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Mitochondrial autophagosomes as a mechanism of drug resistance in breast carcinoma

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Abstract

We have previously described the process by which mitochondria donate their membranes for the formation of autophagosomes, and in this study we show that the same process could be involved in drug sequestration and exocytosis resulting in multidrug-resistant cancerous cells. We examine the implications of mitochondrial vesicle formation of mitoautophagosomes (MAPS) in response to the cytotoxic drug MKT-077, which targets mortalin, in a drug-resistant breast carcinoma cell line overexpressing P-glycoprotein (P-gp). The breast cancer cell line MCF-7Adr is derived from MCF-7, but differs from its ancestral line in tolerance of MKT-077-induced mitochondrial toxicity. Our ultrastructural observations suggest that autophagy in the MCF-7Adr cells entails regional sequestration of MKT077 in multilamellar LC3-labeled MAPS, which then separate from their mitochondria, and fuse with or engulf each other. MAPS appeared to be migrating through the cytoplasm and fusing with the plasma membrane, thus carrying out exocytotic secretion. This mechanism, which seems ineffective in the ancestral cell line, provides a resistance mechanism for MKT-077 by enhancing the efflux process of the cells. After 8 hr of MKT-077 exposure, a fraction of the resistant cells appeared viable and contained larger number of smaller sized mitochondria. Mitoautophagosomes, therefore, provide a potentially novel model for multi-drug resistance in cancerous cells and may contribute to the P-gp efflux process.

Keywords

Breast carcinoma; drug resistance; mitochondria; mitoautophagosomes; MKT-077; multidrug resistance; P-glycoprotein; P-gp efflux pump

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Disclosure statement

The authors declare they have no conflicting financial interests.

Introduction

Drug resistance in cancer patients, which is a common occurrence, has not been associated with autophagy before.^{1,2} It can be attributed to the heterogeneous nature of the disease and the selective pressures of chemotherapies that produce resistant variants.³ An emerging solution is the administration of a cocktail of drugs directed towards multiple therapeutic targets.⁴ However, adverse side effects as well as emerging resistance are common issues of combination therapies.⁵ Although drug resistance is associated with the P-gp efflux pump at the plasma membrane,^{2,6} its association with mitochondrial mitophagosomes (MAPS) has not been suggested before.⁷ This study examines a novel phenomenon of drug resistance which involves the induction of MAPS in response to treatment with the rhodacyanine MKT-077.

MKT-077 is a cytotoxic compound to a variety of cancerous cells.^{8,9} It affects cells through accumulating specifically within mitochondria, and subsequently disrupting mitochondrial membranes, DNA, and oxidative functions.^{10–14} MKT-077 also increases mitochondrial fragmentation by inhibiting mortalin (GRP75; *HSPA9*) in a Drp1-dependent manner.¹⁴ Mitochondria possess high negative-internal trans-membrane potential which renders them a target for lipophilic cations, such as rhodamines. The intramitochondrial concentration of these compounds tends to be 100- to 1000-folds higher than the rest of the cell.^{15–17} MKT-077 adversely affects breast (MCF-7), colon (CX-1), melanoma (LOX), pancreas (CRL1420), and transitional carcinoma (EJ) cell lines.^{8,9,18} However, all these cell lines exhibited a range of sensitivity to the drug. When treated with MKT-077, normal cells showed none of the symptoms reported in cancerous cell lines.¹⁵ However, it caused eventual irreversible renal toxicity in clinical trials.¹⁹

We examined the cytotoxicity of MKT-077 in MCF-7 and its derivative MCF-7Adr line because the latter overexpresses P-glycoprotein (P-gp) related multidrug resistance. Rhodamines are an effective substrate for P-gp efflux pump; therefore, this efflux mechanism may render MCF-7Adr resistant to MKT-077.^{20–22} The multidrug-resistant phenotype observed in cultured tumor cells is poorly understood on molecular and ultrastructural levels. Published studies have so far only speculated on the location and association of the P-gp efflux pump within a few cellular organelles including the plasma membrane and non-endocytic cytoplasmic vesicles.^{23–25}

In this study, we use time series electron microscopy (EM) images to describe a model of mitophagosome involvement in the resistance of the breast cancer cell line MCF-7Adr to MKT-077 in comparison to its sensitive parental cell line MCF-7. We previously provided evidence for a mitochondrial origin of MAPS.^{1,7} The mechanism described in this study differs from the regulatory mitophagy process of damaged and dysfunctional mitochondria.²⁶ Our ultrastructural observations suggest a novel mitochondrial P-gp–MAPS mechanism that explains varying resistance among cell lines and could place the P-gp efflux pump in the inner mitochondrial membrane and MAPS.

