the anticancer mechanisms of YXM110 both in vitro and in vivo. Protein level of 4E-binding protein 1, which is crucial in cap-independent translation, was decreased significantly after YXM110 treatment via c-Jun N-terminal kinases-mediated proteasomal degradation. Moreover, the effects of YXM110 were associated with several characteristics of autophagy, including accumulation of autophagic vacuoles, elevation of Atg12-Atg5 and light chain 3 (LC3)-II, and levels of GFP-LC3 puncta. The results suggest that depletion of McI-1 contributes to YXM110-triggered autophagy, whereas downregulation of lysosomal-related genes could cause autophagy impairment. Furthermore, YXM110-induced cell death was prevented by autophagy inhibitor 3-methyladenine and Atg5 silencing, indicating that YXM110-mediated autophagy impairment leads to cancer cell death. These data reveal key mechanisms that support the further development of YXM110 as a promising anticancer agent.

Introduction

Cancer is currently one of the most life-threatening diseases, and identifying better anticancer agents is a major focus of biopharmaceutical research and development. Phenanthroquinolizidines are natural alkaloids isolated from the Asclepiadaceae and Moraceae families, which have significant antimicrobial, anti-inflammatory and antiangiogenic effects (1,2). Cryptopleurine is a phenanthroquinolizidine alkaloid and has been reported as a potent and promising anticancer compound. Moreover, this type of structure also exhibits excellent anticancer activities in drug-resistant cancer cells (3). Several biological functions of cryptopleurine have previously been elucidated, including antiproliferation and antiangiogenesis effects, but many specific targets remain unknown (4).

However, the potential development of this natural product has been limited due to its ability to penetrate the blood–brain-barrier. YXM is a series of derivatives of cryptopleurine containing more hydrophilic structures to prevent the CNS toxicity (5). Among the cryptopleurine derivatives that have been screened, YXM110 was the most potent agent against five cancer cell lines, and against human colorectal cancer HCT116 cells and non-small cell lung cancer A549 cells, in particular (Figure 1B). The GI50 values of this agent against most cell lines are ~3–25 nM. However, the mechanisms underlying the activity of this compound are not fully understood and require further investigation.

Nucleoli, where rRNA synthesis and ribosomal assembly take place, are often larger in highly transformed cells. Moreover, a higher protein synthesis rate is required for proliferative cells, such as cancer cells. Thus, enlargement of nucleoli is an indicator of increased transformation and could be an index for cancer malignancy (6). Inhibition of protein synthesis could effectively decrease the proliferation rate of cancer cells and correlates highly with apoptosis (7).

In translation signaling, the messenger RNA (mRNA) 5′ cap-binding protein, eIF4E and 4E-BP1 modulate the cap-dependent protein translation process (8). When 4E-BP1 is hypophosphorylated, it becomes inhibitory and binds to eIF4E, thus preventing protein elongation, whereas hyperphosphorylated 4E-BP1 is inactive and prone to degradation (9). 4E-BP1 also mediates cap-independent translation in hypoxic conditions. Hypophosphorylated 4E-BP1 not only sequesters eIF4E but also suppresses cap-dependent mRNA translation; however, in certain conditions, it activates the translation of internal ribosome entry site-containing mRNAs that are involved with various processes including angiogenesis, hypoxia responses and inhibition of apoptosis (8,10). Therefore, 4E-BP1 plays a dual role in regulation of protein translation.

Macroautophagy (hereafter referred to as autophagy) is a highly regulated catabolic process that results in degradation of long-lived proteins and recycling of damaged organelles, in order to maintain cellular homeostasis and in some cases such as adaptation to starvation or nutrient replete situation (11,12). During autophagy, parts of cytoplasm or organelles are engulfed in large double-membrane vesicles (autophagosomes) and are delivered to lysosomes for bulk degradation (13). A set of autophagy-related genes that are conserved from yeast to mammals, called ATG genes, are responsible for autophagosome formation. The role of autophagy as a pathway for cell survival or cell death is still debatable, and could differ among specific cell types (14). Although autophagy was initially defined as a survival strategy, impaired or prolonged activation of this self-degradation process could also lead to cell death (15–17). Additionally, B-cell lymphoma 2 homologues are known to regulate the autophagy pathway (12). One of the B-cell lymphoma 2 family members, McI-1, which acts as a stress sensor, has been reported to sequester Beclin-1 (Atg6) from initiating autophagosome nucleation and regulating the balance between autophagy and apoptosis (18).

