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Relationship of mitochondrial function and cellular adenosine triphosphate levels to pMC540 and merodantoin cytotoxicity in MCF-7 human breast cancer cells

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In previous studies we have reported that preactivated merocyanine 540 (pMC540) and its chemically synthesized isolates merocil and merodantoin mediate their preferential cytotoxicity towards certain types of malignant cells including human breast cancer cells *in vitro* and *in vivo*. The mechanism of cytotoxic action appears to be, in part, via initial interaction with topoisomerase II leading to apoptosis. To further build upon these findings we now show that pMC540 and merodantoin disrupt mitochondrial morphology and function in intact MCF-7 human breast cancer cells as seen by their causing the release of rhodamine 123 from prestained cells, a rapid reduction in ATP levels, inhibition of succinate dehydrogenase activity and oxygen consumption. These data suggest that mitochondria may also be an important target for the cytotoxic action of pMC540 and merodantoin mediated through disruption of the energy balance.

Key words: ATP, breast cancer, merocyanine 540, merodantoin, mitochondria, ultrastructure.

Introduction

An ideal requirement of any cancer therapy is that the chemotherapeutic agent must spare the host while destroying the malignancy. Systematic experiments, ongoing for the past 9 years, have resulted in the evolution of pMC540 and merodantoin (*N,N'*-dibutyl-2-thio-4,5-imidazolidion), which remain the most effective and easily tolerated agents produced by this effort. The evolution of pMC540 and merodantoin is based on a patented technology called 'preactivation'. Preactivation is a process in which a photoactive compound is illuminated prior to its use in biological systems. Singlet oxygen generated during the process of pre-illumination reacts with the dye molecule itself and results in the formation of heretofore unknown products.[1–3] Photoproducts of merocyanine 540 formed under the conditions of preactivation have been termed pMC540.[1] Other researchers in this field have now reported that pre-illumination of merocyanine 540 produces photoproducts which could play a role in cytotoxicity.[4] Formation of stable and unstable photoproducts from other photoactive compounds has also been reported.[4–7]

Active photoproducts merodantoin and merocil present in pMC540 have been isolated and characterized. Merodantoin has been chemically synthesized and is currently in use in comparison to pMC540. We have reported that these photoproducts are effective mediators of preferential cytotoxicity towards enveloped viruses and certain types of tumor cells *in vitro* and *in vivo*.[1,2,8–13] Three photoproducts from preactivated merocyanine have been isolated and characterized.[14] Studies of the *in vivo* effects of pMC540 and merodantoin against solid human MCF-7 breast tumor xenografts have shown that these compounds are effective inhibitors of breast tumor growth and that they do not produce any organ specific toxicity.[15]

Recent data shows that pMC540 and merodantoin block the rejoining reaction of DNA topoisomerase II by stabilizing a reversible enzyme–DNA complex, termed cleavable complex. Protein denaturant treatment of the cleavable complex leads to double-stranded DNA breakage. These compounds also prevent DNA, RNA, protein synthesis, cause imbalance of intracellular calcium resulting in compromised membrane integrity with marked swelling and induction of apoptosis leading to cell death.[16]

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Early, potentially irreversible swelling, of an injured cell may be due to direct membrane damage or to ATP depletion and disturbance of membrane pump activity.

However, the effect of these compounds on mitochondrial morphology or function is not known. In this paper we report that treatment of human breast cancer MCF-7 cells with pMC540 and merodantoin causes marked alterations in mitochondrial morphology and function leading to a rapid reduction in ATP levels, inhibition of succinate dehydrogenase activity and oxygen consumption. Taken together, these data suggest that mitochondria represent an important intracellular target for pMC540 and merodantoin mediated cytotoxicity.

Materials and methods

Chemicals

Merocyanine 540 was purchased from Eastman Fine Chemicals, Eastman Kodak Co. (Rochester, NY). Merodantoin (*N, N'*-dibutyl-2-thio-4,5-imidazolidion) was originally isolated and purified from pMC540 and synthesized in our laboratories.[14] Rhodamine 123 was purchased from Sigma (St Louis), enzymes were purchased from Boehringer Mannheim (New York, NY). Protein assay kit was purchased from BioRad laboratories (Hercules, CA).

