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Bongkrekiic Acid induces Selective Cytotoxicity in Tumor Cells, revealed by CCK-8

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Bongkrekiic acid (BKA) is shown to inhibit adenine nucleotide translocator in the mitochondrial inner membrane, which is bifunctional and facilitates ATP/ADP exchange and permeability transition pore opening in mitochondria. We demonstrate that BKA reduces the viability of tumor cells depending on the cell number in the culture. This reduction was first demonstrated by Cell Counting Kit-8 (CCK-8), which indicates cellular viabilities based on the reduction of a formazan dye by cellular NADH. Reduced viability was not seen in normal cells. The glycolysis inhibitor 2-deoxyglucose enhanced the effect of BKA. BKA is thought to work as a mitochondrial inhibitor; however, its action is different from mitochondrial uncouplers. We further demonstrate that BKA suppresses autophagy induction, directing cells to death in nutrient-depleted conditions. Together these data indicate that BKA is a new type of mitochondrial inhibitor that does not disrupt the mitochondrial membrane and that is thus used together with a glycolysis inhibitor as an anticancer agent.

Keywords: bongkrekiic acid (BKA), cellular viability, tumor cells

1. Introduction

Bongkrekiic acid (BKA) was, originally, identified as a cause of the poisoning of the coconut-fermented food (Fig. 1). Welling et al. reported in their article 50 years ago that "oral administration of BKA initially produces hyperglycaemia, and soon followed by hypoglycaemia" ¹. Interestingly, the paper also reported that "just before death all glycogen had disappeared from liver and heart". Later, it was revealed that BKA inhibits the adenine nucleotide exchange in mitochondria by binding to adenine nucleotide translocator (ANT) was revealed ^{2,3}. The fundamental role of ANT is the exchange of ATP and ADP across the inner mitochondrial membrane. In addition, it forms the inner membrane channel of the mitochondrial permeability transition pore (MPTP) complex, which is associated with apoptosis induction ^{4 5}. Several papers demonstrated that BKA also suppresses apoptosis through inhibiting MPTP opening ^{6,7}. However, it is thought that BKA also inhibits ATP production, which may result in adverse effects on cell viability. So far, BKA has been well studied with isolated mitochondria, yet, is hardly considered in cultured tissues.

The increased glycolysis in cancer cells even under aerobic conditions is known as the "Warburg effect" ⁸. Altered tumor metabolism, like increased uptake of

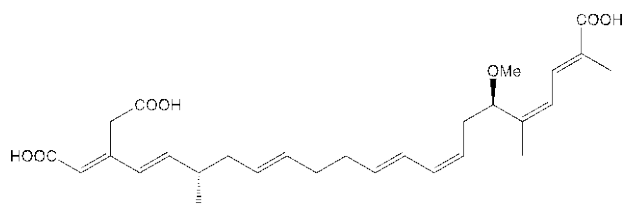


Figure. 1: Chemical structure of BKA

glucose, is one of the considerable targets of cancer chemotherapy ⁹. For instance, glucose derivative, 2-deoxyglucose (2-DG), has long been known as a glycolysis inhibitor and exhibits cytotoxic effects in cancer cells ¹⁰. However, it has also been considered that combined treatment with mitochondrial inhibitors is required for sufficient cytotoxicity.

In this study, we aimed to clarify the effect of BKA on culture cells. We show BKA causes loss of viability in a murine mammary tumor cell line, 4T1, but not in normal cell lines, such as NIH3T3, which was first demonstrated by Cell Counting Kit-8 (Dojindo Lab, Kumamoto, Japan). The CCK-8 indicates cellular viabilities based on the reduction of a formazan dye by cellular NADH. Unlike other similar kits, CCK-8 consists of a water-soluble formazan dye and an electron carrier. Due to the hydrophilic property of a formazan, CCK-8 is

thought to indicate the level of cytosolic NADH¹¹). The effect of BKA on the viability of tumor cells is dependent on the seeded number in culture. The dependence on the cell number is thought to arise from the difference in some metabolic activities. As well, the sensitivity to BKA observed in tumor cells is thought to be due to the higher metabolic activities in cancer cells. The combined treatment with 2-DG showed enhanced cytotoxicity in 4T1 tumor cells, which further indicates that BKA affects energy production in cultured cells. BKA may be expected to new type anti-cancer drug that targets cancer metabolism.