Materials and methods

MKT-077 (formerly known as FJ-776).

It was obtained from Fuji Photo Film Company Ltd. (Tadao Shishido, Kanagawa, Japan). Aqueous solution of MKT077 was used at 1–50 µg/mL concentrations.

Mortalin siRNA.

It was purchased from Origene (Rockville, MD, USA). MCF7-LCC9 cells were transfected with 1-µM siRNA using RNAiMAX lipofectamin (Invitrogen, Carlsbad, CA, USA).

Cell cultures.

MCF-7 and MCF-7Adr cells were grown in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Walkersville, MD, USA) containing 10-mM glutamine, 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). MCF7-LCC9 cells were grown in phenol red free IMEM with 10% charcoal stripped calf serum.^{27,28} Cultures were incubated at 37°C, under 5% CO₂, and 100% humidity.

Clonogenic assay.

Cells were plated at 1000 cells/well in 6-well plates (Becton Dickinson Labware, Lincoln Park, NJ, USA). After 24 hr, old medium was replaced with a fresh medium containing the various concentrations of MKT-077 (1–50 µg/mL). Cells were incubated for 5 days with continuous exposure to the drug, washed with Dulbecco's phosphate buffered saline (PBS) (+Ca [1 mM] and Mg [0.5 mM]), and stained with crystal violet (2%) for 10 min. Counting was done with Artek 1880 counter (Dynatech Laboratory Inc., Chantilly, VA, USA). All assays were performed in triplicates.

Electron microscopy (EM).

Cells were grown as above and exposed to 50 µg/mL of MKT-077. Cultures from both cell lines were fixed in glutaraldehyde (2.5% in PBS, pH 7.4) at the following intervals: 30 min, 1 hr, 1.5 hr, 2 hr, 3 hr, 4 hr, 5 hr, 6 hr, 7 hr, and 8 hr. Also, two controls were fixed, one at 0 min and 8 hr. Processing for EM was carried out according to Ogilvy et al.²⁹ Briefly, cells were post-fixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections (~90 nm thick) were mounted on 200-mesh uncoated copper grids, doubly stained with uranyl acetate and lead citrate, and examined and photographed with a Phillips CM10 transmission electron microscope.

Western blot hybridization.

MCF7/LCC9 cells were transfected with a scrambled control siRNA or GRP75 (mortalin) targeting siRNA for 72 hr before protein was harvested. Proteins were size fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated overnight with primary antibodies to either LC3A/B, p62, mortalin, ATG5, or PINK1 (Cell Signaling Technologies, Danvers, MA, USA) to determine the effect of mortalin silencing (the target protein of MKT-077) on autophagosome formation, flux, and autophagy. Protein levels were visualized by chemiluminescence and quantified by

densitometry. Protein loading was visualized by probing membranes with an antibody against β -tubulin (Sigma-Aldrich, St. Louis, MO, USA).

Results

MCF-7Adr surviving high concentrations of MKT-077

When exposed to the same concentration of MKT-077, the two cell lines exhibited different survival curves (Figure 1). Survival of MCF-7 cells were severely affected by the drug compared to MCF-7Adr. At a concentration of 10 $\mu\text{g/mL}$, MCF-7 had less than 5% survival rate, while MCF-7Adr had 85% survival. At a higher concentration, 50 $\mu\text{g/mL}$, the parental cell line had zero survival and the resistant line bordered on 3%.

MKT-077 inducing mitoautophagosomes in breast carcinoma cell lines

Mitochondrial morphology of control cell lines—Both untreated cell lines appeared healthy with dense cytoplasm filled with glycogen, intact nuclei, and viable mitochondria (Figure 2A & B, Figure 3A & B). The controls of both cell lines possessed mitochondria with intact outer and inner membranes; however, they lacked vacuoles, constrictions, and budding MAPS (Figure 2A & B, Figure 3A & B). The mitochondria of MCF-7 had tightly packed uneven cristae with scant intermembrane space (IMS) between them (high ratio of cristae [C] to IMS), and the same morphology was observed after 8 hr (Figure 2C). In contrast, the mitochondria of the MCF-7Adr cells possessed narrower tubular bodies, thin cristae, and wider IMS areas than those of MCF-7 (low C/IMS ratio); the cristae were arranged in various directions (Figure 3A & B).