Cells

Human breast cancer cell line MCF-7 was purchased from ATCC (Rockville, MD), and monolayer cultures were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 0.25 mmol/l L-glutamine, 25 mmol/l HEPES and 25 μg/ml gentamicin sulfate (Gibco, Grand Island, NY), and kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Rhodamine 123 retention assay and fluorescence microscopy

Retention of rhodamine 123 in mitochondria of pMC540 and merodantoin treated MCF-7 cells was determined as described earlier.[17] Briefly, MCF-7 cells, 5×10^5/ml were plated in 12-well culture dishes, 1 ml per dish for 48 h. The cells were incubated with 10 μg/ml rhodamine 123 for 10 min and washed in PBS. The cells were treated with 210.5 μM pMC540 and 103.3 μM merodantoin for 10, 20 and 30 min, and 1 and 2 h. After the treatment, rhodamine retained in the cells was dissolved in 1 ml of 1% sodium dodecyl sulfate in distilled water for more than 1 h. Intensity of fluorescence was determined with a fluorescence spectrophotometer (model 204A, Perkin-Elmer, Norwalk, CT) with excitation and emission filters of 490 and 530 nm, respectively. For fluorescence distribution studies rhodamine 123 loaded MCF-7 cells were either left untreated or treated with 210.5 μM pMC540 and 103.3 μM merodantoin for 10, 20 and 30 min, and 1 and 2 h, and observed by using a fluorescence microscope (Olympus Vanox-AHBT equipped with fluorescence illuminator AH2-RFA, Lake Success, NY).

Electron microscopy

MCF-7 cells (1×10^6) were plated in a 60 × 25 mm Petri dish. The cells were pre-cultured for 24 h and then treated with pMC540 and merodantoin for 1, 2, 4 and 8 h. After the treatment, cells were washed in phosphate buffered saline (PBS), fixed in PBS containing 2.5% glutaraldehyde for 1 h and washed in PBS overnight. After being post-fixed in 1% osmium tetraoxide for 30 min, the cells were dehydrated and embedded in spurr medium (Electron Microscopic Service, Fort Washington, PA). Thin sections were cut on Sorvall Ultracut. Ultrastructures of the cells were observed with a Phillips 300 electron microscope.

Oxygen consumption

Oxygen utilization in whole cells was measured using a Clarke oxygen electrode (YSI, Yellow Springs, OH). The oxygen solubility in the chamber was calibrated at 234 nmol/ml and 35°C. Cell dilutions ranged from 2×10^5 to 3×10^6 cells/ml. Linear oxygen consumption in the cells was measured in PBS. The micro chamber (0.6 ml volume) was used to estimate the basal rate of oxygen consumption. Values used to calculate the nanomoles of oxygen consumption per milligram protein fell within the linear range of the rate curve for oxygen consumption. Oxygen consumption of both control and treated cells at various time points was determined alternatively.

ATP content

MCF-7 cells were treated for various time periods ranging between 15 min and 24 h with pMC540 and merodantoin. Cells (5×10^6) were collected by scraping and mixed with equal volumes of 5% perchloric acid and kept at 4°C on ice. After extraction for 30 min with intermittent manual stirring, the samples were centrifuged and the precipitated protein was removed. The acid extracts were neutralized with a solution of 0.5 M triethanolamine and 3.0 M K_2CO_3. The insoluble potassium perchlorate was removed by centrifugation and extracts were analyzed at once. All steps were carried out at 4°C. ATP was determined enzymatically essentially as described by Adams *et al.*[18] Briefly, ATP was determined on 0.5 ml samples of neutralized extracts with phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase. Changes in the absorbance of the added NADH in these determinations were measured at 340 nm with a spectrophotometer (Perkin-Elmer). In each measurement, triplicate samples were used. The chemiluminescence of four dilutions per sample was measured in order to ensure that the ATP concentrations obtained fell within the linear range of the curve. An ATP standard curve was used to determine total ATP content/mg protein. Quantitation of proteins in each sample was determined with BioRad protein assay reagents.[19]