In this study, we aimed to elucidate the mechanisms of action of YXM110. We demonstrated that YXM110 suppresses protein synthesis, induces proteasomal degradation of 4E-BP1 and MCI-1, autophagy-associated cell death and apoptosis. We also show the anticancer activity of YXM110 in the mice xenograft models. These results suggest that YXM110 has the potential to be an effective anti-tumor agent.

Materials and methods

Materials

The synthesized compound YXM series was designed by K.-H.L. and X.-M.Y. (Natural Products Research Laboratories, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, NC), and the purity is >98%. RPMI 1640, 1-methionine-free RPMI 1640, fetal bovine serum, penicillin, streptomycin and all other tissue culture reagents were obtained from Life Technologies (Grand Island, NY). Antibodies against various proteins were
listed as following: α-tubulin, p21, poly(ADP-ribose) polymerase, McI-1, horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); caspase-3 was from Ingentex (San Diego, CA); caspase-9, 4E-BP1, phospho-4E-BP1 (Thr37/46), phospho-mammalian target of rapamycin (mTOR) (Ser2448), phosphor-p70S6K (Thr42/Thr44) and Atg5 were from Cell Signaling Technology (Beverly, MA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphor-p70S6K (Thr389) were from Epitomics (Burlingame, CA). LC-3B was from Novus biological (Littleton, CO). Click-IT AHA Alexa Fluor® 488 Protein Synthesis HCS Assay kit was from Life Technologies. Trizol, Atg5 small interfering RNA (siRNA) and scramble siRNA were from Invitrogen (Carlsbad, CA). RNAse mini kit was from Qiagen (Valencia, CA). Green fluorescent protein (GFP)-LC3 and McI-1 plasmids were obtained from Addgene (Cambridge, MA). Enhanced chemiluminescence detection kit was from Amersham (Fairfield, CT). Trizol reagent. Lipofectamine 2000 and RNAiMax were from Invitrogen. MG132 and 3-MA were from Calbiochem (Billerica, MA). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforhodamine B (SRB), SP600125, rapamycin, dapsylcavadinone (MDC) and all of the other chemical reagents were obtained from Sigma Chemical (St Louis, MO).

Cell culture

Human colorectal cancer cell line HCT116, human non-small cell lung cancer cell line A549, human prostate cancer cell line DU145 and human oral cancer cell line KB were purchased from American Type Culture Collection (Manassas, VA). KBvin is the vincristine-resistant KB subline used as a P-gp multidrug-resistant system. p53-null HCT116 cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum (vol/vol) and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cells were maintained in a humidified incubator at 37°C in 5% CO2/95% air.

Cell proliferation assay (sulforhodamine B assay)

Cells were seeded in 96-well plates in medium with 5% fetal bovine serum overnight. Cells were fixed with 10% trichloroacetic acid representing cell viability (%) = average optical density of wells/average optical density of control. Percentage growth inhibition was calculated as 100 − [(T0 − Tt) / (C − T0)] × 100. Growth inhibition of 50% (GI50) was determined at the drug concentration that results in 50% reduction of total protein increase in control cells during the compound incubation.

Cell viability assay (MTT assay)

HCT116 cells were incubated with vehicle (0.1% DMSO) or YXM110 for 48h. Cells were fixed with 10% trichloroacetic acid and then stained with SRB at 0.4% (wt/vol) in 1% acetic acid. Excess SRB dye was washed away by 1% acetic acid, and dye-containing cells were lysed with 10 mmol/L Trizma base. The absorbance was read under a wavelength of 515 nm. By measuring time zero (T0), growth rate and cell growth in the presence of the drug (Tt), the percentage growth was calculated. Percentage growth inhibition was calculated as 100 − [(T0 − Tt) / (C − T0)] × 100. Growth inhibition of 50% (GI50) was determined at the time point. Transfected cells were trypsinized and reseeded in 96-well or 6-well plates to further experiments.

Microarray analysis

HCT116 cells were treated with 0.3 μMYXM110 for 24h. The total RNA was extracted with Trizol reagent by the manufacturer’s protocol and then purified by RNA mini kit for further studies. The Human Whole Genome OneArray® (Phalanx Biotech, Hsinchu, Taiwan) was used to analyze the changes of RNA expression after treatment compared with control group. The data were loaded into Rosetta Resolver® System (Rosetta Biosoftware), and the selection criteria were established at |Fold changes| ≥ 2 and P-values <0.05. Then we analyzed the gene annotation by DAVID bioinformatics resources and KEGG database.