MKT-077 inducing mitoautophagosomes in MCF-7 and causing the destruction of mitochondria—After 30 min of 50 $\mu\text{g/mL}$ MKT-077 exposure, there was only visible damage to the mitochondrial outer and inner membranes (Figure 2D & E). Internal translucent vesicles were formed within the mitochondria, while others budded out; some can be seen still attached to the mitochondria and others separated (Figure 2D & E). These MAPS vesicles had varied number of membranes, some were single-membrane bound while others were multi-membrane bound. Lipid droplets were present in the cytoplasm; but both nuclei and cytoplasm lacked other visible changes.

After 60 min of exposure to treatment, there was an increase in translucent MAPS vesicles lacking normal dense mitochondrial matrix budding out and separating from the mitochondria (Figure 2F–H). Furthermore, the size, shape, location, and number of membranes varied among the affected mitochondria. Some vesicles formed in the middle of the mitochondria, thus separating each mitochondrion into two parts. At this stage, these vesicles, internal, or budding out, seem to be a sign of autophagy at various degrees of development (Figure. 1F–H). Impact appeared localized to particular areas inside the mitochondria with the remaining structure intact.

At 90 min, further damage emerged in the mitochondria (Figure 2I–L). A significant number of the mitochondrial population had completely disintegrated, while others exhibited disorganized or degenerate outer and inner membranes. MAPS vesicles became more ubiquitous and were present at various stages of development. MAPS vesicles with multi-

concentric membranes pinched off from the mitochondrial body, thus retaining healthy portion of mitochondria and producing a free MAPS vesicle (Figure 2I & K). Some vesicles were observed to contain cytoplasmic material that they engulfed from the cytoplasm (Figure 2K & L upper right corner).

After 3 hr, all mitochondria of MCF-7 manifested greater degrees of damage and degeneration (Figure 2M & N). Inner membrane organization was entirely lost and mitochondria appeared as dense vesicles. Multi-membrane MAPS vesicles were ubiquitous and many were attached to degenerate mitochondria.

After 7 hr, the mitochondria had lost most of their internal organization, resembling lysosomes in appearance, and contained numerous vesicles; some of these vesicles were multi-membraned (Figure 2O & P). The nuclei as well as cytoplasm had diffused and degenerate appearances.

The EM images showed two types of MAPS vesicles. The first were simple translucent vesicles that most likely originated from the inner membrane of the mitochondria with a single or multi-lamellar membrane (Figure 2F, G, N, & O). The second type of vesicle, found sparingly, seemed to have originated from the outer membrane. They formed by extending a multilamellar arm, a phagophore, that sequestered cytoplasm and then fused again at its distal margin with the mitochondrial outer membrane (Figure 2I, K, L & M). Their contents were a defining feature between the two types; the first was translucent and the second always contained cytoplasmic material.

MKT-077 inducing mitoautophagosomes in MCF-7Adr and causing the selection of resistant cells—The mitochondria of the untreated MCF-7Adr control cell line had narrow tubular cristae, wide IMS, as well as intact outer and inner membranes (Figure 3A & B). There were no vesicles within or budding out from the mitochondria; moreover, the cellular cytoplasm was relatively dense and loaded with glycogen.

After 60 min of exposure to 50 µg/mL MKT-077, some of the mitochondria produced MAPS vesicles of various sizes within and outside of the mitochondria, while large MAPS were visible within the cytoplasm (Figure 3C & D). From 90 min to 2 hr (Figure 3E–G), the mitochondria produced many MAPS vesicles. MAPS formation was also associated with an elongation of mitochondria resulting in a dumbbell morphology (Figure 3D–G). These elongated mitochondria were frequently observed to produce their MAPS vesicles in the middle, whereas others appeared to have split into two smaller mitochondria while releasing the MAPS vesicle (Figure 3E–G).

After 5 hr of exposure to the drug, the mitochondria of MCF-7Adr produced a large number of MAPS vesicles and preserved the healthy portions that yielded smaller sized mitochondria (Figure 3H, and G: 5 hr). Additionally, some lipid droplets appeared in the cytoplasm signifying hypoxic conditions (Figure 3F: 90 min, H: 5 hr, & I: 6 hr). During the period of 6–8 hr (Figure 3I–L), the number of MAPS vesicles in the cytoplasm was drastically reduced and the MCF-7Adr cells maintained a population of healthy looking mitochondria. The emergent mitochondria appeared smaller in size than their predecessors

(Figure 3K & L v. control A & B). Furthermore, some of the mitochondria did not produce vesicles but swelled into dense degenerative spheroids that resembled lysosomal morphology (Figure 3I lower right corner).