Succinate dehydrogenase assay

Succinate dehydrogenase activity was measured by the reduction of 2,6-dichlorophenol indophenol as described earlier.[20] Briefly untreated and pMC540 (210.5 μM) treated MCF-7 human breast cancer cells were washed twice and re-suspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.8. After sonication, an aliquot of 75 μl of 0.6 M succinate was added, the mixture was kept on ice for 1 h and then 1 N NaOH was used to adjust the pH to 9.0. To this mixture 200 μl of *n*-butanol was added and stirred continuously for 15 min on ice. The samples were then centrifuged at 2000 r.p.m. for 45 min and the clear middle layer was removed. The pH of this layer was adjusted to 6.0 with 2 N acetic acid. The samples were then added to 0.16 mM 2,6-dichlorophenol indophenol, 2.0 mM KCN, 0.9 mM PMS, 1 μg antimycin A and 50 mM potassium phosphate buffer, pH 7.8, to a final reaction volume of 1 ml. The reaction was followed at 600 nm by the addition of 10 mM succinate to the control and read by using a Perkin-Elmer 522A spectrophotometer.

Statistical analysis

All values reported are means ± SD. Student's *t*-tests were used to determine statistical significance. Calculations were performed using the Biostatistics program version 1.12, Graph Pad computer program for Macintosh (San Diego, CA) and values of $p < 0.05$ were considered significant.

Results

Fluorescence microscopy and rhodamine 123 retention assay

The effects of pMC540 or merodantoin on mitochondrial structure in living cells was determined with the aid of the mitochondrial vital stain rhodamine 123. This fluorescent cation is specifically taken up by active mitochondria maintaining a potential gradient across the inner membrane of the organelle.[21] In untreated cells, the fluorescence of the rhodamine probe was distributed mainly in the mitochondria of the cells, which appear as brightly fluorescent cytoplasmic bodies (Figure 1A). In contrast, the fluoresence intensity retained in the mitochondria was remarkably diminished in the cells treated with pMC540 for a period of 30 min as determined by fluorescence microscopy (Figure 1B). The reduction in fluorescence intensity was easily visible after as little as a 10 min period of drug treatment.

Similar results were obtained when cells were treated with merodantoin (not shown). Spectrophotometric analysis of similarly treated cells shows (Figure 2) that as compared with the untreated controls, there was a significant loss of fluorescence from drug treated cells which continued to decline over a period of 7 h. This loss of fluorescence occurred in a time and drug dose dependent manner. A 50% reduction in fluorescence intensity occurred after 4 h of treatment with the highest dose (210.5 μM) of pMC540 used.

Effect of pMC540 and merodantoin on mitochondrial structure

Electron microscopy data show that pMC540 and merodantoin caused a significant effect on the morphology of MCF-7 mitochondria. The ultrastructure of MCF-7 cells treated with pMC540 or merodantoin was examined on spurr-embedded thin sections. In control (untreated) MCF-7 cells, euchromatin and