Western blot analysis

After the treatment of vehicle or compounds at indicated concentrations, cells were harvested and tumor tissues were homogenized. For total lysate, cells were lysed with the 120 μl ice-cold lysis buffer [10 mmol/L Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l ethyleneglycol-bis(aminoetethlyer)-tetraetacetic acid, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM NaF and 1% Triton X-100]. Cell lysates were centrifuged at 13 000 r.p.m. for 30 min. Western blot analysis, the amount of protein (40–60 μg) was separated by electrophoresis in 10–15% polyacrylamide gels and transferred to polyvinylidene difluoro membranes. After 1h incubation at room temperature in PBS/5% nonfat milk, the membrane was washed with PBS/0.1% Tween 20 and incubated with the indicated antibodies at 4°C overnight. After washing with PBS/0.1% Tween 20, the corresponding secondary antibodies were applied to the membranes for 1h at room temperature. The membranes were then washed with PBS/0.1% Tween 20, and the detection of signal was done with an enhanced chemiluminescence detection kit.

Protein synthesis assay

The methionine analog t-azidohomoalanine (AHA) containing an azide moiety was added to cell culture for incorporation into nascent proteins synthesis. The HCT116 cells were seeded in a 96-well plate overnight and treated vehicle or YXM110 in t-methionine-free medium containing AHA for 30 min. After treatment, medium was removed followed by cell fixation and permeabilization. Then the AHA reaction cocktails were added to detect the AHA signaling according to manufacturer’s protocol. The final results were obtained by fluorescence microplate reader (495/519 nm), and the inhibition percentages were calculated compared with control group.

Transient transfection

HCT116 and A549 cells were seeded in six-well plates overnight and then transfected with vector, GFP-LC3 and McI-1 plasmids or scramble and Atg5 siRNAs using Lipofectamine 2000 and RNAiMax according to the manufacturer’s protocol. After 8h transfection and reserum, cells were treated with vehicle (0.1% DMSO) or YXM110 for the indicated times. Cells were harvested and lysed and the cell lysates were collected for western blot analysis; for GFP-LC3 puncta detection, cells were fixed with paraformeldehyde and directly observed by Leica DM IRB microscope; for survival and MDC staining test, transfected cells were trypsinized and reseded in 96-well or 6-well plates to further experiments.

Tumor xenograft models

Nude mice were implanted with HCT116 tumor cells subcutaneously. When the tumors reached the average volume of 100 mm3, the mice were divided into four groups (n = 5) and the agent treatment was initiated. Vehicle (5% DMSO + 5% Creomoph + 90% dextrose 5% in water) or YXM110 (5 and 10 mg/kg) were administered intravenously/intraperitoneally every other day, whereas irinotecan was administered intravenously once a week. The length (L) and width (W) of the tumor were measured every 3–4 days, and the tumor volume was calculated as LW2/2. The protocols of the in vivo study were approved by the Animal Care and Use Committee at National Taiwan University.

Statistical analysis

Data were expressed as mean ± SEM of the indicated number for separate experiments. Statistical analysis of data was performed with one-way analysis of variance followed by the Student’s t-test, and P-values <0.05 were considered significant.

Results

Evaluating the anticancer effects of YXM110 in cancer cell lines

Crytoplearine analogue, YXM110 (Figure 1A), shows potent anticancer effects among various cancer cell lines. As shown in Figure 1B, the values of GI50 against these five cancer cells were ≤25 nm. In HCT116 cells, the GI50 was even lower as 3 nm. Most importantly, in the KB/KBvin multidrug-resistant system, YXM110 exhibited excellent growth inhibition capacity in multidrug-resistant KBvin cells.

