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Abstract. KillerRed is a unique phototoxic red fluorescent protein that can be used to induce local oxidative stress by green-orange light illumination. Here we studied phototoxicity of KillerRed targeted to cytoplasmic surface of lysosomes via fusion with Rab7, a small GTPase that is known to be attached to membranes of late endosomes and lysosomes. It was found that lysosome-associated KillerRed ensures efficient light-induced cell death similar to previously reported mitochondria- and plasma membrane–localized KillerRed. Inhibitory analysis demonstrated that lysosomal cathepsins play an important role in the manifestation of KillerRed-Rab7 phototoxicity. Time-lapse monitoring of cell morphology, membrane integrity, and nuclei shape allowed us to conclude that KillerRed-Rab7-mediated cell death occurs via necrosis at high light intensity or via apoptosis at lower light intensity. Potentially, KillerRed-Rab7 can be used as an optogenetic tool to direct target cell populations to either apoptosis or necrosis. [DOI: 10.1117/1.JBO.19.7.071403]

Keywords: green fluorescent protein; photosensitizer; phototoxicity; cell death; fluorescence microscopy.

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1 Introduction

Photosensitizers are dyes capable of producing reactive oxygen species (ROS) in response to visible light illumination.[1] ROS can easily oxidize various biological molecules and thus are very cytotoxic. Typically, ROS have a short lifetime and act within a limited distance. Therefore, subcellular distribution of a photosensitizer determines the target of the oxidative damage. Among chemical photosensitizers, there are molecules targeting preferentially plasma membrane, endoplasmic reticulum (ER), and Golgi membranes, as well as nucleus, mitochondria, and lysosomes. Some of them distribute broadly between these targets, some are more specific, and some relocalize upon light irradiation.[1,2] Localization, along with cellular ATP level, light dose, and photosensitizer concentration, is a key factor in determining the modality of light-induced cell death.[3]

In the classic cell death paradigm, lysosomes were considered only to be involved in necrotic and autophagic cell death, and the role of lysosomal proteases was limited to the nonspecific protein degradation. However, it is now becoming evident that lysosmes play a far more sophisticated role in cell death than was previously thought.[4] There are models of apoptosis that depend on either cathepsins or caspases, and also models that require both these enzymes for apoptosis initiation and execution.[5] It has been shown for several models that lysosomal permeabilization followed by release of lysosomal proteolytic enzymes into the cytosol contributes to the apoptosis execution.[6] As suggested by the experiments with lysosomotropic detergents, the extent of lysosomal permeabilization acts as a switch between alternate cell death pathways. While moderate permeabilization initiates apoptotic cell death, extensive destruction of lysosomes results in necrosis.[7,8] In an acridine orange model, severe photo-oxidation, which resulted in strong lysosomal damage, caused cellular necrosis, whereas moderate stress, resulting in only partial lysosomal leakiness, led to apoptosis with TUNEL-positive nuclei and shrunken cytoplasm.[9] Lysosomal damage was shown to trigger mitochondrial membrane permeabilization and cell death via Bax and Bak—central executioners of apoptotic mechanism. Remarkably, inhibition of caspases did not affect cell death in this model.[10] Thus, lysosomal death pathway may offer an opportunity to efficiently kill cells with impaired caspase-dependent apoptotic cascade, which is not uncommon among cancer cells.

In 2006, we described the first genetically encoded photosensitizer—red fluorescent protein KillerRed capable of ROS production in response to green light illumination.[11] Recent studies demonstrated that KillerRed produces superoxide anion radical and H_2O_2 but not singlet oxygen.[12] KillerRed can be used for light-induced protein inactivation,[13–15] killing specific cell populations *in vivo*,[13,16–18] and studying intracellular local oxidative stress.[19–21]

Similar to other fluorescent proteins, KillerRed can be targeted to specific cell compartments using well-known protein localization signals. Importantly, it was demonstrated that KillerRed induces clearly different cell responses at different locations. For example, mitochondria-localized KillerRed induces morphological changes and depolarization of the mitochondria and triggers caspase-dependent or caspase-independent cell death.[11,20] KillerRed at plasma membrane evokes mainly necrotic cell death by damage of the membrane.[13,16–18] Chromatin-associated KillerRed mediates light-induced DNA damage, activation of reparation machinery, and temporary blockage of cell division.[22,23]

Lysosomes represent a promising target for photodynamic treatment. Here, we studied phototoxicity of KillerRed localized

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to the cytoplasmic surface of lysosomes. We found that in this localization, KillerRed efficiently mediates light-induced cell death via either necrosis (at higher light intensity) or apoptosis (at lower light intensity).

