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Esterase Activatable Synthetic M^+/Cl^- Channel Induces Apoptosis and Disrupts Autophagy in Cancer Cells

Javid Ahmad Malla,[a] Virender Kumar Sharma,[b] Mayurika Lahiri[b] and Pinaki Talukdar[*,a]

Abstract: The supramolecular esterase activatable synthetic M^+/Cl^- ion channel is reported which can form self-assembled rosette ion channel in the bilayer membrane by the action of esterase enzymes. The channel formation switches the cancer cells to apoptosis via an intrinsic pathway. The more interesting feature is that along with apoptosis this system disrupts autophagy in cancer cells via alkalization of lysosomes, which was confirmed through cell imaging and western blot analysis.

Cell death plays a pivotal role in the normal pathophysiological processes in the biological systems. Dysregulation of cell death results in pathogenesis of a wide range of diseases like neurodegenerative diseases and cancer.[1] Three classical forms of cell death include necrosis, apoptosis, and autophagy. The necrosis is often considered as accidental cell death while as apoptosis is a well-known programmed pathway to regulate normal functioning by wiping out the damaged cells.[2] In contrary autophagic cell death (ACD) refers to cell death that is inhibited *via* specific blockage of the autophagic pathway. The cancer cells have decreased apoptosis rate and high autophagy rate than normal cells, helping cancer cells to proliferate at high rate. The anticancer treatments engage autophagy as a cytoprotective answer in response to toxic dose to mitigate cellular stress, whereas in some cases the results are opposite. So, the apoptosis-inducing agents such as e.g. obatoclax, cannabinoids, etc which target autophagy have great applications to evade cancer-related problems.[3],[4] Obatoclax is an analog of prodigiosin, and its mechanism of action is linked to the alkalization of lysosomes, thereby, disturbing the autophagy process.[5] The synthetic ion transporters have apoptosis inducing activity in cancer cells.[2b, 6] However, synthetic transporters that play a dual role in inducing apoptosis and disrupting autophagy are very rare.[2b, 7]

Very recently, considerable efforts have been invested in the design of stimuli-responsive ion transporters aiming to target cancer cells selectively. In this regard pH,[8] light,[9] enzymes,[10] glutathione,[11] etc. have drawn significant attention. Rotello and coworkers reported that the site of activation of prodrugs is very important in the development of biomedicine.[12] According to them, the extracellular activation is important when cell impermeable substrates are used as prodrugs and are activated by extracellular machinery. In contrast, intracellular activation is required when intracellular machinery is targeted for activation. Jeong and coworkers reported a series of glycoside tethered urea molecules as enzyme-responsive procarriers.[10] However, the crucial limitation of these systems is associated with their choice of enzymes for activation. It is well-known that the levels of intracellular glycosidases are higher compared to their extracellular levels.[13] As a result, the release of active transporters was done by adding the respective enzymes in the extracellular media. Moreover, if applied *in vivo* these water soluble protransporters are expected to be excreted easily by the kidney.

Therefore, we considered a strategy for activating amphiphillic protransporters by intracellular enzyme, and selected esterase enzyme for activating the synthetic channel-mediated ion transport. This enzyme is overexpressed in cancer cells,[14] and therefore, applied significantly in the activation of prodrugs and drug delivery.[15] The inactive form **1** of the channel forming molecule was designed by connecting methyl pivalate at the C-2 position of N^1,N^3-dihexyl-2-hydroxyisophthalamide **2** so that the system fails to proper self-assembly (Figure 1). We have recently demonstrated the ion channel formation by **2** activated by glutathione.[11a] The ion channel activation by glutathione was confirmed using MCF-7 and *INS-1E* cells. The detailed mechanistic study corroborated to the apoptosis of MCF-7 cells due to the disruption of ion homeostasis. Therefore, we realized that upon reaction with intracellular esterases, methyl pivalate moiety of compound **1** would be cleaved[16] releasing **2** that would form self-assembled M^+/Cl^- ion channels in the membrane resulting in cancer cell death via the perturbation of ion homeostasis. Therefore, the strategy would

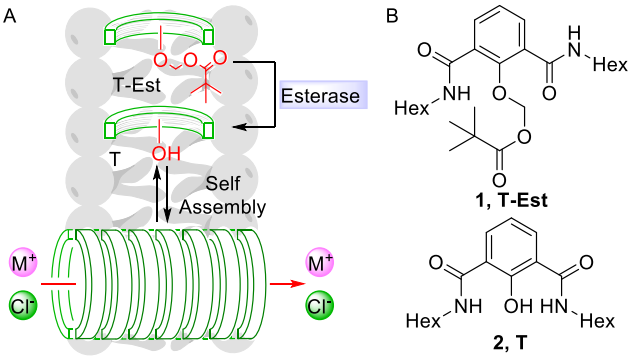

Figure 1. Representation of esterase activatable M^+/Cl^- channel (A). Structure of inactive ester form **1** (**T-Est**) and channel forming form **2** (**T**) (B).