Subsequently, we focused mainly on the inhibitory effect of YXM110 on growth of HCT116 and A549 cell lines. YXM110 significantly inhibited HCT116 cell viability in a concentration-dependent manner, as determined by MTT assay (Figure 1C). To elucidate the effect of YXM110 on cell proliferation, the cell cycle was observed

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Fig. 1. YXM110 inhibits cancer cell growth and delays the cell cycle in various cell lines. (A) Chemical structure of YXM110. (B) KB, KBvin, DU145, A549 and HCT116 cell lines were seeded in 96-well plates and treated with YXM110 for 48 h. The Gl_{50} values were obtained using SRB assay. (C) HCT116 cells were incubated with the indicated concentrations of YXM110 (0.003–0.3 μM) for 48 h. Cell viability was then determined by MTT assay. (D) HCT116 cells were starved overnight, and then incubated with vehicle (0.1% DMSO) or YXM110 (0.03 or 0.1 μM) for the indicated periods. DNA content was subsequently analyzed by PI staining using a FACScan flow cytometric assay. Mean ± SE values from three independent experiments are shown. **P < 0.01 and ***P < 0.001, compared with non-treated cells.
after different treatments (concentrations or incubation times); we found that YXM110 prolonged the doubling time of HCT116 cells. Figure 1D shows that although the control cells completed a cell cycle within 24 h, the drug-treated cells tended to stay longer in the G1 phase as the concentrations of YXM110 increased. The analysis of G1 population was shown in Supplementary Figure 1, available at Carcinogenesis Online, to demonstrate the cell cycle alteration of HCT116 by YXM110. These data demonstrated that YXM110 inhibits cancer cell proliferation.

**YXM110 triggers apoptosis and autophagy pathways in HCT116 xenograft models**

To further investigate the anticancer activity of YXM110 in vivo, HCT116 xenograft models were established in nude mice. The volumes of HCT116 xenograft tumors were profoundly inhibited by YXM110 treatment (10 mg/kg, intravenously/intraperitoneally, every other day), as well as by Irinotecan (100 mg/kg, intraperitoneally, every week; Figure 2A, upper panel) without significant changes in body weight (Figure 2A, lower panel). Immunoblotting results revealed that tumor cells underwent apoptosis after YXM110 treatment. Interestingly, the Atg12-Atg5 conjugate, which is highly involved in autophagy pathways and promotes protein–lipid conjugation (20), also increased (Figure 2B). These data indicated that YXM110 could induce apoptosis and autophagy pathways in HCT116 cells in vivo.

**YXM110 suppresses the protein synthesis and induces late-onset apoptosis**

To analyze the anticancer effects of YXM110, we further used HCT116 cells to elucidate the mechanisms underlying its mode of action. After treatment with YXM110, certain proteins including Mcl-1, 4E-BP1 and p21 were depleted within a short period (Figure 3A). The results of previous studies have shown that natural products with similar structures (e.g. tylophorine) can affect protein synthesis (21). Therefore, we examined whether YXM110 affects protein synthesis in cancer cells. Using an AHA Alexa Fluor® 488 Protein Synthesis HCS assay, the inhibitory effect of YXM110 on protein synthesis was tested in HCT116 cells. The result showed that YXM110 decreased the protein synthesis levels significantly at 0.03 µM within 30 min (Figure 3B). Because Akt/mTOR signaling is known to regulate protein synthesis, we assessed the impact of YXM110 on this pathway. Although YXM110 downregulated the activation of eIF4E (Figure 3C), we found that mTOR was not inhibited. Moreover, p70S6K and 4E-BP1 were phosphorylated after YXM110 treatment. These data indicate that YXM110 suppresses protein synthesis without inhibiting mTOR pathway. Interestingly, the protein level of 4E-BP1 was decreased substantially, while the protein became hyperphosphorylated (Figure 3C). To elucidate the impact of YXM110 on mTOR signaling pathway, the cells were treated with YXM110 or rapamycin (a mTOR inhibitor) for short periods of time. After 30 min, 1, 2 or 4 h treatment, YXM110 did not inhibit the phosphorylation status of mTOR and the downstream signaling including p70S6K and 4E-BP1. Rapamycin as a positive control did inhibit the mTOR pathway significantly (Figure 3D). Therefore, we suggest that YXM110 did not affect mTOR signaling pathway.

Apoptotic proteins were found in YXM110-treated tissue (Figure 2B); therefore, we then examined the evidence for apoptosis in vitro by flow cytometry. Although YXM110 did not trigger significant apoptosis within 48 h, apoptotic cells were detected after prolonged incubation (96 h) or at a higher concentration (1 µM) for 72 h (Figure 3E). The intrinsic apoptotic signaling caspase-9 and the effector caspase-3 were activated at 72 and 96 h after the treatment of YXM110, respectively (Supplementary Figure 2A, available at Carcinogenesis Online; Figure 3F).