2 Materials and Methods

2.1 *Genetic Manipulations*

The vector pKillerRed-C (Evrogen, Moscow, Russia) was used to construct the vector pKillerRed-Rab7, encoding KillerRed protein fused to Rab7A protein via SGLRSRAE linker. cDNA encoding human Rab7A protein was amplified using cDNA from HeLa cells as a template and cloned into XhoI-EcoRI digested KillerRed-C vector in frame with KillerRed to generate Rab7A fused to the C-terminus of KillerRed. Enhanced green fluorescent protein (pEGFP)-N1, pEGFP-Bax vector (both from Clontech, Mountain View, California), and pCasper3-GR (Evrogen, Russia) were used for cotransfection experiments.

2.2 *Cell Culture*

HeLa and rat embryonic fibroblast REF52 cell lines were used. Cells were plated at $\sim 5 \times 10^4$ cells per 35 mm glass-bottomed culture dish and grown in Dulbecco's modified Eagle's medium (DMEM, PanEco, Moscow, Russia) with 10% (v/v) fetal bovine serum (FBS, Sigma, St. Louis, Missouri) for 24 h before transfection. Transient transfections were performed with the FuGene® 6 reagent (Roche, Mannheim, Germany), according to the manufacturer's protocol using 1 μg of plasmid DNA per transfection. Stable cell line HeLa-TurboGFP (Marinepharm, Berlin, Germany) was used as control cells.

2.3 *Microscopy*

Live cell imaging was performed 24 to 48 h after transfection in MEM without phenol red (Sigma) supplemented with 10% (v/v) FBS at 37°C in a 5% CO_2 atmosphere. For fluorescence microscopy, a Leica (Wetzlar, Germany) AF6000 LX imaging system, based on a DMI 6000 B inverted microscope equipped with a Photometrics (Tucson, Arizona) CoolSNAP HQ charge-coupled device camera, was used. A 120 W HXP short arc lamp (Osram, Munich, Germany) was used as a light source. A standard TX2 filter set [excitation band pass (BP) 560/40 nm, emission BP 645/75 nm] was used to acquire a red fluorescence signal and to illuminate the cells; green fluorescence was imaged using GFP filter set (excitation BP470/40, emission BP525/50). A Laser Power Meter LP1 (Sanwa, Tokyo, Japan) was used to measure the total power of the excitation light. Light power density (W/cm^2) was estimated by dividing the total power by the area of the illuminated region.

3 Results and Discussion

To avoid low pH-induced denaturation and degradation of KillerRed by proteases in lysosomal lumen, we directed KillerRed to the cytoplasmic surface of the lysosome membrane. KillerRed was fused to the small GTPase Rab7, which is attached to membranes of late endosomes and lysosomes via a lipid anchor.[24,25] KillerRed-Rab7 fusion transiently expressed in mammalian cells showed expected punctuate pattern of red fluorescence well colocalized with EGFP-Rab7 green signal (not shown). Thus, we concluded that KillerRed did not affect localization of Rab7.

We first compared phototoxicity of KillerRed-Rab7 with that of KillerRed localized to mitochondria (KillerRed-mito) or plasma membrane (KillerRed-mem), which were previously shown to efficiently mediate light-induced cell death.[11,13,16–18,20] HeLa cells were transiently transfected with KillerRed-Rab7, KillerRed-mito, KillerRed-mem, or KillerRed-C (the latter as a negative control with low phototoxicity) and mixed with HeLa stable cell line expressing green fluorescent protein TurboGFP. Culture dishes with mixed populations of cells were illuminated with green LED array (530 nm, 30 mW/cm^2 for 1 h) or kept in the dark (control cells) and left to grow for 24 h. Then several random fields of view were analyzed by fluorescence microscopy for each dish to compare number of KillerRed- and TurboGFP-expressing cells. We found that green light illumination practically did not affect cells expressing free