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provide a new being higher in lysosome compared to the cytoplasm,[17] an activation of such ion transport across lysosomal membranes would also perturb the functions of these organelles. So the channel can act as a double-edged sword for inducing apoptosis and disrupting autophagy in cancer cells.

The synthesis of **1** was carried out by reaction of compound **2** with iodimethyl pivalate presence of triethylamine[18] (Scheme S1, ESI). The compound was characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry.

After the successful synthesis of compound **1**, the release of compound **2** from **1** upon enzymatic cleavage was studied using ^{1}H NMR studies. The compound **1** (1 mM) in phosphate buffer saline (PBS) was treated with 4 U/mL of porcine liver esterase enzyme (Sigma Aldrich) prepared in PBS at pH 7.2 for different time intervals. The disappearance of the peak for $H_{b'}$ at δ = 5.6 ppm, the shift of peak for H_a at δ = 8.0 ppm to 7.98 ppm as well as the reappearance of the peak for H_c around 14.4 ppm was monitored and it was found that almost 95% of the cleavage takes place within three hours of reaction time (Figure 2). These results suggest the release of **2** from compound **1** is a very feasible process when incubated in cells due to higher expression of esterases.

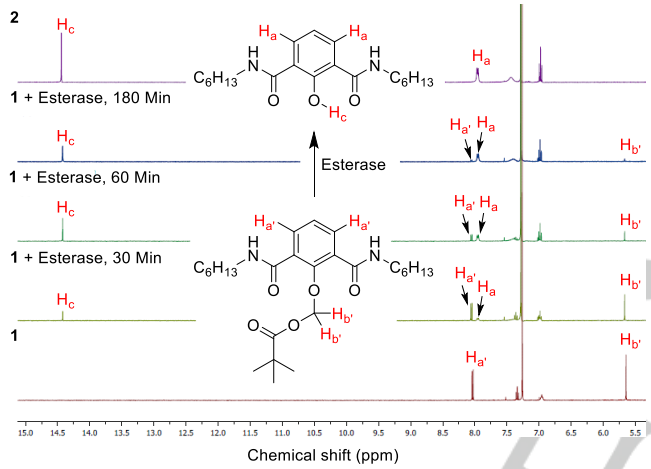

Figure 2. Esterase mediated release of **2** from **1** monitored by ^{1}H NMR experiments.

The ion transport properties of **1** and **2** were evaluated using lucigenin based fluorescence assay using large unilamellar vesicles (LUVs) composed of egg-yolk phosphatidylcholine (EYPC).[19] The compound **1** was found to be almost inactive than compound **2** for ion transport at identical concentrations (Figure 3). However, upon treatment with esterase enzyme, the transport activity was regained showing the efficient release of the active channel forming compound **2** from **1**. These studies encouraged us to further carry out the cellular experimentations. The compound **1** was incubated in the human epithelial breast cancer cells MCF-7, for 30 min and then the cells were observed under a confocal microscope (Leica SP8). The results suggested that there is an enhancement of blue fluorescence in the cells (in the cytoplasm and near the membrane) incubated with **1** compared to control (Figure 4) suggesting that compound **2** gets released from **1** by intracellular esterases.

The results of the cell-imaging assay encouraged us to evaluate the cell viability of cancer cells in the presence of compound **1**. MCF-7 cells and HeLa cells were incubated with compound **1** in a dose-dependent manner for 24 hours and then the cell viability was evaluated by MTT assay.[6b] The results suggested that compound **1** displayed enhanced cytotoxicity with increase in concentration of the compound giving IC_{50} values of 15 μM and 20 μM for MCF-7 and HeLa cells respectively. The higher IC_{50} value of the esterase activatable compound (IC_{50} value of 15 μM for MCF-7 cells) compared to our previously reported glutathione activatable compound (IC_{50} value of 1 μM for MCF-7 cells)[11a] may be corroborated to (i) better permeability of the 2,4-dinitrobenzenesulfonyl (DNS) linked protransporter and (ii) better cleavability of DNS group (*i.e.*, faster activation. The effect of *in situ* generated by-products (formaldehyde and pivalic acid as 4-methoxyphenol is proved to be non-toxic to cells)[20] on cell viability was evaluated independently in MCF-7 cells and the compound **6** showed negligible cytotoxicity compared to **1** (Figure S2).

