Photoactivation Enhances the Mitochondrial Toxicity of the Cationic Rhodacyanine MKT-077

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ABSTRACT

In this study, the mitochondrial phototoxicity of the cationic rhodacyanine MKT-077 was investigated by comparing its effects on the inhibition of mitochondrial respiration and the structural integrity of mitochondrial DNA (mtDNA) in the presence and absence of added high-intensity visible light (7.5 J/cm²). Results indicate that photoirradiation significantly enhances the mitochondrial toxicity of MKT-077 at both the biochemical and DNA levels. For example, the concentration of MKT-077 required to achieve one-half maximal inhibition of ADP-stimulated respiration was observed to be 6-fold lower in the presence versus absence of high-intensity light (one-half maximal inhibition at 2.5 versus 15 µg MKT-077/mg, respectively). In addition, photoirradiation produced a 25-fold increase in inhibition of succinate-cytochrome c reductase activity by MKT-077 (one-half maximal inhibition at 2 versus 50 µg MKT-077/ml, ±light, respectively) and a 6-fold increase in inhibition of cytochrome oxidase activity (one-half maximal inhibition at 5 versus 30 µg MKT-077/ml, ±light, respectively). Furthermore, the combination of 25 µg/ml MKT-077 and 7.5 J/cm² visible light caused significant degradation of mtDNA in isolated rat liver mitochondria, whereas the same concentration of dye in the absence of light had only a modest effect on mtDNA. Evaluation of light-induced MKT-077 lipid peroxidation in mitochondrial membrane fragments by the thiobarbituric acid test and by measurement of nonrespiratory-linked oxygen uptake suggests that mitochondrial phototoxicity by MKT-077 may be the result of lipid peroxidation via reactive oxygen species. These results have important implications with regard to the potential use of MKT-077 in photochemotherapy.

INTRODUCTION

PCT² is an investigational cancer treatment involving light activation of a photoreactive drug, or photosensitizer, that is selectively taken up or retained by malignant cells (1—3). Whereas ideally the photosensitizer is relatively innocuous to the surrounding normal tissue, local irradiation of the tumor site converts the drug to a toxic or reactive species capable of destroying the malignant cells. PCT, therefore, has the potential to provide a means of highly specific tumor cell killing without injury to normal cells.

The mitochondrion has been implicated as an important, perhaps general perturbation of mitochondrial membranes and consequent nonspecific damage to membrane-bound enzymes (18). MKT-077 also produced a mild-to-moderately degradative effect on mtDNA, but not on nuclear DNA, of various cancer cell types (18). In this study, the mitochondrial phototoxicity of MKT-077 was investigated by measuring the inhibitory effect of the compound on mitochondrial respiratory activity and its effect on the structural integrity of mtDNA in the presence and absence of added high-intensity visible light. Our data demonstrate that the selective mitochondrial toxicity exhibited by MKT-077 is significantly enhanced by photoactivation of the compound and that this effect is most likely the result of lipid peroxidation via reactive oxygen species. These results have important implications with regard to the use of MKT-077 in alternative strategies for treatment of carcinoma.

MATERIALS AND METHODS

Materials. MKT-077 was provided by Fuji Pharmaceuticals and was dissolved in water at a stock concentration of 1 mg/ml. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Cultures. The monkey kidney epithelial cell line CV-1 was grown in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Hyclone) and antibiotics (10,000 IU/ml penicillin and 10,000 µg/ml streptomycin). All cells were maintained at 37°C in a 5% CO₂ atmosphere.

Isolation of Mitochondria. Liver mitochondria were isolated from male Sprague Dawley rats by differential centrifugation as described previously (19). Briefly, approximately 5 g of tissue were minced and homogenized in STE (250 mM succrose, 1 mM Tris-HCl, and 1 mM EDTA (pH 7.4)) and then centrifuged at 600 × g for 10 min at 4°C. The supernatant was collected and centrifuged at 8,000 × g for 10 min at 4°C. The resulting mitochondrial pellet was washed twice in STE and then washed once in 250 mM sucrose and 1 mM Tris-HCl (pH 7.4) to reach a final concentration of approximately 20 µg protein/ml.