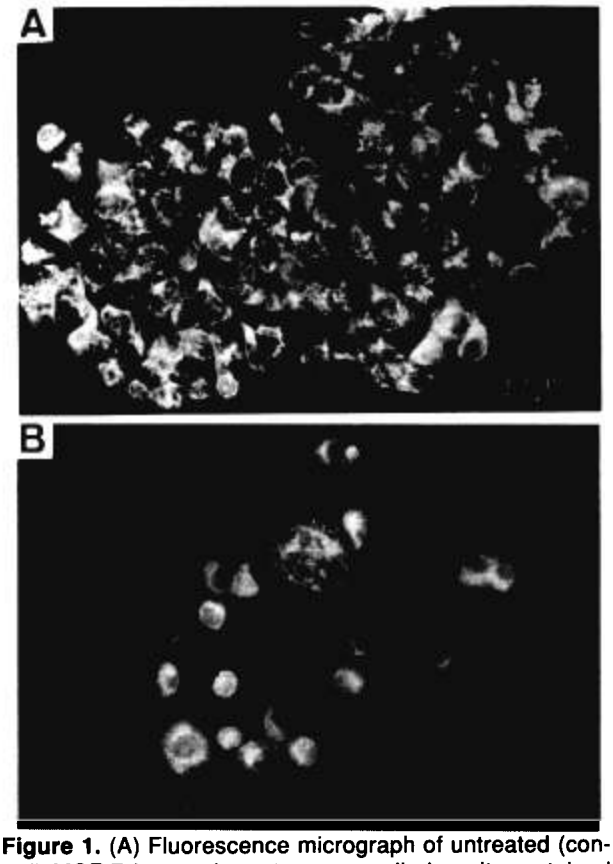

Figure 1. (A) Fluorescence micrograph of untreated (control) MCF-7 human breast cancer cells in culture stained with rhodamine 123. Bright fluorescence is predominantly distributed in the mitochondria of cells which fluoresce as small cytoplasmic bodies. (B) Fluorescence micrograph of pMC540 (210.5 μM) treated MCF-7 human breast cancer cells. Cells were stained with rhodamine 123 and examined following a 10, 20, 30, 60 or 120 min treatment with pMC540. The photograph was taken after a 30 min treatment showing less fluorescence of the majority of mitochondria as well as loss of nuclear detail. Similar results were obtained after treatment of MCF-7 cells with 103.3 μM merodantoin (photograph not shown).

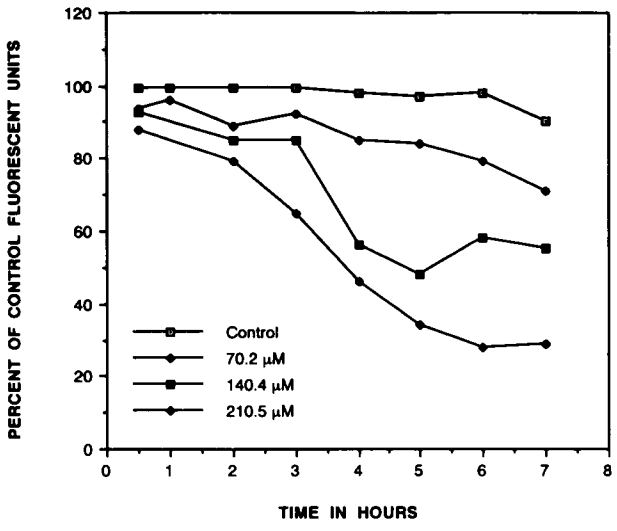

Figure 2. Mitochondrial retention of rhodamine 123 in the absence and presence of different doses of pMC540. MCF-7 human breast cancer cells (5×10^5/ml) were plated in 12-well culture dishes, 1 ml per dish, for 48 h. The cells were loaded with 10 μg/ml rhodamine 123 for 10 min, washed in PBS and then treated with 210.5 μM pMC540 and 103.3 μM merodantoin for 10, 20 and 30 min, and 1 and 2 h. After the treatment, rhodamine retained in the cells was dissolved in 1 ml of 1% sodium dodecyl sulfate in distilled water for more than 1 h. At the time point of interest retained fluorescence was measured by a fluorescence spectrophotometer. Mean values of three separate experiments calculated as a percent of control fluorescent units are shown.

