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Facile synthesis of autophagonizer and evaluation of its activity to induce autophagic cell death in apoptosis-defective cell line

Jennifer Nguyen, Luxi Chen, Dhiraj Kumar, Jiyong Lee *

Department of Chemistry and Biochemistry, University of Texas at Dallas, 800 W. Campbell Rd., Richardson, TX 75080, USA

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ABSTRACT

Some cancer cells are resistant to apoptosis, rendering them irresponsive towards apoptosis-inducing chemotherapy drugs. Another mode of action to kill these apoptosis-defective cells is essential and autophagy, a dynamic process that degrades cytoplasmic contents for cellular maintenance, has been considered as one of the alternate routes. A small molecule inducer of autophagy, autophagonizer was reported to induce cell death through a novel process that is independent of extrinsic apoptosis and the normal signaling pathways of autophagy. Here, we describe an efficient synthetic procedure for the autophagonizer. The newly synthesized autophagonizer (DK-1-49) resulted in an accumulation of autophagy-associated LC3-II and enhanced levels of autophagosomes and acidic vacuoles. Furthermore, cell viability was inhibited by autophagic cell death in not only human cancer cells but also Bax/Bak double-knockout cells. These findings highlight that intrinsic apoptosis is not also involved in the induction of cellular death by the autophagonizer suggesting the autophagonizer is a promising candidate for anticancer therapeutics for cancer cells that are resistant to apoptosis-inducing chemotherapy.

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Autophagy is a homeostatic process that involves the rearrangement of membrane to sequester intracellular contents into double-membrane vesicles called autophagosomes. The cargo is then transferred into lysosomes for degradation by acidic hydrolases and recycled to sustain cellular metabolism.[1,2] Increasing evidence suggests for the involvement of autophagy in tumorigenesis. In 40–75% of human breast, prostate, and ovarian cancer, the autophagy-essential gene *beclin 1* is monoallelically deleted.[3] Partial disruption of *beclin 1* decreases autophagy and increases the cell's susceptibility to developing into spontaneous tumors such as lymphomas, lung carcinomas, hepatocellular carcinomas, and mammary precancerous lesions.[4] While reduced levels of beclin 1 increase the likelihood of cancer development, elevated levels of beclin 1 result in autophagic cell death.[5–8] Since autophagy is commonly associated as a survival strategy, its main role in tumorigenesis remains controversial and the signaling pathways from the onset of autophagy to cellular death remain unclear.[9,10]

Nevertheless, the prospect of actuating cellular death through the induction of autophagy has attracted considerable attention as a potential approach towards cancer treatment. Current chemotherapy drugs are ineffective to some cancer because they are resistant to a programmed cell death called apoptosis.[11–14] This resistance may be due to DNA mutation in essential apoptosis genes, overexpression of anti-apoptosis genes, and/or silencing of pro-apoptotic genes.[14–16] The capability of these cancer cells to evolve and adopt intrinsic survival strategies is an obstacle that current researchers face when developing cancer-killing drugs that rely on apoptotic pathways. Hence, another mode of action to kill these apoptosis-defective cells is highly desirable.

Cell death by excess autophagy has become one of the main contenders as an alternate therapeutic strategy against cancer. Natural alkaloid molecules,[17] clozapine,[18] arsenic trioxide,[19] resveratrol,[20] apigenin,[21] lapatinib,[22] and histone deacetylase inhibitors have demonstrated to induce autophagic cell death and inhibit cell growth in human cancer cell lines.[23] A novel small molecule called autophagonizer was recently reported to induce autophagic cell death via a unique mechanism that diverges from the traditional pathways of autophagy.[24] Various cancer cell lines were shown to exhibit sensitivity to autophagonizer. EC_{50} of the autophagonizer-induced autophagic cell death was estimated to be in the range of 3–4 μM. Intriguingly, autophagonizer was described to promote such activity without confiding in apoptotic machinery, particularly the machinery involved in the extrinsic pathway.[24] Because cancer cells develop insensitivity to apoptosis in varying degrees, current cancer research aims to develop anti-cancer drugs that are ideally independent of both extrinsic and intrinsic apoptosis.[11–14] Therefore, we desire to promote

* Corresponding author.
E-mail address: jiyong.lee@utdallas.edu (J. Lee).