Mitochondria were isolated from cultured cells by a modification of the procedure of Maltese and Aprile (20). Typically, 5–10 × 10⁶ cells were harvested in Dulbecco’s modified Eagle’s medium. Cells were pelleted and washed once with homogenization buffer (250 mM sucrose, 1 mM Tris-HCl, 1 mM EDTA, and 1 mg/ml BSA (pH 7.4)) at low speed in a tabletop clinical centrifuge. The final pellet of cells was resuspended in homogenization buffer to a volume of 7 ml and homogenized in a Dounce tissue grinder with a tight pestle until at least 95% of the cells were disrupted (approximately 125 up/down strokes). The homogenate was centrifuged at 300 × g for 10 min at 4°C. The supernatant was removed and saved, and the pellet was resuspended and centrifuged again at 800 × g for 10 min. The supernatants were then pooled and centrifuged at 9400 × g (10 min, 4°C); the pellet was resuspended in homogenization buffer and centrifuged again at 9400 × g, and the final pellet was suspended in homogenization buffer to a volume of about 5 mg protein/ml. Protein concentration was determined by the method of Lowry et al. (21), using BSA as the standard.

Respiration. Oxygen consumption was measured polarographically with a Clark electrode in a 1-ml water-jacketed chamber maintained at 30°C (22). The basic respiratory assay medium consisted of 225 mM succrose, 10 mM KCl, 1 mM EDTA, 10 mM KH₂PO₄—KH₂PO₄, 5 mM MgCl₂, and 10 mM Tris-HCl (pH 7.4); 1 mg/ml BSA was included to stabilize the membranes. An initial rate of oxygen consumption (state 2 rate) was recorded after the addition of substrate (5 mM each glutamate plus malate), MKT-077 (0–35 µg/mg mitochondrial protein).

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1 The abbreviations used are: PCT, phototherapeutic; mtDNA, mitochondrial DNA; TBA, thiobarbituric acid; MDA, malondialdehyde.

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protein), and approximately 0.15 mg of isolated CV-1 mitochondria. Two min after addition of mitochondria, ADP (120 nmol) was added, and a state 3 rate was obtained. After recording a measurable state 4 rate (i.e., rate after ADP is phosphorylated), 80 μM 2,4-dinitrophenol were added to obtain a respiratory rate in the absence of coupled oxidative phosphorylation.

Enzyme Assays. Succinate-cytochrome c reductase activity was determined spectrophotometrically by measuring the rate of increase in absorbance at 550 nm due to the oxidation of cytochrome c (24). Freeze-thawed sonicated rat liver mitochondria were added to a cuvette containing 2 mM KCN, 50 mM potassium phosphate (KH₂PO₄/K₂HPO₄; pH 7.4), and 20 mM succinate at 37°C. The reaction was initiated by adding 1 mg of oxidized cytochrome c to the cuvette, and a change in the absorbance was recorded over time. The reaction was terminated by the addition of 5 μg of antimycin A, which provided a background rate. The assay was repeated in triplicate.

Cytochrome c oxidase activity was determined spectrophotometrically by measuring the decrease in absorbance at 550 nm due to the oxidation of cytochrome c (24). Freeze-thawed sonicated rat liver mitochondria were added to a cuvette containing 40 mM potassium phosphate (KH₂PO₄/K₂HPO₄; pH 7.0) at 37°C. The reaction was initiated by adding 0.7 mg of reduced cytochrome c, and a linear rate was recorded. The assay was repeated in triplicate.

Citrinate synthetase was recorded spectrophotometrically at 412 nm (25). A background rate was obtained by adding freeze-thawed rat liver mitochondria to 0.1 mM 3,5'-dithiobis-2-nitrobenzoate and 0.3 mM acetyl-CoA. This initial rate was subtracted from the rate obtained on addition of the substrate, 0.1 mM succinate.