2. Experimental

2.1 Cell culture and assay reagents

Murine mammary carcinoma 4T1 cells were maintained with RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum in a 5% CO₂ atmosphere at 37 °C. BKA was purchased from Sigma-Aldrich Japan K.K (Tokyo Japan) or kindly gifted from Prof. Y. Shinohara, Institute for Genome Research, the University of Tokushima. There is no fundamental difference in the activity between the purchased BKA and the gifted one. The cell viability test was performed by Cell Counting Kit-8 (CCK-8, Dojindo Laboratories Kumamoto, Japan) according to the manufacture's instruction. The cell death assay was performed by Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Corp., WI, USA) based on the activities of lactose dehydrogenase (LDH) in the media that was compared to the lysed cells by freeze-thawing and indicated as "percent cell death".

2.2 Tissue staining

The CYTO-ID Autophagy Detection Kit¹²) and LYSO-ID Red Detection Kit were purchased from Enzo Life Sciences Inc. (NY, USA) and used to evaluate autophagosomes and lysosomes respectively according to the manufacture's instruction. The intensity of stained fluorescence was analyzed by Ensign Multimode Plate Reader (PerkinElmer Inc., Waltham, MA).

2.3 Western Blotting

Tissues were washed briefly with PBS and lysed with RIPA (10 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 0.5 mM phenylmethanesulfonyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). The cell lysates were centrifuged, and the supernatants were recovered and assayed for protein amounts using the bicinchoninic acid protein assay kit (Pierce Chemical Co.). Cell lysates were separated on SDS-PAGEs, transferred to polyvinylidene difluoride membranes, and incubated with anti-LC3 antibody (MBL Co. Ltd.

PM036). Anti-rabbit antibody conjugated with horseradish peroxidase was used as secondary antibody and visualized using chemiluminescent substrate (ImmunoStar LD; Wako Pure Chemical Industries, Ltd.)

2.4 Data managements

All experiments were performed at least three times and the typical results were shown.

3. Results and Discussion

3.1 BKA selectively reduces viabilities of 4T1 tumor cells but not NIH3T3 normal cells

Nature has been shown to be a powerful source of materials useful for different applications. For instance, seeds of *Moringa oleifera* have been shown to be a good source of lectin, which can be used as a natural coagulant¹³). In this study, we investigated cytotoxicity of BKA, which was first isolated from *Pseudomonas cocovenans*. After the initial evaluation of viability tests in 4T1 tumor cells, we found that BKA reduces cellular viabilities depending on the seeded number of cells, which was demonstrated by CCK-8 (Fig. 2A). The evaluation with CCK-8 reflects the level of NADH, an energy carrier in a cell¹¹). Interestingly, the cytotoxicity of BKA was not observed in normal cells, NIH3T3. The dose-dependent cytotoxicity of BKA was observed in 4T1 tumor cells but not in NIH3T3 cells as well (Fig. 2B).

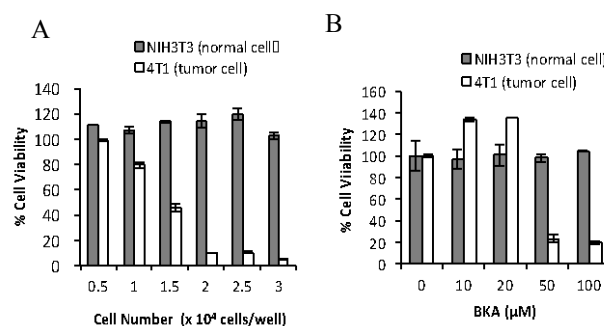


Figure 2. BKA selectively reduces the viabilities of tumor cells demonstrated by CCK-8. A) The dependency of the initial seeded cell number at 100 µM of BKA. B) The dose dependency of BKA at 2.5 x 10⁴ cells/well of initial seeded number.