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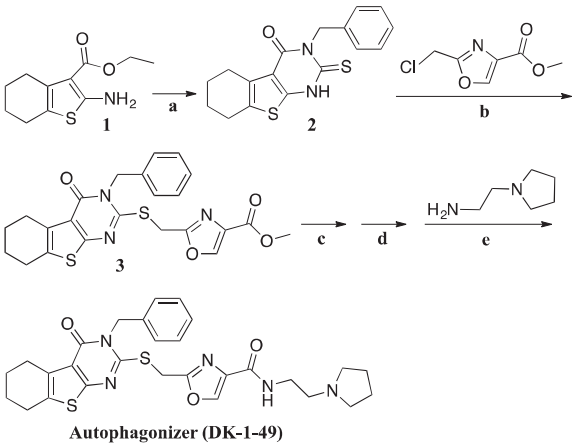
autophagonizer as a potential therapeutic drug by investigating its unique activity in regards to not only autophagy but also intrinsic apoptosis. In order to achieve this goal, we have developed the first synthetic procedure of the autophagonizer because a synthetic procedure of the compound has not been reported yet. The newly synthesized autophagonizer was then tested on cancer cells and apoptosis-defective cells for autophagic activity and cell viability. Studying the autophagonizer and its peculiar mode of action not only allows for more discoveries of potential cancer therapeutic drugs but also brings a new understanding to the complexities of cellular survival and death.



Scheme 1. Synthesis of autophagonizer (DK-1-49). Reagents and conditions: (a) BnNCS, KOH, EtOH, Reflux, 16 h, 90%; (b) K_2CO_3, THF, RT, 16 h, 70%; (c) 1 M LiOH, THF/MeOH (9:1), RT, 16 h, 94%; (d) Oxalyl chloride, DCM, DMF (cat.), RT, 4 h, quantitative; (e) TEA, THF, RT, 16 h, 55%.

The synthetic pathways depicted in Scheme 1 outline the chemistry of the present study. Ethyl 2-amino-4,5,6,7-tetrahydrobenzo [*b*]thiophene-3-carboxylate (**1**) was easily prepared from the well-established procedure.[25] Compound **1** was then converted to the corresponding benzylthiourea by reaction with benzylisothiocyanate in ethanol, which were cyclized using potassium hydroxide to give compound **2**. Compound **2** underwent nucleophilic substitution with methyl 2-(chloromethyl)oxazole-4-carboxylate[26] under basic condition to afford compound **3**, which was hydrolyzed and converted to acid chloride intermediate which was finally coupled with *N*-(2-aminoethyl)pyrrolidine giving target compound autophagonizer (DK-1-49). The overall yield to prepare the autophagonizer from the starting material (**1**) was 33%.

In order to validate biological activity of the newly synthesized autophagonizer (DK-1-49), the compound was then tested on HeLa cervical cancer cells for autophagic cell death activity. After 24 h treatment of HeLa cells with DK-1-49 at various dosages, autophagosomes and DNA were stained with Cyto-ID reagent and Hoechst nuclear stain, respectively (Fig. 1A). Fluorescence images reveal that DK-1-49-treated cells exhibited higher fluorescent intensity by Cyto-ID-labeled autophagosomes compared with vehicle control (DMSO)-treated cells, suggesting that autophagy was induced upon treatment with DK-1-49. A sigmoidal dose-response curve indicates that DK-1-49 promotes autophagosome formation in a dose-dependent manner, and the EC_{50} was approximated to be 3.5 μM (Fig. 1B). Monodansylcadaverine (MDC), a specific marker for acidic vacuoles, was also used to confirm the induction of autophagy in DK-1-49-treated cells. Prominent punctate patterns of blue fluorescence representing the presence of acidic vacuoles were observed (Fig. 1C), further supporting autophagic activity by DK-1-49. The EC_{50} was calculated to be approximately 1.3 μM (Fig. 1D). Immunoblot analysis was finally utilized to assess the conversion of light chain 3-I (LC3-I) for additional validation of DK-1-49 for its autophagy induction activity. Upon