Photoirradiation. In the assays requiring photoirradiation, intact mitochondria or freeze-thawed mitochondrial fragments were added to a 12 × 75-mm clear glass test tube and incubated in the appropriate assay mixture at the desired dye concentration. Phototreatment was accomplished by using a Kodak slide projector continuously delivering 25 mW/cm² visible light for a period of 5 min (7.5 J/cm²), during which there was no measurable change in temperature of the assay mixture. After phototreatment, the assay mixture was transferred to an appropriate vessel for further treatment and/or analysis.

mtDNA Analysis. Nine mg of isolated intact rat liver mitochondria were resuspended in a final volume of 1.5 ml of H₂O ± 75 μl of MKT-077 (1 mg/ml) ± phototreatment. After the 5-min incubation, mitochondria were pelleted by centrifugation and resuspended in 0.1 ml of ice-cold potassium acetate, 25 mM Tris-CI (pH 8.0), and 10 mM EDTA (pH 8.0). mtDNA was then extracted using an alkaline lysis mini-prep procedure. Briefly, 0.2 ml of SDS/NaOH was added, and the tubes were inverted to mix. After an additional 5 min, 0.15 ml of ice-cold potassium acetate (3 M potassium-5 M acetate) was added, and the samples were centrifuged. The mtDNA was precipitated from the resulting supernatants by addition of isopropanol, washed once with ethanol, and resuspended in 10 mM Tris (pH 8) and 1 mM EDTA. Ten μg of mtDNA extracted from each sample were digested with BamHI (1 h; 37°C), run out on a 1% agarose gel (95 V; 1.5 h), and observed by staining with ethidium bromide. The mtDNA was then transferred to nitrocellulose by Southern blotting (26) and probed with [³²P]dCTP-labeled total rat mtDNA.

TBA Test. The TBA test was used to measure the extent of lipid peroxidation induced by phototreatment of MKT-077 in isolated rat liver mitochondria (27). Three mg of freeze-thawed mitochondrial fragments were resuspended in water to a final volume of 0.5 ml ± 25 μl of MKT-077 (1 mg/ml) in the presence or absence of high-intensity light for 5 min. After incubation, 0.25 ml of a solution of 20% trichloroacetic acid containing 1% FeSO₄ and 0.5 ml of a solution of 0.67% (w/v) TBA were added to each sample. The mixture was then incubated at 80°C for 10 min and cooled to room temperature, and the precipitated protein was removed by centrifugation. The amount of MDA, a by-product of lipid peroxidation, that was present in each sample was determined spectrophotometrically by measuring the absorbance of the corresponding supernatants at a wavelength of 530 nm. The extinction coefficient of MDA = 1.56 × 10³ cm⁻¹/mmol (27).

Polarographic Determination of Lipid Peroxidation. The extent of lipid peroxidation was also evaluated by measurement of nonrespiratory-linked oxygen consumption in freeze-thawed rat liver mitochondria (27). Approximately 3 mg of mitochondrial protein were added to 0.8 ml of buffer solution containing 175 mM KCl, 10 mM Tris-base (pH 7.4), and 0-100 μg MKT-077/mg mitochondrial protein and maintained in a water-jacketed chamber at 37°C. Using a Clark oxygen electrode, the basal rate of oxygen consumption of the sample was recorded. The sample was then incubated at room temperature in the presence or absence of high-intensity light (25 mW/cm²) for 2.5 min. The sample was immediately returned to the water-jacketed chamber, and oxygen consumption was again recorded. The amount of oxygen consumed as a result of lipid peroxidation was determined for each sample by calculation of the difference in oxygen content as measured before and after incubation (and after subtraction of any basal level of oxygen consumption).

RESULTS

Mitochondrial Respiration. The effect of photoactivation of MKT-077 on mitochondrial respiration was determined polarographically, using functionally intact CV-1 mitochondria and glutamate/malate as the respiratory substrate. As shown in Fig. 1, MKT-077 inhibited mitochondrial respiration in a dose-dependent manner in both the presence and absence of added high-intensity light, with one-half maximal inhibition of respiration achieved at concentrations of 2.5 and 15 μg MKT-077/mg mitochondrial protein, respectively. These data indicate a 6-fold potentiation of inhibition of mitochondrial respiration by photirradiated MKT-077 as compared to the nonirradiated control.