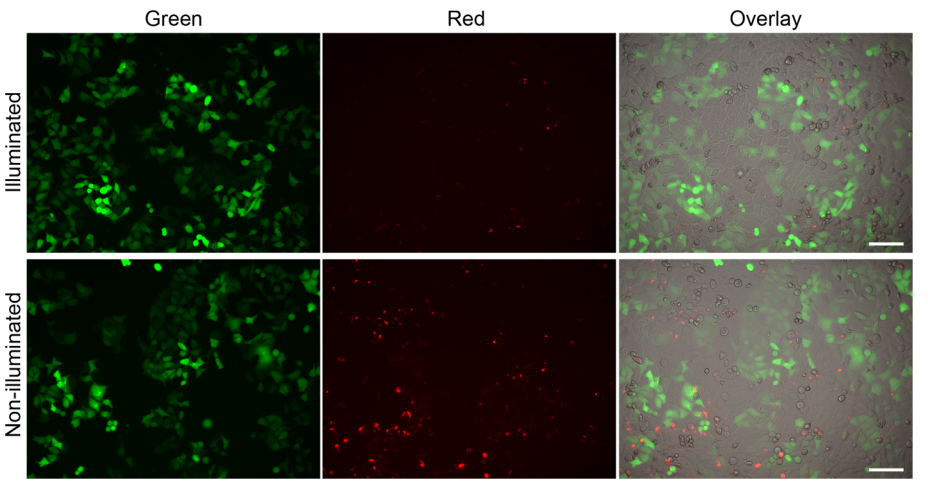

Fig. 1 Phototoxicity of KillerRed-Rab7 in HeLa cells. Green light induced strong decrease in the number of KillerRed-Rab7-containing cells in a mixed population of HeLa cells expressing TurboGFP (stably) or KillerRed-Rab7 (transiently). Shown are representative fields of view in green and red channels and their overlay with transmitted light images for green light illuminated (upper panels) and nonilluminated (bottom panels) dishes (24 h after illumination). Scale bars 100 μm.