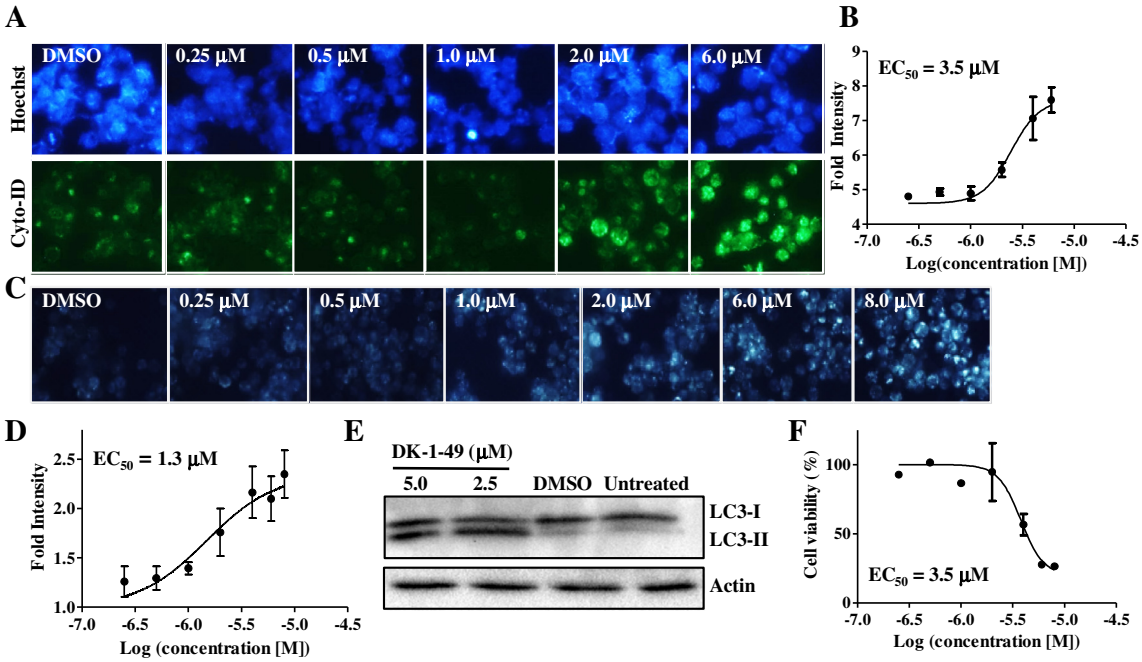


Figure 1. Autophagonizer (DK-1-49) induces autophagic cell death of cancer cells. HeLa cells were treated with DK-1-49 for 24 h at various dosages. (A) Treated cells were stained with the Hoechst 33342 nuclear stain and green Cyto-ID reagent. Images were taken under the DAPI and GFP filter. (B) Fluorescence was read at 340/480 nm (Ex/Em) for Hoechst nuclear staining and 480/539 nm (Ex/Em) for Cyto-ID autophagosome staining. Fluorescence per cell was taken relative to DMSO-treated cells and used to construct a sigmoidal dose–response curve. (C) Treated cells were stained with MDC for acidic vacuoles. Images were taken under the DAPI filter. (D) MDC fluorescence was read at 335/512 nm (Ex/Em). Fluorescence was first normalized against the number of viable cells measured by MTT assay. Fluorescence per cell was then taken relative to DMSO-treated cells to obtain fold intensity and a sigmoidal dose–response curve was constructed. (E) Immunoblot analysis for conversion of LC3-I to LC3-II. (F) MTT cell viability assay. The number of viable cells was normalized against DMSO-treated cells to obtain the percentage of viable cells.

activation of autophagy, the microtubule-associated form of LC3, LC3-I, is converted to the autophagy-associated form, LC3-II.[1] After 24 h of treatment with DMSO or DK-1-49, changes in LC3 levels were evaluated (Fig. 1E). DK-1-49-treated cells exhibited an accumulation of LC3-II while DMSO-treated cells exhibited no significant conversion of LC3-I to LC3-II. Note that the increase of MDC fluorescence and LC3-II accumulation of cancer cells by DK-1-49 was consistent with the previous Letter.[24]

The autophagonizer was originally reported to induce autophagic cell death. To validate that our autophagonizer exerts similar cytotoxic effects, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay was employed. MTT results showed that cell death occurred in cells after 24 h treatment with DK-1-49 (Fig. 1F). The percentage of viable cells dramatically declined by 5-fold after compound treatment at a dosage of 8 μM. From the dose–response curve, EC_{50} was estimated to be 3.5 μM, which is similar to the previously reported value.[24] Taken together from these results of autophagic activity and cell viability, the DK-1-49 synthesized using the scheme described above was validated to induce autophagic cell death in cancer cell line.

Autophagonizer was previously reported to undergo a mechanism that is unique from the traditional pathways towards the onset of autophagy. In order to gain additional insight, we are currently performing structure–activity relationship (SAR) analysis. Derivatives of autophagonizer were generated, each bearing a modification in various regions of the structure. Additional detailed studies on SAR are necessary and will be reported in due course. However, we have identified a less-active derivative of the autophagonizer, DK-1-71 (Fig. 2A). The nitrogen of pyrrolidine moiety of DK-1-49 was replaced with a carbon in DK-1-71. This modification resulted in a marked reduction in autophagic cell death activity in HeLa cells. No sufficient increase in MDC fluorescence was observed in cells treated with DK-1-71 compared to cells treated with DK-1-49 (Fig. 2B), suggesting that DK-1-71 induces weaker autophagy. This was also reflected by DK-1-71's autophagic cell death activity as the EC_{50} of the compound was calculated to be approximately 25 μM, about 7-folds higher than DK-1-49's EC_{50} of 3.5 μM (Fig. 2C). Because DK-1-71 proves to be less active in inducing autophagic cell death than DK-1-49 after the replacement of the nitrogen of pyrrolidine moiety, DK-1-71 was assessed alongside DK-1-49 in the following studies.