Enzyme Assays. MKT-077 was previously shown to inhibit electron transfer activity in freeze-thawed mitochondrial membrane fragments. In this study, the effect of light activation of MKT-077 on electron transfer reactions was examined via determination of succinate-cytochrome c reductase activity (which is a measure of electron transfer from complex II through coenzyme Q to complex III) and cytochrome c oxidase activity (which is a measure of the complex IV enzyme) in freeze-thawed mitochondrial membrane fragments. According to Fig. 2, without photoirradiation, mitochondrial membrane preparations exposed to varying concentrations of MKT-077 exhibited one-half maximal inhibition of succinate-cytochrome c reductase activity at a concentration of 50 μg MKT-077/ml. When photoirradiated, however, one-half maximal inhibition of activity was achieved at 2 μg MKT-077/ml, indicating a 25-fold potentiation of the inhibitory effect of the drug by photoactivation. Fig. 3 shows that photoirradiation of MKT-077 produced a 6-fold increase in the inhibition of cytochrome oxidase activity over that which was produced in the absence of added light, with one-half maximal inhibition obtained at a concentration of 5 and 30 μg/ml, respectively. Photoirradiation of MKT-077 had a minimal effect on citrate synthetase activity, producing only 35% inhibition at a concentration of 100 μg/ml, whereas in the absence of light, the compound had no effect on the activity of this enzyme, even at the highest concentration of MKT-077 tested (0-100 μg/ml; data not shown).

![Fig. 1. Effect of MKT-077 on respiratory activity in isolated CV-1 mitochondria. ADP-stimulated respiration was determined polarographically using glutamate/malate as the respiratory substrate in the presence (●) and absence (○) of 7.5 J/cm² visible light. Data presented are the results from a typical experiment.](image-url)
DISCUSSION

Démonstrated that MKT-077 selectively inhibits mitochondrial respiration than in its absence. Of MKT-077, more oxygen was consumed in the presence of light under the condition of +MKT-077/—light at any given concentration MKT-077/mg mitochondrial protein caused a dose-dependent increase in respiratory-linked oxygen uptake in mitochondrial fragments using a chondria versus mitochondria that had been treated with 25 μg/ml MKT-077. There was no significant difference in MDA production in untreated mitochondrial treated controls (Fig. 5). Furthermore, in the absence of light, there was no significant difference in MDA production in untreated mitochondrial versus mitochondria that had been treated with 25 μg/ml MKT-077 (data not shown).

Evaluation of Lipid Peroxidation. The TBA test was used for direct determination of the lipid peroxidation degradation product, MDA, produced in mitochondrial membrane fragments that had been treated with 25 μg/ml MKT-077 in the presence or absence of high-intensity light. Our results indicate that photoirradiation of MKT-077-treated mitochondria induced a 5-fold increase in MDA production over that which is produced in nonirradiated MKT-077-treated controls (Fig. 5). Furthermore, in the absence of light, there was no significant difference in MDA production in untreated mitochondrial fragments versus mitochondria that had been treated with 25 μg/ml MKT-077 (data not shown).

The extent of MKT-077-induced lipid peroxidation in the presence and absence of added light was further assessed by measuring nonrespiratory-linked oxygen uptake in mitochondrial fragments using a Clark oxygen electrode. As shown in Fig. 6, our data demonstrate that when photoirradiated at a constant light intensity, a range of 0–100 μg MKT-077/mg mitochondrial protein caused a dose-dependent increase in nonrespiratory-linked oxygen consumption. Furthermore, although there was a slight background level of oxygen consumption under the condition of +MKT-077/—light at any given concentration of MKT-077, more oxygen was consumed in the presence of light than in its absence.

mtDNA Analysis. Electrophoresis and Southern blot analysis were used to investigate the effect of light-activated MKT-077 on the structural integrity of mtDNA. According to Fig. 4, the BamHI-digested, —dye, —light control (A and B, Lane 1) produced, as expected, two distinct bands corresponding to approximately 11 and 5 kb in length. A concentration of 25 μg/ml MKT-077 in the absence of light (A and B, Lane 2) had a modest effect on the structural integrity of mtDNA, producing two bands that were slightly less intense than that of the control. Photoirradiation of mtDNA in the absence of MKT-077 was shown to have no effect (A and B, Lane 3), whereas the combination of 25 μg/ml MKT-077 and 7.5 J/cm² visible light (A and B, Lane 4) produced a smear indicating significant degradation of mtDNA.