prominent nucleoli in the nucleus and mitochondria with intact cristae were found in the cytoplasm (Figure 3A). After treatment of cells for 60 min and later time points with pMC540 or merodantoin, the nuclei were convoluted, the nucleoplasm was more diffuse and the mitochondria were considerably swollen. Various stages of damage were observed in the morphological degeneration of these organelles in cells treated with pMC540 and merodantoin. The following four stages of sequential alterations were observed: (i) the matrix turned less granular; (ii) electron translucent, mitochondria were swelling and the cristae were discontinuous; (iii) cristae penetrated only a small portion of the interior of the mitochondria while extensive swelling greatly stretched the outer double mitochondrial membrane, as the mitochondrial volume increased significantly; and (iv) the integrity of the outer mitochondrial membrane was lost, resulting in the gradual collapse of the organelle and the eventual disruption of all membranous material (Figure 2B). The observed changes in the mitochondrial structure were time and drug concentration dependent. Mitochondria reached stage 2 with a drug incubation time as short as 60 min, though the full range of changes manifest after several hours. The maximum amount of morphological changes was seen in cells treated with 210.5 μM of pMC540 or 103.3 μM of merodantoin (Figure 3B).

Oxygen consumption

The amounts of total and CN^- resistant respiration were compared in untreated control and pMC540 and merodantoin treated MCF-7 cells for various time points (15 min to 24 h). Mitochondrial

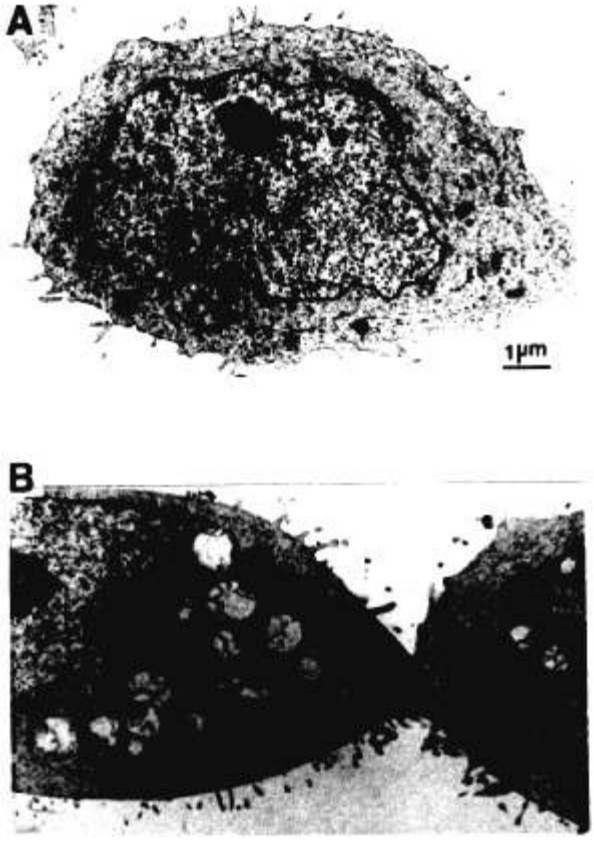

Figure 3. (A) Electron micrograph of MCF-7 human breast cancer cells. A prominent nucleus (N) with euchromatin and a prominent nucleolus (Ni) is centered in cytoplasm containing small ovoid mitochondria (arrows) in condensed form occupied by cristae that traverse the mitochondrial matrix. (B) Electron micrograph of two MCF-7 human breast cancer cells following 8 h incubation with 210.5 μM of pMC540. The nuclei (N) are more irregular in contour, the chromatin is more dispersed and the nucleoli (Ni) are present. The cytoplasm contains swollen mitochondria showing disorientation or total loss in some cases of cristae (arrows).

respiration (values obtained by subtracting the CN^- resistant respiration from total cellular respiration) was dramatically reduced within 15 min of treatment with pMC540 or merodantoin. This initial reduction in respiration was followed by a period of gradual recovery to near basal levels over a period of 4 h. In MCF-7 cells treated with pMC540 this recovery leveled off at 4 h (Figure 4). In merodantoin treated cells, this recovery was virtually complete by 4 h. However, in both cases by 6 h the respiration dropped back to levels comparable to those obtained at 15 min. Respiration at the 24 h time point was not taken into consideration due to significant cell death induced by pMC540 and merodantoin treatments.