An obstacle to chemotherapy is some cancer cells' resistance against apoptosis, rendering them unresponsive to anti-cancer drugs that target the apoptosis pathway. In order to determine whether DK-1-49 holds suppressive effects against apoptosis-defective cells through autophagic cell death, Bax/Bak double-knockout (DKO) mouse embryonic fibroblasts (MEFs) were used.[27] Bax and Bak are pro-apoptotic Bcl-2 family members that permeabilize mitochondria to induce apoptosis.[28] Therefore, Bax/Bak DKO MEFs are defective in activating mitochondrial intrinsic apoptosis. We have expected that, if DK-1-49 induces cell death through the intrinsic apoptosis pathway, DK-1-49 may induce less cell death in Bax/Bak DKO MEFs compared to wild type MEFs. After 15 h of treatment, wild-type and DKO MEFs were stained with MDC and Cyto-ID reagent (Fig. 3A). Upon treatment with DK-1-49, an accumulation of acidic vacuoles and autophagosomes was observed in both wild-type and DKO MEFs. Furthermore, LC3-I to LC3-II conversion was detected by Western blot, confirming that autophagy was induced in DK-1-49-treated cells (Fig. 3B). The wild-type and DKO MEFs were subsequently assessed with MTT assay for effects on cell viability (Fig. 3C). DK-1-49 was shown to have potent cytotoxicity in both wild-type and DKO MEFs. The apparent EC_{50} calculated from the dose-response curve for the wild-type and DKO MEFs were essentially identical, 2.1 μM and 2.2 μM respectively. These results further suggest DK-1-49's potentiality as an anti-cancer drug against apoptosis-defective cells. As mentioned previously, DK-1-71 stimulates weaker autophagy compared to DK-1-49 in HeLa cells. Similar results were observed in both wild-type and DKO MEFs. Minimal MDC and Cyto-ID fluorescence were seen (Fig. 3A) and low conversion of LC3-I to LC3-II was detected (Fig. 3B). Additionally, cytotoxic effects of DK-1-71 were weaker than those of DK-1-49 by approximately 10-fold in both wild-type cells and apoptosis-defective cells. The EC_{50} was determined to be 28.6 μM and 24.2 μM respectively (Fig. 3C). These results on apoptosis-defective MEFs further accentuate that the modification of the pyrrolidine nitrogen to a carbon markedly reduced autophagic cell death, suggesting that this nitrogen may be vital for autophagonizer activity. According to a previous study, autophagonizer promotes cell death in cancer cells even under the presence of Z-VAD-FMK, a pan-caspase inhibitor.[24] This finding implicates that the extrinsic pathway of apoptosis is not involved in the mechanism towards autophagonizer-induced cell death. Based on our results regarding Bax/Bak DKO cells, the intrinsic pathway of apoptosis is also not involved because cell death still occurred after treatment with the autophagonizer.

In summary, in order to gain a further understanding of autophagonizer-induced autophagic cell death, we have successfully developed the first synthetic procedure of the autophagonizer. The synthesized autophagonizer (DK-1-49) was validated to induce autophagic cell death in cancer cells as similarly described in the previous study. We have also shown that DK-1-49 can induce cell death in Bax/Bak DKO MEFs in which the intrinsic apoptosis was disabled. This result, combined with the result in