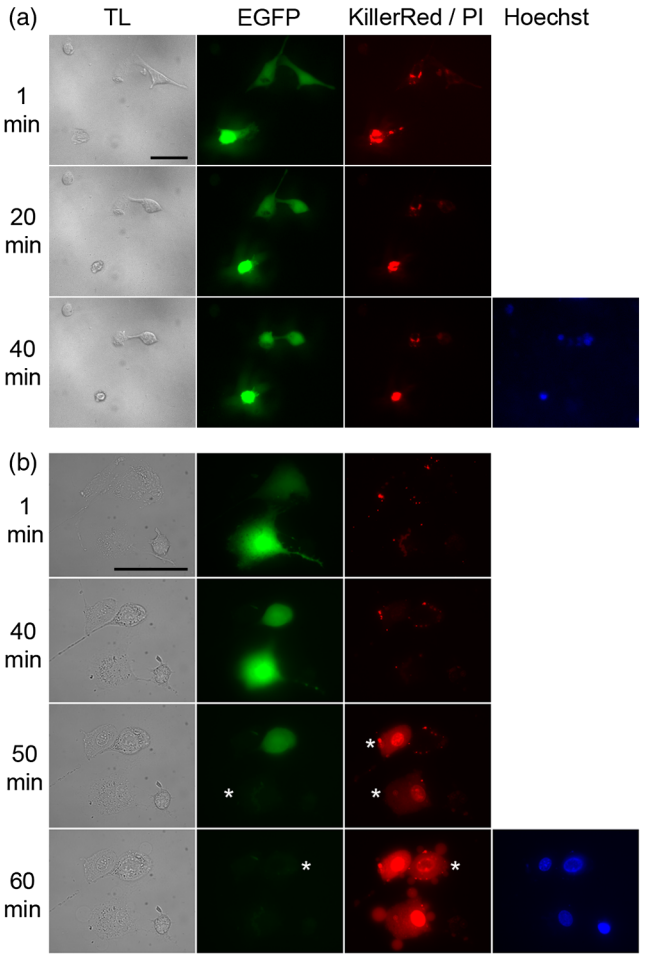

Fig. 2 Changes of cell membrane integrity and nucleus shape during KillerRed-Rab7-mediated cell death. Fluorescence microscopy of REF52 cells coexpressing KillerRed-Rab7 and EGFP and stained with propidium iodide (PI) and Hoechst33342. Shown are representative cells ($n = 17$ to 20) after illumination with 75 mW/cm^2 for 20 min (a) or 700 mW/cm^2 for 5 min (b). Time after illumination is designated on the left. Events of EGFP leakage and incorporation of PI are marked by stars. Scale bars 50 μm.

cytoplasmic KillerRed-C but resulted in a dramatic decrease of the KillerRed-expressing cells numbers for KillerRed-Rab7 (7.9 ± 1.9-fold, $N = 3$, Fig. 1), KillerRed-mito (10.5 ± 2.5-fold, $N = 3$), and KillerRed-mem (6.3 ± 1.2-fold, $N = 3$). As the observed differences between KillerRed-Rab7, KillerRed-mito, and KillerRed-mem were not statistically significant, we concluded that lysosome-associated KillerRed ensures efficient light-induced cell death similar to previously reported localizations to mitochondria and plasma membrane.

Next we studied phototoxicity of KillerRed-Rab7 in more detail using rat embryonic fibroblasts REF52. Cotransfection with EGFP was used to monitor cell morphology and membrane integrity. To induce phototoxic effects, cells were illuminated with green light (530 to 550 nm) using a fluorescence microscope; cell fate was then tracked by time-lapse imaging. At a relatively low light intensity and dose (75 mW/cm^2 for 20 min), we observed morphological changes (shrinkage and blebbing) in the transfected cells 40 to 60 min after illumination [Fig. 2(a)]. At the same time, nontransfected cells remained intact after illumination. All transfected cells ($n = 17$) excluded propidium iodide (PI) and retained EGFP green fluorescence 1 h after illumination, indicating that the plasma membrane remained intact. Hoechst33342 staining revealed compaction of chromatin in the dying cells. Altogether, these features are characteristic of the apoptotic cell death.[26] In contrast, transfected cells ($n = 20$) illuminated with higher light intensity and dose (700 mW/cm^2 for 5 min; these conditions did not kill control nontransfected cells) incorporated PI within 30 to 60 min after illumination and simultaneously lost EGFP green fluorescence [Fig. 2(b)]. In this case, Hoechst33342-stained nuclei were round-shaped with smooth edges. Thus, we concluded that intense green light illumination results in necrosis[26] of KillerRed-Rab7-expressing cells.

In order to assess whether lysosomal proteases are involved in KillerRed-Rab7-mediated photodamage, we incubated REF52 cells coexpressing KillerRed-Rab7 and EGFP with cathepsin inhibitors. Cells were preincubated with 10 μM pepstatin A (inhibits cathepsin D) or 10 μM E-64 (inhibits cathepsins B, L, H) for 1 h prior to the illumination, and then the cells were illuminated with 700 mW/cm^2 green light for 3 min. About 60% of the control cells (w/o inhibitor) died within 90 min after illumination, compared to only 30 and 35% dead cells with pepstatin A and E-64, respectively ($n = 20$ to 25 cells for conditions). Thus, lysosomal cathepsins play important role in KillerRed-Rab7-mediated cell death.

To conclude, KillerRed-Rab7 localized to the cytoplasmic surface of lysosomes delivers a considerable phototoxicity to mammalian cells, comparable to that of membrane- and mitochondria-localized KillerRed. Similar to lysosome-localized chemical photosensitizers, KillerRed-Rab7 induces either apoptosis or necrosis depending on light intensity and dose. This feature of KillerRed-Rab7 differs from previously studied localizations of KillerRed. Thus, KillerRed-Rab7 is particularly useful for models where controllable switching between apoptotic and necrotic cell death is desirable. Being genetically encoded, KillerRed with different intracellular localization tags represents a useful optogenetic tool to study local oxidative stress and eliminate specific cell populations.

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