**YXM110 induces proteasome-dependent degradation of 4E-BP1 and Mcl-1, whereas c-Jun N-terminal kinases mediates 4E-BP1 depletion**

Hyphosphorylated 4E-BP1 traps eIF4E and prevents its association with eIF4G, thereby disturbing the cap-dependent translation process.

**Fig. 2. Anticancer activity of YXM110 as observed in HCT116 xenograft models.** HCT116 cells were injected subcutaneously into the flanks of male nude mice. The mice were divided into four groups (n = 5) and when the tumors reached the average volume (90 mm³), treatment was initiated. The mice were killed on 30 days thereafter. (A) Curves show the mean (±SE) tumor volumes measured within each group. Differences in tumor size between the control and mice treated with 10 mg/kg YXM110 (solid triangles) or 100 mg/kg irinotecan (hollow circles) were statistically
Although 4E-BP1 has been identified as an inhibitor of protein translation in cancer cells, some researchers have recently reported that 4E-BP1 is overexpressed in certain types of cancer and mediates cap-independent translation, which promotes cancer growth (10,22,23). Previous data have shown that YXM110 could deplete protein levels of 4E-BP1 and Mcl-1. It has been shown that, when phosphorylated, 4E-BP1 is degraded (9). We then pretreated cells with the proteasome inhibitor MG132 to block the degradation process, and found that the YXM110-induced 4E-BP1 depletion was reversed. Additionally, the YXM110-triggered decrease of Mcl-1 was recovered by MG132 in HCT116 and also A549 cells (Figure 4A). Moreover, we used the protein synthesis inhibitor cycloheximide to observe the effect of YXM110 on the half-lives of 4E-BP1 and Mcl-1. Figure 4B shows that 4E-BP1, whose protein half-life was shortened by YXM110 was shortened by YXM110 treatment. Interestingly, cycloheximide alone already affected the protein level and phosphorylation status of 4E-BP1, whereas combination with YXM110 did not enhance 4E-BP1 depletion further. These findings revealed that both 4E-BP1 and Mcl-1 depletion are proteasome dependent. Because phosphorylation of 4E-BP1 is critical for its degradation, we screened some possible kinases that may be involved in phosphorylation of 4E-BP1. Mitogen-activated protein kinases have been reported to mediate the phosphorylation status of 4E-BP1. After examining extracellular signal-regulated kinases, c-Jun N-terminal kinases (JNK) and p38, we found that JNK was activated by YXM110, as well as 4E-BP1 was hyperphosphorylated and depleted, within a short time (Figure 4C). To further elucidate the correlation between JNK and 4E-BP1, the JNK inhibitor, SP600125, was introduced to block JNK activation. SP600125 could effectively prevent the hyperphosphorylation and depletion of 4E-BP1 (Figure 4D). As previously reported, hypoxia can trigger cap-independent translation through mTOR inhibition, resulting in 4E-BP hypophosphorylation, and leading to its interaction with eIF4E and subsequent cap-independent translation initiation. We found that YXM110 still downregulated 4E-BP1 levels under hypoxic condition and inhibition of cell survival still occurred (Supplementary Figure 3A and B, available at Carcinogenesis Online). These results proved that the YXM110-induced 4E-BP1 and Mcl-1 depletion were proteasome dependent, and that the total protein level and phosphorylation status of 4E-BP1 was mediated through JNK activation.

**YXM110 regulates autophagy pathway via lysosomal pathway and Mcl-1 downregulation**

Figure 2 shows that autophagic signaling was detected in YXM110-treated tumor tissue. The evidences that YXM110 regulates autophagy were further confirmed in HCT116 and A549 cells in vitro. We first determined the effect of YXM110 on autophagolysosome levels by staining the treated cells with MDC, which could detect the autophagic vacuole (AV). We found that the mean fluorescence increased up to 1.5- and 2.6-fold in HCT116 and A549 cells, respectively, after treatment with YXM110 for 48h (Figure 5A). These data indicated that YXM110 enhances autophagolysosome accumulation. Accumulation of autophagolysosomes could be due to autophagy initiation or impaired degradation of autolysosomes (24). Figure 5B shows the results of RNA array from YXM110-treated HCT116 cells. We observed that RNA levels of 12 proteins in the lysosomal pathway were suppressed by YXM110; this could lead to impaired autophagolysosome degradation.