3.2 Recovery of reduced cellular viabilities of 4T1 tumor cells by changing media

Interestingly, BKA reduces viabilities of 4T1 cells depending on cell number in the culture. We speculated that this is due to the secondary effect of changes in the composition of the media while 4T1 cells are being cultivated. To evaluate this hypothesis, the culture media were changed to the fresh one with or without BKA at 12 h in the total 24 h culture. Figure 3 demonstrates that the viabilities were recovered at least partially by changing media freshly. This result suggests either loss of nutrient,

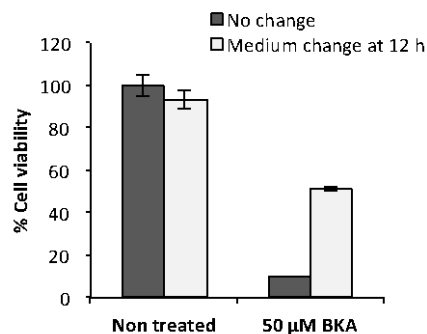


Figure 3. Recovering of cellular viability of BKA-treated 4T1 cells by changing to fresh media with/without BKA at 12 h in 24 h culture, demonstrated by CCK-8.

accumulation of excreted metabolites, or secretion of some bioactive substances in the culture media. Using isolated mitochondria, It has been demonstrated that binding activities of BKA on mitochondria increase in lower pH^{3,14}. Indeed, the culture media of 4T1 cells show reduced levels of pH due to the increasing lactate that is secreted as a metabolite of glycolysis, which is further facilitated by increased cell number. Therefore, we next tested the effect of pH of culture media on the cytotoxicity of BKA using 4T1 cells (Fig. 4). The viabilities of 4T1 cells were decreased with lowering of pH. However, this observation was the same in conditions with or without BKA. Therefore, it is thought that the observed reducing viabilities in the lower pH are not effects of BKA activities. In the higher pH, pH 7.6 and 7.8, the viabilities were decreased in the BKA-treated but not in control. The enhanced metabolism and growth of cultured cells in higher pH media have been shown^{15,16}, which may reflect the result of the reduced cell viabilities with BKA. However, the further investigation is required to clarify the reason for this observation.

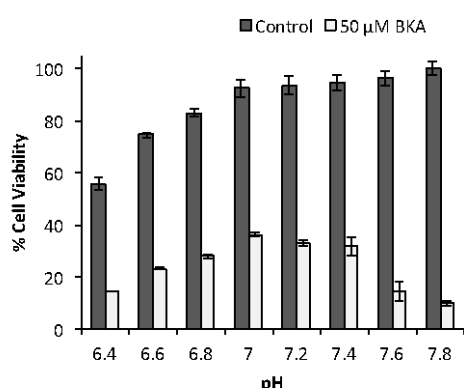


Figure 4. The effect of pH of culture media on cell viabilities of 4T1 cells with or without BKA, demonstrated by CCK-8. The pHs were controlled in the indicated values with HEPES buffer.

