In Vivo Administration of MKT-077 Causes Partial yet Reversible Impairment of Mitochondrial Function

Ellen L. Weisberg, Keizo Koya, Josephine Modica-Napoliitano, Yang Li, and Lan Bo Chen

ABSTRACT

The effects of in vivo administration of a pharmacologically toxic dose of the lipophilic cationic compound, MKT-077, were investigated in selected vital organs of the rat. MKT-077 (15 mg/kg body weight), administered by bolus i.v. injection every day for 5 days, did not detectably influence rat heart and kidney mitochondrial respiration. Although the same dosage of MKT-077 significantly decreased respiratory rates in rat liver mitochondria relative to untreated controls, complete recovery was evident within 3 days following drug withdrawal. Whereas the mitochondrial DNA of rat kidney and liver appeared to be unaffected by MKT-077 treatment, levels of heart mtDNA were noticeably less than control levels in the immediate interval following drug administration. However, this latter effect was partially reversed as early as 10 days following treatment and completely reversed within a 30-day posttreatment period. These results strongly suggest that a pharmacologically toxic dose of MKT-077 minimally affects the overall functional integrity of mitochondria in such critical, although highly vulnerable, tissues as the heart, liver, and kidney.

INTRODUCTION

MKT-077 (formerly known as FJ776) is a novel rhodacyanine dye found to be selectively taken up by the mitochondria of carcinoma cells (1) and demonstrated both in vitro and in vivo to display anticaneroma activity. MKT-077 is currently undergoing investigation as a potential anticancer agent in Phase III clinical trials.

In contrast to the vast majority of clinically available anticanceroma compounds for which nuclear DNA is the primary molecular target, the lipophilic cation MKT-077 pinpoints the mitochondria of tumor cells and subsequently influences the morphological and functional integrity of these structures (1–4). Delocalization of the positive charge of lipophilic cations enables them to pass through the hydrophobic barriers of plasma membrane and mitochondrial membrane lipid bilayers, and the high negative charge existing inside the mitochondria strongly attracts the positively charged drug (5). The attraction of lipophilic cations is greater for tumor cell mitochondria than for the mitochondria of normal cells, primarily because the mitochondrial membrane potential of carcinoma cells is higher than that of normal epithelial cells (2, 3, 6, 7).

Among the lipophilic cations that display a concentration-dependent mitochondrial toxicity are rhodamine 123, which inhibits mitochondrial ATP synthesis at the level of F$_{1}$/F$_{0}$ ATPase (8), and decaquium chloride, which inhibits NADH-ubiquinone reductase (9). MKT-077 has been demonstrated to inhibit the mitochondrial respiration of cancer cells, most likely via a nonspecific perturbation of the mitochondrial membrane, and to have a mild-to-moderately degradative effect on the mtDNA, but not nuclear DNA, of a variety of cancer cell types (10). Since mitochondria are the primary sites of ATP-generating aerobic metabolism, selective mitochondrial toxicity in carcinoma cells that results from the enhanced uptake and retention of lipophilic cations provides the basis for the selective anticaneroma activity displayed by these compounds (1–3, 6, 7). However, since high concentrations of lipophilic cations can be toxic to all mitochondria, it is conceivable that in vivo administration of certain lipophilic cations at high concentrations and/or for prolonged periods of time might induce adverse effects in the mitochondria of normal cells as well as of the targeted carcinoma cells.

The purpose of this study was to investigate the in vivo effects of a toxic dose of MKT-077 on several selected vital tissues. This was accomplished by examining both mitochondrial bioenergetic function and the integrity of mtDNA in the heart, liver, and kidneys of Sprague-Dawley rats treated with a previously determined toxic dose of MKT-077. Our results, which indicate a partial yet completely reversible impairment of mitochondrial function in several critical, yet highly vulnerable, rat organs, have important positive implications with regard to the clinical use of MKT-077 in the treatment of carcinoma.

MATERIALS AND METHODS

Drug Treatment for Analysis of Mitochondrial Respiratory Activity and Measurement of mtDNA Levels. Female Sprague-Dawley rats, average weight of 120–125 g, received i.v. injections of a bolus dose of 15 mg MKT-077/kg body weight dissolved in D$_{2}$W solution every day for 5 days. Control rats received i.v. injections of a bolus dose of the vehicle, D$_{2}$W solution, every day during this 5-day period. Following sacrifice, the liver, heart, and kidneys were removed for analysis of mitochondrial respiratory activity on the fifth day of treatment, after 3 days of recovery following treatment, and after 10 days of recovery following treatment. For analysis of mtDNA levels, the heart and kidney were removed from animals receiving i.v. injections on the fifth day of treatment, after 3 days of recovery following treatment, after 10 days of recovery following treatment, and after 30 days of recovery following treatment. The liver was removed on the fifth day of treatment, after 2 days of recovery following treatment, and after 22 days of recovery following treatment.