To further elucidate whether YXM110 could also initiate autophagy, we next examined the conversion and distribution of the microtubule-associate protein 1 LC3. Under normal circumstances, LC3 is evenly distributed throughout the cytoplasm. During autophagy, LC3 undergoes conversion of LC3-I to LC3-II and participates in the formation of autophagosome, forming LC3 puncta. To define the cellular localization of LC3, we transiently transfected HCT116 and A549 cells with GFP-LC3 for 24h and then treated cells with YXM110 for 18h. Figure 5C shows that, in certain YXM110-treated cells, translocated GFP-LC3 formed GFP-LC3 puncta, in contrast with the diffuse pattern seen in non-treated cells. To further verify the autophagic markers induced by YXM110, the conversion of LC3 and the Atg12-Atg5 autophagy-related protein conjugate were also investigated using immunoblotting. Significant induction of LC3-II and Atg12-Atg5 were observed after YXM110 treatment for 12h in HCT116 and after 18h in A549 cells (Figure 5D).

Previous data have shown that YXM110 decreased the protein level of Mcl-1. Mcl-1 has been documented as a stress sensor that interacts with Beclin-1 and prevents the subsequent autophagic signaling (25). In this study, we used transient transfection to overexpress Mcl-1 and clarify the role in YXM110-regulated autophagy. As shown in Figure 5E, when Mcl-1 depletion was abolished, the level of AV accumulation was partially and significantly reversed. On the basis of this result, we suggested that Mcl-1 depletion increased the level of autophagy and contributed to YXM110-induced AV accumulation. These results indicate that YXM110 could activate autophagy via Mcl-1 downregulation and impair the autophagosome degradation by lysosomal effects.

**YXM110-regulated autophagy is involved in cancer cell death**

Recently, the existence of autophagic cell death has been debated because of discrepant findings. In some studies, dysfunctional autophagy led to cell death. We, therefore, verified whether YXM110-regulated, impaired autophagy was involved in YXM110-induced cell death. When the class III PI3K inhibitor 3-MA was used to block autophagy, we found that the YXM110-induced LC3 conversion and AV accumulation were reversed (Figure 6A and B). To further elucidate the impact of autophagy on cell viability, we used MTT assay to measure cell death after cotreatment of cells with YXM110 and 3-MA; we found that YXM110-induced inhibition of HCT116 and A549 cell survival was rescued when combined with 3-MA (Figure 6C). When we used transient transfection to knock down Atg5 protein levels in HCT116 cells, the YXM110-induced inhibition of cell viability was also reversed (Figure 6D). These data indicate that YXM110-induced impaired autophagy is indeed involved in cell death. Taken together, YXM110 could inhibit cell growth and induce cell death through novel mechanisms including depletion of 4E-BP1 and Mcl-1, inhibition of protein synthesis and regulation of autophagy (Figure 6E).

**Discussion**

YXM110 is a novel and potent anticancer agent with in vitro and in vivo effects. Our results demonstrated that the YXM110-induced anticancer effects are associated with the following mechanisms: (i) mTOR-independent inhibition of protein synthesis (Figure 3); (ii) late-onset apoptosis (Figure 3E and F); (iii) proteasome-dependent degradation of 4E-BP1 (Figure 4) and (iv) Mcl-1-related autophagy initiation (Figure 5C and D) and impaired autolysosome degradation (Figure 5A and B). In mice xenograft models, YXM110 significantly inhibited HCT116 tumor volumes without causing apparent body weight loss. Western blotting results of tumor tissue indicated that YXM110 induced apoptosis and autophagy pathways; caspase-9 and poly (ADP ribose) polymerase were cleaved and the autophagy-related Atg12-Atg5 conjugate was increased (Figure 2B). In vitro data also proved that YXM110 could induce both autophagy-associated cell death and apoptosis within 24 and 96h, respectively (Figures 6D and 3E and F).