Differences and similarities between the two cell lines in response to MKT-077

—In both cell lines, the mitochondria responded to MKT-077 by producing MAPS. The ontogenesis of these MAPS vesicles in both lines followed similar phases. A vesicle frequently appeared on one side of the mitochondria and presumably became enlarged. In certain cases, the whole mitochondrion was transformed into an MAPS vesicle with disintegrated inner membranes. Following separation from the mitochondria, vesicles appeared to be bound by one or more membranes. The shape and size of the MAPS vesicles varied initially, and later seemed to become uniformly spheroidal. MAPS vesicles may have fused with each other and with the plasma membrane in what could be exocytosis. Yet, the most pronounced difference between the two cell lines was a pattern of MAPS production in response to the drug treatment. MAPS formation in MCF-7Adr was found to be more extensive at the beginning and subsided later, resulting in a new persistent mitochondrial population. In comparison, the mitochondria of MCF-7 continued to produce vesicles and after 8 hr of exposure the mitochondrial population was entirely abnormal and deteriorating.

MKT-077 targeting mortalin and inducing autophagy through parkin—

Previously, we have demonstrated that vesicles induced by anti-cancerous drugs are indeed mitoautophagosomes.^{1,7} Since MKT-077 binds specifically to mortalin³⁰, we show that targeting mortalin with RNAi induced autophagy. When mortalin was reduced by RNAi in drug-resistant MCF7-LCC9 breast cancer cells, autophagy was induced as indicated by elevated LC3-II formation and reduced p62 (Figure 4). Mortalin-targeting induced autophagy appears independent of the classical autophagy-related gene (ATG) mediated pathway because transfection of MCF7-LCC9 cells with GRP75 siRNA significantly inhibited ATG5 protein levels (Figure 4). Moreover, silencing of mortalin was associated with a potent induction of PTEN-induced putative kinase 1 (PINK1), a mitochondrial protein that translocates to the mitochondrial outer membrane which then interacts with parkin to stimulate autophagy (Figure 4). Taken together, these data support and confirm our ultrastructural data indicating that targeting mortalin with MKT-077 specifically induces autophagy.

Discussion

Chemotherapy-resistant cancer is an evolutionary process that is not yet fully understood.³¹ Therefore, an integral understanding and elucidation of this phenomenon requires an ultrastructural examination to reveal the cellular organelles' alterations associated with its molecular events. Studies of chemotherapy resistance that used EM have not gauged treatment effects through comparative ultrastructural examination, time series sampling, and none focused on mitochondrial population dynamics.

Vesicle formation due to drug treatment has been previously reported in the cytoplasm of several multidrug-resistant cell lines.^{6,32} A mitochondrial origin of these vesicles as MAPS has never been suggested before; however, the endoplasmic reticulum³³, plasma

membrane^{6,34}, or non-endoplasmic origin²⁴, were hypothesized as the primary sources of these vesicles. Other publications regarding these vesicles failed to resolve the nature of their origin because they did not correspond to endoplasmic vesicles^{24,35}, and a non-mitochondrial autophagy was suggested.³⁶ Our results reveal for the first time the mitochondrial origin of drug-induced vesicles using time series sampling to demonstrate that they are MAPS. Furthermore, we show the probable impact on chemo-resistance in cancerous cells as well as mitochondrial division and regeneration under chemotherapy.

Vesicular formation of MAPS followed by mitochondrial division appears to be employed by MCF-7Adr cells to sequester and expel MKT-077. Therefore, the mechanism is likely related to the P-gp efflux pump because the resistant cell line, MCF-7Adr, overexpresses the P-gp protein. This suggests that the MAPS may contain P-gp within their membranes and could be laden with MKT-077. Indeed, Gong et al. have demonstrated that “micro-particles,” 0.1–1µm vesicles, sequester drugs; however, they proposed a plasma membrane origin without providing ultrastructural data.^{6,37}

While many studies have suggested that the P-gp pump functions at the plasma membrane^{24,37}, or are native membrane glycoprotein³⁸, our results suggests a mitochondrial membrane site. The P-gp molecules may be functioning in the mitochondria and their presence at the plasma membrane is due to the fusion of the MAPS membranes with the plasma membrane during exocytosis. This hypothesis is supported by published observations that the vesicles are loaded with drugs and are shed outside the cell.⁶