Isolation of Mitochondria. The liver, kidneys, and heart of MKT-077-treated and control rats were excised immediately following the sacrifice of the animal and placed in the appropriate ice-cold isolation medium. Liver mitochondria were isolated by differential centrifugation at 4°C as described previously (11). Briefly, tissue was minced and homogenized in STE at 600 × g. The mitochondrial pellet was resuspended and washed twice in STE, followed by one wash in ST (250 mM sucrose, 1 mM Tris-HCl, pHi 7.4), and centrifuged at 600 × g. The mitochondrial pellet was resuspended and washed twice in STE, followed by one wash in ST (250 mM sucrose, 1 mM Tris-HCl, pH 7.4), and resuspension of the final pellet in ST. Mitochondria were isolated from whole kidney tissue according to the same procedure, except that there were only two washes, one in STE and one in ST.

Mitochondria were isolated from heart ventricular tissue (bottom 2/3 of organ) by a modification of the procedure by Palmer et al. (12). Heart tissue was placed in ice-cold buffer I (10 mM KCl, 50 mM 4-morpholinepropanesulfonic acid, and 2 mM EGTA, pH 7.4, plus 0.2% BSA) and rinsed 5–10 times to remove blood. The tissue was quickly weighed, minced finely with a razor blade, and placed in a beaker with ice-cold buffer I at a final concentration of 10 ml buffer/kg tissue. Nagarse (5 mg/g wet weight tissue) was added to the beaker, and the mixed solution was allowed to sit at 4°C for 2 min. The tissue was homogenized using a Polytron, and the resulting homogenate was diluted two times with buffer I and centrifuged at 5000 × g for 5 min. After discarding the supernatant,
The supernatant was removed and combined with the previously
each day for MKT-077-treated animals; 0, weights recorded each day for untreated
and centrifuged at 500 \( \times \) g for 10 min. The supernatant was collected and
various tissues of MKT-077-treated and control rats was measured polaro-
consumption (state 2) was recorded following the addition of a substrate, either
chamber maintained at 30°C (14). The assay medium consisted of 225 mist
resuspended and washed two times in buffer I. The final pellet was resus-
chloroform/isooamyl alcohol, spun for 10 mm at 8000 rpm, extracted again with
DNA was partially depurinated in 0.25 M HCl for 10 min, treated with 0.4 M NaOH-0.6 M NaCl for 30 min for hydrolysis of the phosphodiester backbone, and finally neutralized in 1.5 M NaCl-0.5 M Tris-HCl (pH 7.5). DNA was transferred to a GeneScreen Plus nylon membrane (NEN, Boston, MA) by overnight capillary blotting in 10× SSC (0.15 M trisodium citrate and 1.5 M NaCl) according to the method of Southern (15).
Filters were soaked briefly in 2× SSC and prehybridized for 15–20 min at 42°C in 50% deionized formamide (pH 7.5), 0.02 M Tris, 1× SSC, 1× Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 10% dextran sulfate, and 1% SDS. Filters were subsequently hybridized in the same hybridization fluid containing 100 μg/ml single-stranded salmon sperm DNA and 1–2 ng/ml of CT32P-labeled probe (3 × 10^6 cpm/μg).
After labeling using the random 14-mer primer method (NEN, Boston, MA), probes were purified by elution over a Select-D, G-50 spin column (5 Prime—3 Prime, Inc., Boulder, CO). Probes were boiled for 5 min and placed on ice for 2 min prior to addition to hybridization fluid. Following overnight hybridization at 42°C, filters were autoradiographed using an intensifying screen at —80°C with Kodak X-Omat AR film.

Probes. The human mtDNA probe [excised and purified from the pGEM-72f(+) vector] consisted of a portion of the mitochondrial genome (8729–10254 bp) containing the coding region for the protein product COXIII and for part of the protein products ATPase 6 and ND3. The 540-bp G3PDH probe, a gift from Dr. Graham Barnard (University of Massachusetts Medical Center, Worcester, MA), was PCR amplified (16) from a cDNA clone using the primers 5′-ATGGGGAAGTTGAAGTCGG-3′ and 5′-GGGTGCTAAG-CAGTTGGT-3′ (17) and was used as an internal control for all experiments.