3.3 Enhanced cytotoxicities of BKA in combination with glycolysis inhibitor

Although BKA reduces viabilities of 4T1 tumor cells, it does not induce cell death, which is shown by evaluation of the released LDH (lactose dehydrogenase), an intracellular enzyme, in the culture media (Fig. 5 control). When the cell dies, the integrity of the plasma membrane is disrupted and the LDH is released eventually to the culture media. The reduced cell viabilities by BKA were further augmented in the higher pH media as shown above, which may be correlated with the changes of some cellular metabolism. In addition, BKA is known as an inhibitor of ANT that plays a role in the supply of ATP from the mitochondria to the cytoplasm. Therefore we attempted to suppress another major path for the ATP production, glycolysis, by a specific inhibitor, 2-deoxy glucose (2-DG)¹⁷. Twenty mM of 2-DG induced cell death of about 20% in 4T1 tumor cells without BKA, which was markedly enhanced to 60% with 100 μ M of BKA (Fig. 5). 2-DG is being investigated as an anti-cancer drug. However, combined treatment with mitochondria inhibitor is considered to be required to exert effective antitumor activities⁹. BKA can thus be a candidate for a treatment of cancer medication.

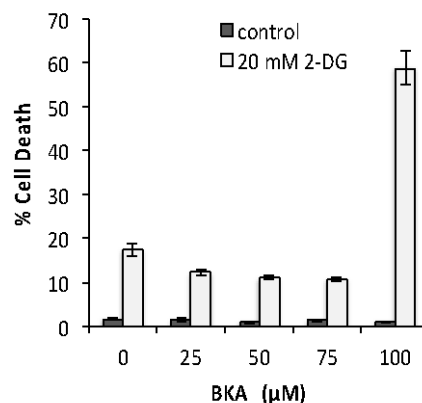


Figure 5. BKA does not induce cell death in 4T1 cells but does with glycolysis inhibitor 2-DG.

3.4 BKA suppresses autophagy induction

We observed emerging of vesicles in BKA-treated 4T1 cells (Fig. 6). Autophagy is a protective mechanism in which cells digest intercellular contents and organelles when the cell is starved of nutrients. Therefore, we first considered that these vesicles were autophagosomes, because the above results suggested that BKA perturbed metabolic balance in the cells. To evaluate autophagic induction, LC3 protein was analyzed by western blotting. LC3 protein is a component of autophagosomes and acetylated in their forms, which is observed as a faster migrating protein in SDS-PAGE¹⁸. The acetylated LC3 (LC3-II) started to increase 24 h after the onset of culture in 4T1 cells (Fig. 7A). It is thought that autophagy is induced at around 24 h due to depletion of nutrients in the

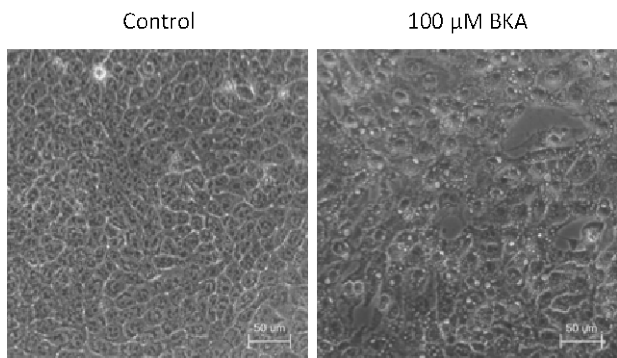


Figure 6. Phase contrast microscopic observation of 4T1 cells treated with or without BKA for 24 h.

media. When 4T1 cells were cultured with BKA, the level of LC3-II was reduced in a dose-dependent manner (Fig. 7B). This result suggests that BKA suppresses autophagy induction. For further confirmation, autophagic and lysosomal vesicles were stained with fluorescent probes and the specific fluorescence was quantified. Hoechst 33342 staining, indicating cell numbers, did not vary across various concentrations of BKA (Fig. 8A). However, the CYTO-ID staining, demonstrating autophagosomes, was reduced with increasing concentration of BKA, while the LYSO-ID staining, demonstrating lysosomes, was slightly increased (Fig. 8B). Taken together with the western blot results of LC3, these results suggest that BKA suppresses autophagy. By suppressing autophagy, BKA seems to slant the cellular digestion to lysosomes. A previous study demonstrated that the intracellular ATP is necessary for the induction of autophagy¹⁹⁾. Thus, the treatment of BKA may cause insufficient levels of ATP in a cell for induction of autophagy, resulting in cell death instead of saving energy by autophagic digestion.