Published photos of EM immunolabeling of P-gp show a sporadic and patchy labeling of exceedingly few regions of the plasma membrane.²⁴ This is an unusual pattern for a presumably overexpressed membrane protein but congruent with our hypothesis of mitochondrial origin and fusion with the plasma membrane. If the membrane of MAPS contains P-gp, then upon its coalescence with the plasma membrane, it integrates its own surface proteins into the plasma membrane, thereby producing a patchy labeling pattern. Additionally, we did not observe any vesicle formation by endocytosis on the plasma membrane, therefore our data do not support the suggestion that vesicle formation can take place inwardly at the plasma membrane.^{24,37}

Our results show that the efficiency of MAPS is correlated with P-gp overexpression and led to higher survival. In the sensitive cell line, MCF-7, after a few hours of high-dose drug exposure, mitophagosome production was exhausted, most of the cells lost the majority of their mitochondria, and some mitochondria were completely transformed into MAPS. Our model may explain the observation that cellular drug efflux cannot be increased in linear correlation dependent on the concentration of P-gp molecules since it leads to mitochondrial depletion.^{39,40}

The identification of P-gp on various structures has led to the conclusion that the distribution of P-gp varies among multidrug-resistant cell lines.^{24,41} However, it is still unknown if the MAPS carry Pgp within their membranes, and if they continue to accumulate the drug in their lumen after budding off from mitochondria and emerging in the cytoplasm.

An additional observation that emerged from our study is the striking difference in mitochondrial morphology between the sensitive and resistant cell lines. The mitochondrial population of each cell line appear to represent a unique phenotype. While the mitochondria of MCF-7 have wider cristae and reduced IMS (high C/IMS ratio), those of MCF-7Adr possess thin cristae and ample IMS (low C/IMS ratio). The wider IMS of MCF-7Adr may be pointing to the importance of the IMS contents in drug resistance and possibly the presence or storage of molecules that are necessary for the production of MAPS. Additionally, the cristae architecture of the MCF-7Adr mitochondria is very different from those of MCF-7. Taken together, these aspects may be at the center of the copious production of MAPS in the resistant cell line. Drug treatments are known to alter the morphology of mitochondria⁴², and may select for a resistant phenotype; yet, many published studies that showed excellent ultrastructural details of cristae have not presented a contrast between mitochondrial phenotypes of drug sensitive and resistant cell lines.^{43,44} Therefore, it is still premature to speculate on whether the observed phenotype of mitochondria in MCF-7Adr is the dominant type in drug-resistant cell lines.

Mitochondrial vesicular formation of MAPS appears to be an evolutionarily conserved primordial process that exists in all cell lines, whether drug sensitive or resistant. The efficiency of the process seems to correlate with the expression of the P-gp efflux protein, thus conferring chemo-resistance and increased survival rates in cell lines overexpressing P-gp. MAPS appear to function as both the sinks for the xenodrugs and their transport vehicles outside of the cell. Thus, the more efficient the production of MAPS and their drug sequestration, the more resistant the cancerous cell line is to the drug. Targeting MAPS to inhibit cancer chemo-resistance in humans may not provide a feasible option because this could result in lethal toxicity in normal cells. However, the morphological differences of the mitochondria between both sensitive and resistant cell lines may reveal vulnerabilities in the resistant cell line that can be exploited to counteract their resistance.

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Figure 1.

Clonogenic cell survival assay of MCF-7 and MCF-7Adr. Survival curves showing rates of the two cell lines at different concentrations of MKT-077 for the MCF-7 and MCF-7Adr cell lines.

Figure 2. Effects of MKT-077 on MCF-7 cell line. Untreated controls (A–C): 0 min (A & B), 8 hr (C). D–P, 50 µg/mL MKT-077 treated cells: 30 min (D & E); 60 min (F–H); 90 min (E–F); 90 min (I–L); 3 hr (M & N); 7 hr (O & P). L: lipid droplet; MAPS: mitoautophagosome.

Figure 3.

Ultrastructural effects of MKT-077 on MCF-7Adr cell line. Untreated controls (**A & B**): 8 hr (**A & B**). C–L, 50 $\mu\text{g/mL}$ MKT-077 treated cells: 60 min (**C & D**); 90 min (**E & F**); 2 hr (**G**); 5 hr (**H**); 6 hr (**I**); 7 hr (**J**); 8 hr (**K & L**). L: lipid droplet; MAPS: mitoauphagosome, N: nucleus.

Figure 4. MKT-077 targets mortalin and induces mitophagosomes in MCF-LCC9. **(A)** Western blot showing the silencing of mortalin with siRNA. **(B)** Silencing of mortalin increased LC3-II and PINK1 and reduced ATG5 and p62.