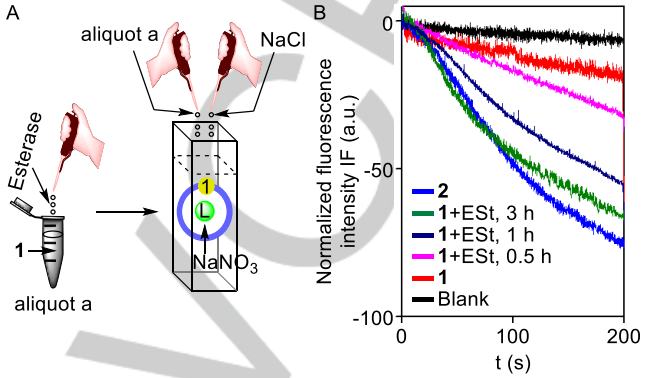

Figure 3. Schematic representation of assay (A) and comparison of Cl^- ion influx into EYPC-LUVs⊃lucigenin by compounds **2** and **1** treated with esterase enzyme for specific time intervals (B).

The above results encouraged us to further evaluate the mechanism of cell death *i.e.*, whether cell death is being mediated by necrosis or apoptosis. We evaluated the apoptotic pathway starting with monitoring the mitochondrial membrane potential (MMP) change on treated cells using JC-1 dye.[21] The enhanced green fluorescence in treated cells compared to control cells indicated that the MMP is damaged (Figure 5A, B, Figure S3). This change in MMP leads to disturbances in the electron transport chain in the mitochondrial respiratory cycles, which ultimately results in the generation of reactive oxygen species (ROS).[22] Therefore, we monitored the ROS produced in the mitochondria using mitosox[23] (Figure S4) and overall cellular ROS using H_2DCFDA probes[24] (Figure S5, S6). These results indicated the production of ROS in the cells treated with compound **1**.

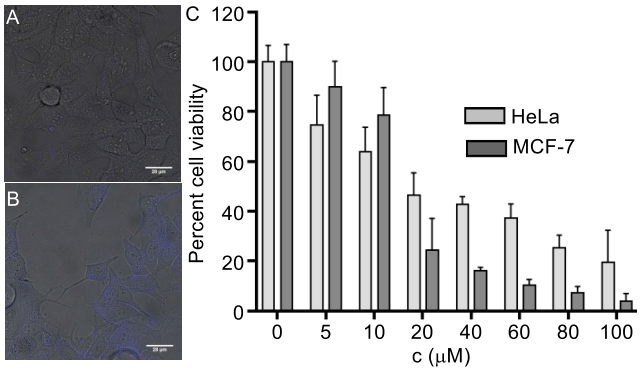

Figure 4. Live cell imaging of MCF-7 cells upon incubation with 0 μM (A) and 15 μM (B) of **1**. Dose-dependent viability of MCF-7 and HeLa cells incubated with **1** evaluated by MTT assay (C).

It is reported that the intrinsic apoptotic pathway initiates from the mitochondria and releases cytochrome c which binds to the Apaf-1 to form an apoptosome.[6b] The apoptosome activates the caspase 9 pathway, which switches the cells to apoptosis. Therefore, to confirm the apoptotic pathway is functional we

probed the activation of the caspase 9 pathway using immunoblot analysis of MCF-7 cells upon treatment with compound **1** (15 μM). GAPDH was used as the loading control. The results showed an increase in expression of cleaved caspase 9 (Figure 5E), which confirms the activation of the intrinsic pathway of apoptosis. To further support, the expression of cleaved poly(ADP-ribose) polymerase (PARP) was also monitored, which showed a significant amount of degradation of full-length PARP-1 (116 KDa) with a concomitant increase of cleaved PARP-1 (86 kDa)[25] (Figure 5F) supporting activation of the caspase 9 pathway of apoptosis.