Our data demonstrate a 6-fold increase in inhibition of both ADP-stimulated respiration and mitochondrial cytochrome oxidase activity and a 25-fold increase in inhibition of succinate-cytochrome c reductase on photoactivation of MKT-077 with 7.5 J/cm² visible light. Although a direct comparison between the effect of photoirradiated MKT-077 on respiratory rates and electron transfer activities is not valid due to vastly different assay conditions, a comparison of the effect of photoirradiated MKT-077 on the two measured electron transfer reactions indicates a 4-fold greater sensitivity of succinate-cytochrome c reductase activity as compared to mitochondrial cytochrome oxidase activity. Interestingly, succinate dehydrogenase activity has previously been found to be much more sensitive than cytochrome oxidase activity to the effects of photosensitization (29).

We previously demonstrated a selective loss of mtDNA, but not nuclear DNA, in cultured carcinoma cells that had been treated with 3 μg/ml MKT-077 over a period of 3 days. In this study, we examined the immediate effect (after a 5-min incubation) of a much higher concentration of MKT-077 (25 μg/ml) ± photoirradiation on the structural integrity of mtDNA extracted from isolated intact rat liver mitochondria. Results showed a modest decrease in the level of mtDNA in +dye/—light samples as compared to —dye/—light controls. This decrease in mtDNA levels was similar in magnitude to that which was observed in cultured carcinoma cells after MKT-077 treatment. A much more pronounced degradation of mtDNA was observed for the combination of +dye/+ light, however. These data suggest that MKT-077 alone, in the absence of light, has a primary and immediate damaging effect on the structural integrity of mtDNA and that this effect can be significantly enhanced by photoirradiation of the compound.

On photoirradiation, certain photosensitizers can react with molecular oxygen to initiate one or more of several types of photochemical processes, each of which can ultimately result in the damage or destruction of important biomolecules. Mitochondria are especially susceptible to the photodamaging effects of lipid peroxidation due to...
the high proportion of polyunsaturated fatty acids comprising their membrane phospholipids. Lipid peroxidation has been previously implicated in the impairment of membrane function and inactivation of membrane-bound enzyme activity (30). There is evidence to suggest that lipid peroxidation induces damage to mtDNA as well (31). The possibility that lipid peroxidation may be involved in MKT-077-induced mitochondrial photodamage was explored by monitoring nonrespiratory-linked oxygen consumption and by measurement of the lipid peroxidation by-product MDA in mitochondria exposed to various concentration of dye plus light. Under these conditions, evidence of lipid peroxidation was detected using both methods. These data suggest that mitochondrial phototoxicity by MKT-077 involves, at least in part, lipid peroxidation via reactive oxygen species. However, because most photodynamic actions are quite complex and can proceed simultaneously by more than one pathway, additional photosensitizing reactions cannot be ruled out. Photoirradiation of MKT-077 did in fact induce a modest inhibition of activity of the mitochondrial matrix enzyme, citrate synthetase, an effect that was not observed in the absence of light (18). These data may also implicate type II photosensitization reactions in the phototoxicity induced by MKT-077. Studies investigating the mechanism(s) of photooxidative damage to mitochondrial proteins by this compound are currently underway.

There recently has been considerable interest in PCT as a form of treatment for locally invasive tumors of the skin, lung, breast, bladder, and brain. Cationic photosensitizers are especially promising as PCT agents for the treatment of carcinoma, because they offer the benefit...
of dual selectivity based on preferential accumulation in carcinoma mitochondria and localized irradiation of a malignant lesion or surgical excision site. Other cationic photosensitizers, including rhodamines, phthalo cyanines, and certain chalcogenopyrpylum dyes (32–35), have been evaluated in preclinical studies for their potential as PCT agents. MKT-077 is the only member of this class of compounds currently undergoing United States Food and Drug Administration-approved Phase 1 clinical trials for the treatment of carcinoma. The favorable pharmacological and photochemical properties of MKT-077 may make this compound an especially attractive candidate for development as a photochemotherapeutic agent as well.

REFERENCES


11. Zanotti, A., and Azzone, G. F. Mitochondria treated with 25 gsg/ml MKT-077 in the presence and absence of added high-intensity light. Results presented are the average of three separate experiments ± SE.

Fig. 6. Nonrespiratory-linked oxygen consumption in freeze-thawed rat liver mitochondria treated with 25 μg/ml MKT-077 in the presence and absence of added high-intensity light. Results presented are the average of three separate experiments ± SE.
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