YXM110 inhibited the protein synthesis pathway and delayed the cell cycle (Figure 1C) without mTOR inhibition and depleted the protein levels of 4E-BP1, an important regulator of cap-independent translation (Figures 3C and 4). Although the eIF4E-binding protein, 4E-BP1, was hyperphosphorylated, instead of being inhibitory form, the activity of eIF4E was still decreased (Figure 3C). Moreover, the protein level of 4E-BP1 was decreased and may impair the 4E-BP1-dependent

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significant (**P < 0.01). Body weight was measured on specific dates from day 1 of administration. There were no significant differences after treatment among any of the groups. (B) Treated and untreated tumor tissues were assessed by western blot for detection of procaspase-9, cleaved caspase-9, Atg12-Atg5, poly (ADP ribose) polymerase and GAPDH.
Anticancer effects of the novel agent YXM110

The cap-independent translation pathway in the hypoxic tumor environment. Ovarian cancer cells become more resistant to paclitaxel because of hypoxia (26). A decrease in cap-independent translation would reduce survival of cancer cells in an oxygen-deprived condition and make them more sensitive to anticancer agents (22). We tested the ability of YXM110 to reduce 4E-BP1 protein levels under hypoxic conditions.

Fig. 3. Protein synthesis is regulated without mTOR inhibition and apoptosis is turned on late by YXM110 in HCT116 cells. (A) HCT116 cells were treated with vehicle (0.1% DMSO) or YXM110 (0.05 or 0.1 μM) for 9, 12 or 18 h. Cells were then harvested for detection of p21, Mcl-1 and 4E-BP1 proteins by immunoblotting. (B) HCT116 cells were incubated with various concentrations of YXM110 (0.03, 0.1 or 0.3 μM) for 30 min, fixed, and assayed using an AHA kit. The percentages of protein synthesis inhibition obtained with each treatment were calculated and compared with the control group. **p < 0.01 and ***p < 0.001 compared with non-treated cells. (C) HCT116 cells were treated with YXM110 (0.3 μM) for the indicated periods and then harvested for detection of protein synthesis-related proteins, viz., p-mTOR, p-p70S6K (T389), p-p70S6K (T421), p4E-BP1 (T37/46), 4E-BP1, p-eIF4E, eIF4E and tubulin by immunoblotting. (D) HCT116 cells were treated with YXM110 (0.3 μM) and Rapamycin (0.1 μM) for shorter periods of time and then harvested for detection of mTOR pathway-related proteins, viz., p-mTOR, p-p70S6K (T389), p-p70S6K (T421), p4E-BP1 (T37/46) and tubulin by immunoblotting. (E) HCT116 cells were incubated with YXM110 (0.3 or 1 μM) for 48, 72 or 96 h. DNA content was subsequently analyzed by PI staining using a FACScan flow cytometric assay. (F) HCT116 cells were treated with various concentrations of YXM110 (0.1–1 μM). After 96 h, cells were harvested for detection of apoptotic signaling-related proteins, viz., caspase-3, poly (ADP ribose) polymerase and their cleaved forms.
conditions; and observed that 4E-BP1 was profoundly decreased by YXM110 under hypoxia (Supplementary Figure 3A, available at Carcinogenesis Online), which implies that cap-independent translation could be blocked by YXM110. Furthermore, compared with paclitaxel, HCT116 cells showed less resistance to YXM110 under hypoxic conditions (Supplementary Figure 3B, available at Carcinogenesis Online). These studies suggested that 4E-BP1 depletion could be beneficial for an anticancer agent development.