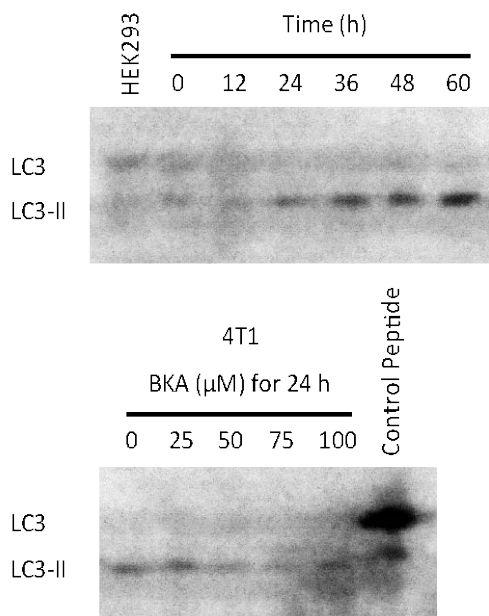


Figure 7. LC3 protein was detected by Western blotting. HEK293; control lysate, Control Peptide; obtained from MBL CO. LTD (Nagoya, Japan)

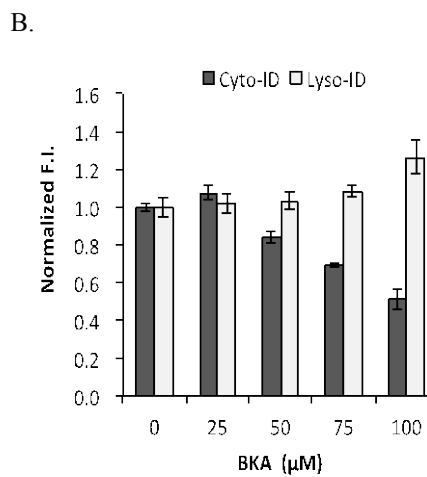
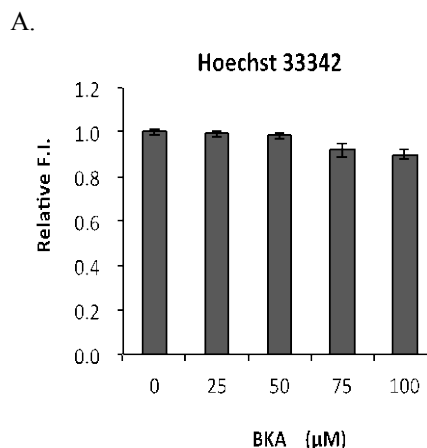


Figure 8. Nuclei, autophagosomes, and lysosomes were stained with specific fluorescence probes and fluorescence intensities (F. I.) were evaluated. Hoechst 33342; nuclear, Cyto-ID; autophagosome, Lyso-ID; lysosome

4. Conclusion

Here, we demonstrated that BKA selectively reduces cellular viabilities in tumor cells. Using the isolated mitochondria, it has been demonstrated that BKA prevents ATP production by inhibiting ATP/ADP exchange reaction. The viability of BKA-treated cells is decreased when it is accompanied by glycolysis inhibition, and the cells eventually died. Many of mitochondrial inhibitors are uncouplers, which cause oxidative stress in the cells and induce apoptosis, whereas BKA blocks mitochondrial pore opening. Recently, it is thought that cancer cells use autophagic mechanisms to survive in nutrient-depleted conditions such as the inside of tumors. In this paper, we showed that BKA inhibits autophagic induction in 4T1 tumor cells. BKA is not an uncoupler, and it is thus, not expected to impair normal tissues. In this milieu, BKA may act as an anti-cancer agent, which is further enhanced by co-treatment with a glycolysis inhibitor, such as 2-DG.

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