RESULTS

Effect of 15 mg/kg of MKT-077 on Average Body Weight of
Rats. To effectively study the extent of mitochondrial impairment that might occur in normal tissues as a result of MKT-077 treatment, it was essential that we used the highest possible nonlethal dosage of the drug. This was determined to be 15 mg MKT-077/kg body weight, a maximally tolerable dose in the rat.3 We assessed the pharmacologi-

capillary blotting in 0.15 M trisodium citrate and 1.5 M NaCl for 30 mm for hydrolysis of the phosphodiester backbone, and finally neutralized in 1.5 M NaCl-0.5 M Tris-HCl (pH 7.5). DNA was transferred to a GeneScreen Plus nylon membrane (NEN, Boston, MA) by overnight capillary blotting in 10× SSC (0.15 M trisodium citrate and 1.5 M NaCl) according to the method of Southern (15).
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nished from the liver, kidneys, and heart of female Sprague-Dawley rats that had been treated with the compound. Fig. 2 shows that the succinate-induced, ADP-stimulated respiratory rate in mitochondria isolated from the liver of rats treated with a bolus i.v. injection of 15 mg MKT-077/kg body weight each day for 5 days is significantly lower than that of untreated controls. However, this inhibition is reversible since respiratory rates returned to and were maintained at (or above) control values in mitochondria isolated from the liver of animals sacrificed on days 3 and 10 following completion of the 5-day treatment regimen. A similar effect was induced by MKT-077 on ADP-stimulated respiration when a combination of glutamate and malate was used as the respiratory substrate and on ADP-stimulated respiratory rates when either succinate or the combination of glutama-
ate and malate was used as the respiratory substrate (data not shown).

Results show no significant effect of in vivo administration of MKT-077 on respiratory activity in mitochondria isolated from the

3 K. Koya, unpublished data.
Effect of In Vivo Administration of MKT-077 on mtDNA Levels. We examined the effect of the in vivo administration of MKT-077 on levels of mtDNA in the liver, heart, and kidneys of treated female Sprague-Dawley rats. Total cellular DNA from tissues of animals that had been treated by i.v. injection with 15 mg MKT-077/kg body weight on designated days and digested with PvuII, which yields a single 16.5-kb band of mtDNA. Following an overnight run on an ethidium bromide-stained gel, the DNA was transferred to a nylon membrane and hybridized to either the 32P-labeled mitochondrial probe or the G3PDH probe, which is representative of nuclear DNA.

In the rat heart, although there was no detectable effect by MKT-077 on mtDNA levels immediately following 5 days treatment, there was an apparent decrease relative to control by the third day of recovery (Fig. 5B). Heart mtDNA levels in treated rats increased slightly by the 10th day of recovery, and by the 30th day of recovery, treated and control levels of mtDNA were equivalent (Fig. 5B). Fig. 5, A and D, which show ethidium bromide-stained, PvuII-digested total DNA, and Fig. 5, C and F, which show 32P-labeled G3PDH in each DNA sample, suggest equivalent loadings of DNA between the lanes. Since there is no detectable degradation of total cellular DNA and no apparent effect of MKT-077 on the nuclear gene, G3PDH, it can be assumed that the effect of MKT-077 on heart mtDNA is a selective effect.

DISCUSSION

The MKT-077-associated impairment of tumor cell mitochondrial function and the marked influence of this drug on the morphological integrity of tumor cell mitochondria have been well documented (10). Levels of drug required to inhibit both ADP- and DNP-stimulated mitochondrial respiration in a dose-dependent manner were found to be significantly less in a variety of cancer cell lines, including CX-1 (colon cancer), MCF-7 (breast cancer), and CRL1420 (pancreatic cancer), than in the normal green monkey kidney cell line, CV-1. Similarly, the fact that MKT-077 was demonstrated to have a selective degradative effect on the mtDNA of the aforementioned cancer cell lines but no detectable effect on normal CV-1 cells further suggests that this compound exhibits a discriminative toxicity toward cells of a malignant nature.

Selective mitochondrial toxicity resulting from increased mitochondrial uptake and retention of MKT-077 has been proposed to be the basis for the selective antitumor effects of this compound. However, the potentially deleterious effects of high concentrations of MKT-077 on the mitochondrial integrity of normal cells as well as of tumor cells could, in theory, have a serious impact on the clinical use of this compound. Consequently, such a critical issue as this is in need of immediate and intensive investigation.