In this study, we also found that JNK is responsible for 4E-BP1 phosphorylation within 15 min, which correlates with its degradation.
Fig. 5. YXM110-regulated autophagy pathway is related to Mcl-1. (A) HCT116 and A549 cells were treated with the indicated concentrations of YXM110 for 48 h and stained with MDC dye for 30 min before harvesting. Fluorescently labeled cells were then trypsinized for flow cytometric assay. (B) HCT116 cells were treated with 0.3 μM YXM110 for 24 h, and the gene expression profile was analyzed by Human Whole Genome OneArray®. Transcript level reduced in genes of lysosome pathway by YXM110 is shown as log₂ ratio. (C) HCT116 and A549 cells were seeded onto chamber slides and transfected with GFP-LC3, followed by treatment with the indicated concentrations of YXM110 for 18 h. Cells were then fixed and observed by microscopy. (D) HCT116 and A549 cells were treated with various concentrations of YXM110 (0.03, 0.1 or 0.3 μM) for the indicated time points. Cells were then harvested for detection of LC3, Atg12-Atg5 and GAPDH or tubulin. (E) HCT116 cells transfected with empty vector or Mcl-1 plasmid were then treated with 0.1 μM YXM110 for 24 or 48 h to detect protein levels (upper panel) and MDC stain signaling (lower panel). *P < 0.05 compared with the vector group.
Fig. 6. Autophagy pathway contributes to YXM110-induced cell death. (A) HCT116 and A549 cells were treated with the autophagy inhibitor 3-MA (5 mM) for 18 h, with or without YXM110 (0.1 or 0.3 μM), and the cells were then harvested for detection of LC3 and tubulin. (B) 3-MA (5 mM) was used to treat HCT116 and A549 cells for 48 h, with or without YXM110 (0.1 μM); cells were then trypsinized for further detection of MDC signaling by flow cytometry. ###P < 0.001 compared with non-treated cells, *P < 0.05 and **P < 0.01 compared with cells treated only with YXM110. (C) Cell viability of HCT116 and A549 cells was determined using MTT assay after treatment with YXM110 alone or in combination with 3-MA (5 mM) for 48 h. Cell viability was evaluated using MTT assay and the percentages shown were calculated according to the corresponding control groups. *P < 0.05, **P < 0.01 and ***P < 0.05 compared with the YXM110 single-treated groups. (D) HCT116 cells were transfected with Atg5 siRNA and then harvested for detection of Atg12-Atg5 and tubulin protein (left panel). The Atg5 knockdown HCT116 cells were treated with various concentrations of YXM110 (0.003–0.1 μM) after which cell viability was evaluated by MTT assay (right panel). **P < 0.01 compared with the scrambled siRNA group. (E) The summary of events leading to YXM110-induced anticancer actions.
(Figure 4C and D). Moreover, when JNK activation was inhibited, the degree of phosphorylation of 4E-BP was reduced, whereas the total protein level remained unaffected. Because 4E-BP1 regulates many biological functions, including cap-dependent and independent translation, further investigations are required to elucidate the function of YXM110 in hypoxia-sensitive and in drug-resistance cells.

Autophagy is a process that allows cells to adapt the stress and to avoid cell death; however, in some cellular settings, autophagy offers an alternative pathway to kill abnormal cells. Mcl-1 degradation has been reported as a cellular response under nutrient deprivation and regulates the activation of autophagy (25). By YXM110 treatment, we found that the protein synthesis was affected and the growth rate of cancer cells was decreased. These results would generate a difficult environment for cancer cells to proliferate and survive. As we mentioned previously that Mcl-1 could respond to stressful situation; after YXM110 treatment, the protein level of Mcl-1 was reduced as an early event (Figure 3A). Mcl-1 could bind to Beclin-1 and inhibit the Beclin-1 initiated autophagy. When we overexpressed Mcl-1, the degree of autophagolysosome accumulation was partially reversed (Figure 5E).

Therefore, Mcl-1 contributes to YXM110-induced autophagy. Moreover, RNA array data revealed that YXM110 decreased the mRNA level of several lysosomal proteins, which is important for lysosomal degradation (Figure 5B), and this action may contribute to impaired autophagolysosome clearance. Impaired autophagy pathway could cause autophagolysosome accumulation and this may explain the remained level of MDC signaling when overexpression of Mcl-1.

The concept of autophagic cell death has been controversial. Although autophagy enhances cell survival in response to some stressful circumstances, autophagy could also play a role leading to cell death. Several literatures have observed that prolonged, excessive or incomplete autophagy results in cell death. Laane et al. (27) have reported that dexamethasone induce cell death through autophagy initiation; Mujumdar et al. (28) also interpreted that Triptolide trigged cytochrome c release, which showed the role of autophagy in cell viability. YXM110 triggered autophagy in late phase (72–96 h) and combination with pan-caspase inhibitor Z-VAD-FMK could not reverse the cell death in 48 h (Supplementary Figure 2B, available at Carcinogenesis Online).

Also when given 3-MA and Atg5 silencing attenuated the YXM110-induced cell death (Figure 6C and D), which showed the role of autophagy in cell viability.

In summary, this study has identified that YXM110 inhibits cancer cell growth and induces cell death in human cancer cells both in vitro and in vivo; the overall mechanisms involve protein synthesis inhibition, proteasome degradation, autophagosome accumulation, autophagic cell death and apoptosis. The tumor suppression with autophagy and apoptosis was also observed in YXM110-treated HCT116 xenograft models. YXM110 also exhibits excellent anticancer activity in multiple drug-resistant cell line KBvin. These observations provide a new insight to understand the mechanisms underlying this novel structure and make YXM110 an attractive anticancer agent against a broad spectrum of cancers.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References


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