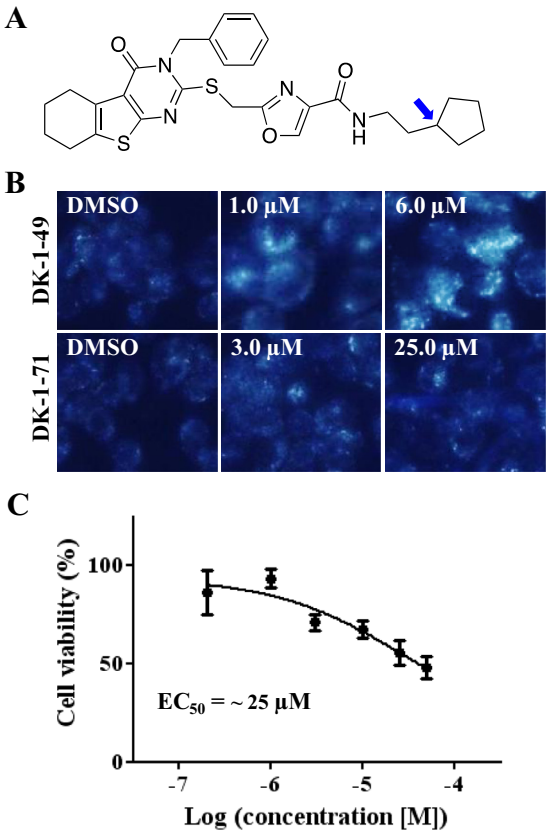


Figure 2. Identification of the less active form of autophagonizer, DK-1-71. (A) Structure of DK-1-71. The arrow indicates the site of modification of DK-1-49 in DK-1-71. (B) After 24 h treatment, cells were stained with MDC for acidic vacuoles. Images were taken under the DAPI filter. (C) Cytotoxicity of DK-1-71 in HeLa cells was measured by MTT assay. Viable DK-1-71-treated cells were normalized against DMSO-treated cells to obtain the percentage of viable cells.

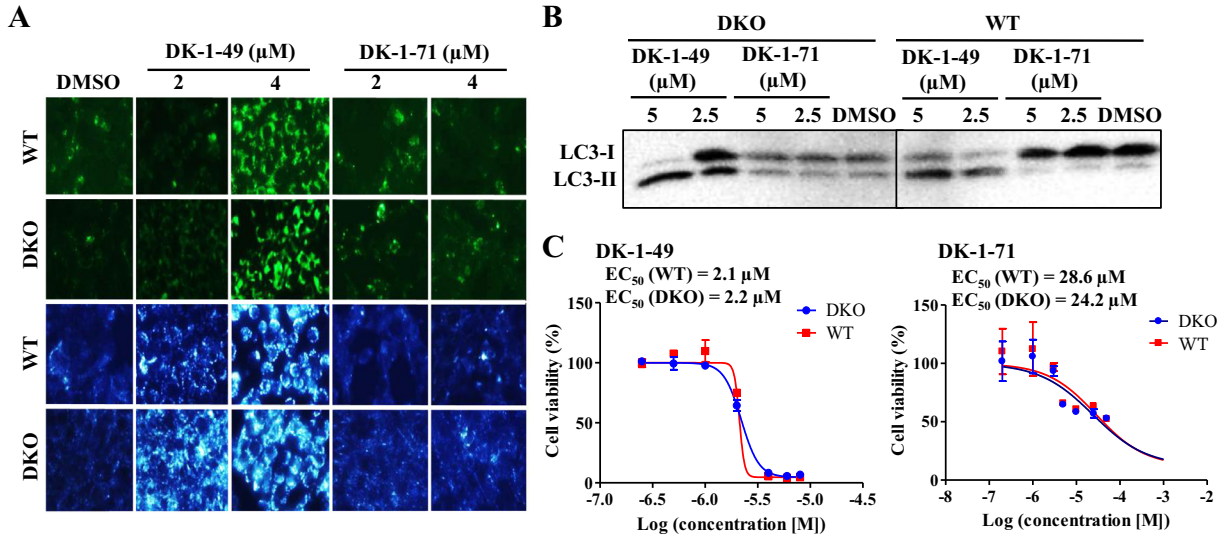


Figure 3. Autophagic cell death of apoptosis-defective cells by DK-1-49. Wild-type (WT) and Bax/Bak double knockout (DKO) MEFs were treated for 15 h with DK-1-49, DK-1-71, and vehicle control (DMSO) at the indicated concentrations. (A) Treated cells were stained with Cyto-ID (green fluorescence) reagent for autophagosomes and MDC (blue fluorescence) for acidic vacuoles. (B) Immunoblot analysis for the conversion of LC3-I to LC3-II. (C) Cytotoxicity of DK-1-49 and DK-1-71 in wild-type and Bax/Bak DKO MEFs. Cell viability was measured by MTT assay and normalized against DMSO-treated cells to obtain the percentage of viable cells.

the previous study that showed that extrinsic apoptosis is not involved in cell death induced by autopagonizer,[24] has led us to conclude that autophagonizer can induce cell death in apoptosis-resistant cells. This revelation further underscores the value of autophagonizer for cancer treatment for cancer cells that are resistant to apoptosis-inducing chemotherapy because the molecule can promote cell death through autophagy without the activation of either the intrinsic or extrinsic pathways of apoptosis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.035. These data include MOL files and InChiKeys of the most important compounds described in this article.

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