The chloride concentration in lysosomes (80 mM) maintains the lysosomal pH (4.5-5.0),[17] and any disturbance in this chloride concentration leads to a change in the lysosomal pH, which causes the disturbance in the activity of the lysosomal enzymes, leading to autophagy disruption.[26] Therefore, to evaluate the effect of compound **1** on lysosomes, we first monitored the pH of lysosomes of treated cells using acridine orange (AO) dye.[27] AO is a cell-permeable dye which accumulates in acidic compartments, such as lysosomes, and shows a characteristic orange fluorescence emission while as emits green fluorescence at higher pH, such as in the cytosol. Upon treatment with **1** followed by AO staining the MCF-7 cells showed complete loss of granular orange fluorescence (Figure 5G) compared to control (Figure 5F), indicating the alkalization of lysosomes.[28]

The alkalization is known to be associated with a decrease in the activity of lysosomal enzymes, hence, autophagy.[7] Therefore, we probed the effect of compound **1** on autophagy using immunoblot analysis. One of the markers of autophagy is Lamin B1, which decreases during autophagy.[29] The treatment of MCF-7 cells with **1** (15 μM) for 24 hours showed a marked decrease in the expression of Lamin B1 (Figure 5H) compared to control, indicating that autophagy is being induced. To further corroborate this data, the expression of microtubule-associated protein 1 light chain 3 (LC3) was monitored. Typically increased expression of LC3 indicate the induction of autophagy in cells.[30] The treatment of MCF-7 cells with **1** showed increased expression of LC3 (Figure 5I) compared to control. Together, these results suggest the activation of the autophagy process upon treatment with compound **1**.

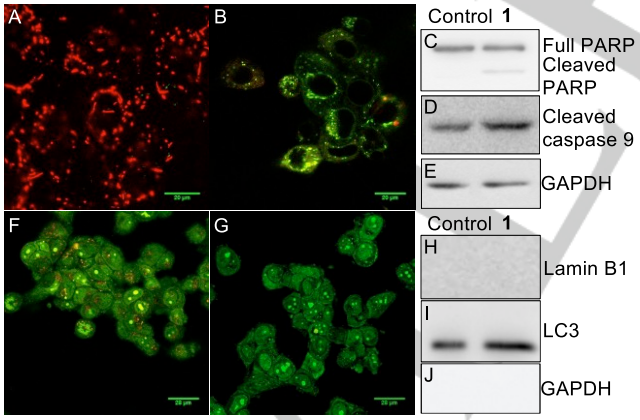

Figure 5. Live cell imaging of MCF7 cells upon treatment with 0 μM (A) and 15 μM (B) of **1** for 24 h followed by staining with JC-1 dye. Red and green channel images were merged to generate the displayed image. Immunoblot assay for PARP cleavage (C) in MCF7 cells, and active caspase 9 (D) after 24 h incubation with 0 μM and 15 μM of **1** with GAPDH as a loading control (E). Live cell imaging of MCF 7 cells incubated with **1** at 0 μM (F), and 15 μM (G) concentrations followed by staining with acridine orange. Immunoblot assay for a decrease in L amin B1 expression (H) and an increase in LC3 expression (I) in MCF 7 cells, with GAPDH as a loading control (J).

In summary, we have reported the novel concept of enzyme activatable synthetic ion channel which can be activated by intracellular esterase enzymes. The successful activation of ion channel inside cells leads to ion transport across the plasma membrane, which switches the cells to apoptosis through the mitochondrial pathway. Furthermore, this transport can dissipate the pH gradient of the lysosomes, which leads to lysosomal dysfunctions, and ultimately leads to autophagy disruption in MCF 7 cells.

Acknowledgements

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Keywords: Supramolecular chemistry • Ion Channel • Enzyme responsive • Apoptosis • Autophagy

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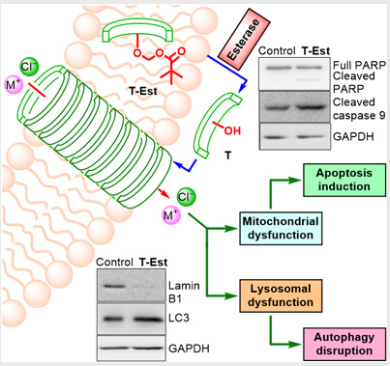
Esterase cleavage of a protransporter leads to ion channel formation in membrane. The activation causes apoptosis and disrupts autophagy in cancer cells.



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