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

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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.

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

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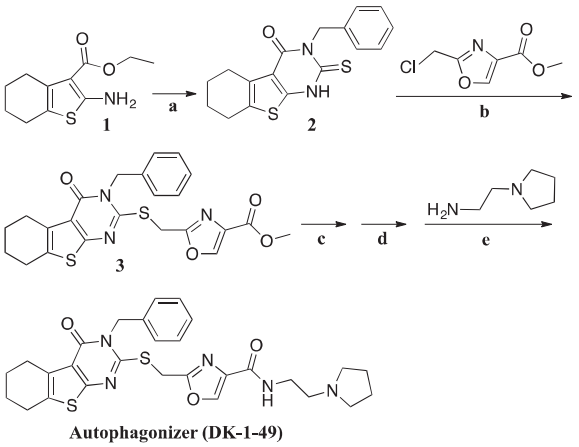
autophagonizer as a potential therapeutic drug by investigating its unique activity in regards to not only autophagy but also intrinsic apoptosis. In order to achieve this goal, we have developed the first synthetic procedure of the autophagonizer because a synthetic procedure of the compound has not 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

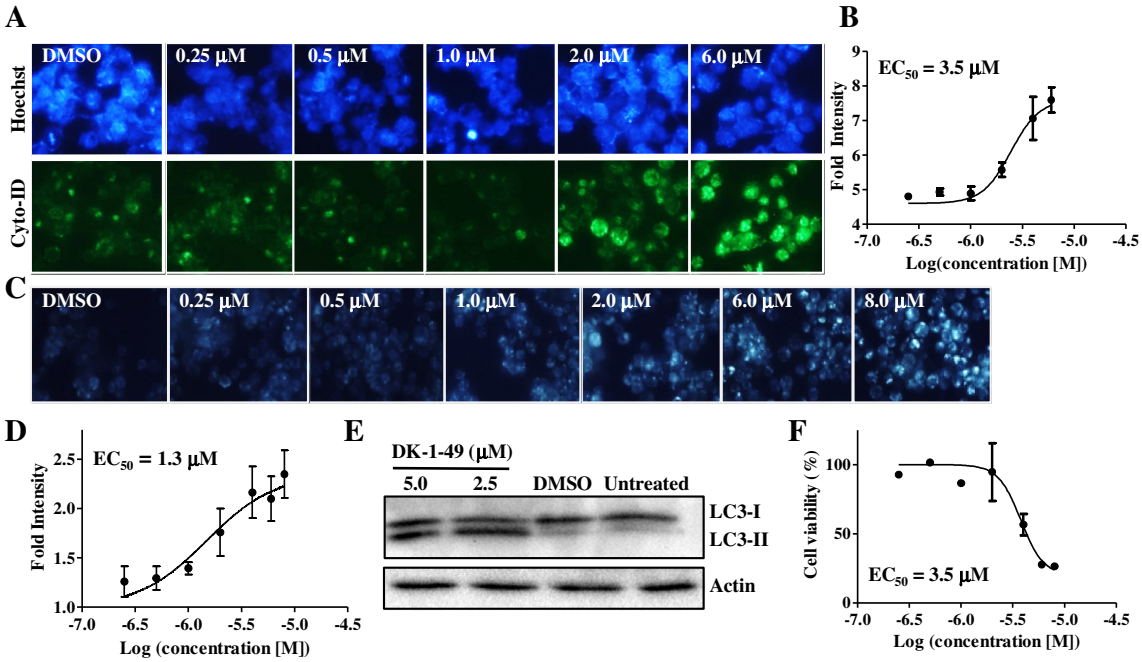


Figure 1. Autophagonizer (DK-1-49) induces autophagic cell death of cancer cells. HeLa cells were treated with DK-1-49 for 24 h at various dosages. (A) Treated cells were stained with the Hoechst 33342 nuclear stain and green Cyto-ID reagent. Images were taken under the DAPI and GFP filter. (B) Fluorescence 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.