We chose to explore the possibility that, when administered in vivo at high doses and/or for prolonged periods of time, MKT-077 might induce mitochondrial toxicity in such normal tissues as the liver, heart, and kidneys of the rat. These organs were chosen in accord with previous findings that show them to be the major sites of accumulation of MKT-077 following drug treatment. In addition, these vital tissues traditionally exhibit the greatest risk for, and consequences of, drug toxicity.

The data obtained show that a dose of 15 mg MKT-077/kg body weight/day for 5 days had no effect on kidney or heart mitochondrial respiration or DNA. In contrast, the same treatment caused a significant decrease in the respiratory rate of mitochondria isolated from rat liver (approximately 33% control values). However, complete recovery from this effect was evident at 3 days following completion of the 5-day treatment regimen (T), n = 3, and from untreated controls (C), n = 3. 5 day treatment, 10 day recovery: mitochondria were isolated from animals 10 days following completion of the 5-day treatment regimen (T), n = 4 and from untreated control (C), n = 4. ADP-stimulated respiration was calculated as the state 3 – state 2 rates (i.e., rate attributed solely to addition of ADP); bars, SE. Succinate was used as the respiratory substrate.

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Fig. 4. Effect of in vivo administration of MKT-077 on mtDNA levels in rat liver. 6/8 L C, 6/8 L TR: total DNA extracted from liver tissue of animals that had received a bolus injection of vehicle or 15 mg MKT-077/kg body weight, respectively, each day for 5 days. 6/10 L TR: total DNA extracted from liver tissue of animals 2 days following completion of 5-day treatment regimen. 6/30 L TR: total DNA extracted from liver tissue of animals 22 days following completion of 5-day treatment regimen. A, ethidium bromide-stained PvuII-digested total tissue DNA (10 μg/lane); B, hybridization of DNA shown in A with 32P-dCTP-labeled rat mtDNA probe; C, hybridization of DNA shown in A with 32P-dCTP-labeled human G3PDH cDNA probe (internal control). Control, n = 1; treated, n = 1.

Fig. 5. Effect of in vivo administration of MKT-077 on mitochondria DNA levels in rat heart. 5/6 H C, 5/6 H TR: control and treated samples, respectively, of total DNA extracted from heart tissue of animals that had received 5-day treatment regimen described in Fig. 4. 5/9 H C, 5/9 H TR: control and treated samples, respectively, of total DNA extracted from heart tissue of animals 3 days following completion of 5-day treatment regimen. 5/16 H C, 5/16 H TR: control and treated samples, respectively, of total DNA extracted from heart tissue of animals 10 days following completion of 5-day treatment regimen. 6/6 H C, 6/6 H TR: control and treated samples, respectively, of total DNA extracted from heart tissue of animals 30 days following completion of 5-day treatment regimen. A and D, ethidium bromide-stained PvuII-digested total tissue DNA (10 μg/lane); B and E, hybridization of DNA shown in A and D, respectively, with 32P-dCTP-labeled rat mtDNA probe; C and F, hybridization of DNA shown in A and D, respectively, with 32P-dCTP-labeled human G3PDH cDNA probe (internal control). Control, n = 2; treated, n = 2.
treatment regimen and was stably maintained 10 days following drug removal. Whereas MKT-077 treatment had no effect on liver and kidney mtDNA levels, it caused a slight decrease in the level of mtDNA, but not nuclear DNA, in the heart as compared to the untreated control. In contrast to the inhibitory effect on mitochondrial bioenergetic function, the effect of MKT-077 on heart mtDNA seemed to lag behind the treatment schedule and did not become apparent until 3 days following completion of drug treatment. By the 10th day following drug removal, mtDNA levels were again approaching control values, and by the 30th day, there was no apparent difference in mtDNA levels between treated versus control samples. The fact that mitochondrial toxicity by MKT-077 was detected only at the level of mitochondrial respiration in the liver and only at the level of mtDNA in the heart is interesting in that it suggests two separate and distinct sites of action of the drug.

The results of this study indicate that although an effectively high dose of MKT-077 administered in the rat did produce some adverse effects in the mitochondria of the liver and heart, these effects were completely and stably reversible within a short period of time following completion of drug treatment. This finding has important positive implications with regard to the severity of potential side effects that may result from clinical use of the drug, since patients will undoubtedly receive well below the dose of MKT-077 comparable to that used in these experiments. Further, these results are in stark contrast to the irreversible mitochondrial toxicity that was observed to result from treatment of patients with the nucleoside analogue and experimental anti-hepatitis drug 1-(2-deoxy-2-fluoro-1-β-D-arabinofuranosyl)5-iodouracil (FIAU) (18) and points to the very different mechanisms of action of these drugs.

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