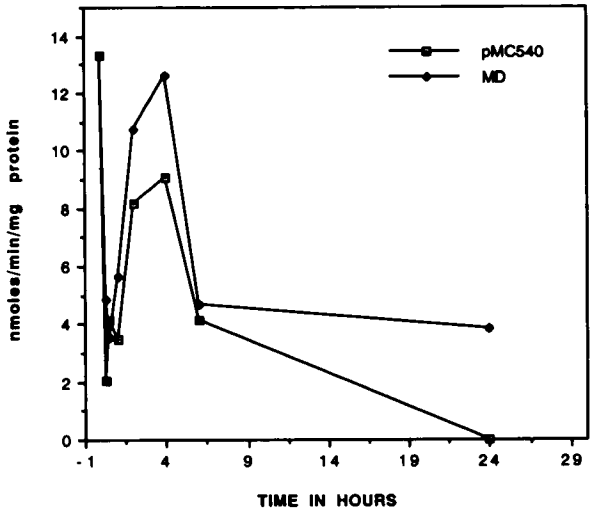

Figure 4. Effect of pMC540 (210.5 μM) and merodantoin (103.3 μM) on oxygen consumption in MCF-7 human breast cancer cells. Three separate experiments were performed in triplicate to determine the total and cyanide resistant respiration. The values of total KCN resistant respiration were subtracted from the total cellular respiration values to obtain total mean values of mitochondrial oxygen consumption shown.

Effect of pMC540 and merodantoin on levels of cellular ATP

Effect of pMC540 and merodantoin on the ATP levels in MCF-7 mammary adenocarcinoma cells was investigated. Results show (Figure 5) that ATP levels dropped off by approximately 50% within 30 min of treatment with pMC540 or merodantoin. Treatment of MCF-7 cells with pMC540 (210.5 μM) caused a 49–57% reduction in cellular ATP levels whereas treatment with merodantoin (103.3 μM) caused a 32–45% reduction over a period of 15 min to 12 h. A drug dose dependent reduction in cellular ATP levels was observed in MCF-7 cells exposed to various concentrations (70.2–210.5 μM) of pMC540 and (41.3–103.3 μM) of merodantoin (data not shown). Treatment of MCF-7 cells with pMC540 also caused a dose dependent reduction in the activity of succinate dehydrogenase (Figure 6). An exception to this trend was a transient stimulation of succinate dehydrogenase activity observed at the lowest dose of 70.2 μM. At the highest dose (210.5 μM) there was a 16 and 49% reduction in the enzyme activity after a treatment period of 30 min and 2 h, respectively.

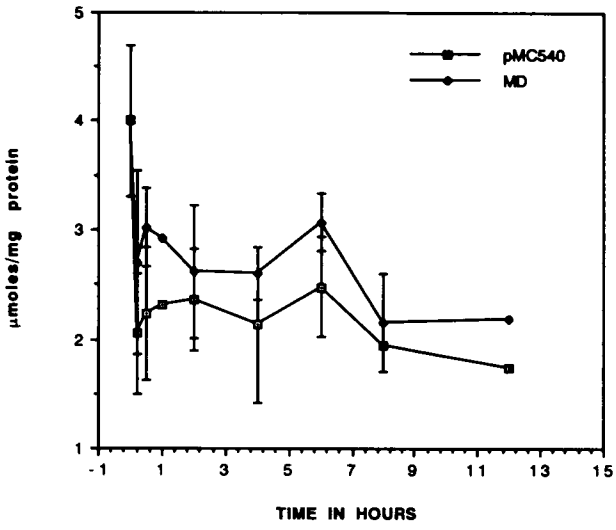

Figure 5. Effect of pMC540 (210.5 µM) and merodantoin (103.3 µM) on the cellular ATP levels in MCF-7 human breast cancer cells treated for indicated periods of time. Mean ± SD of at least three separate experiments are shown.

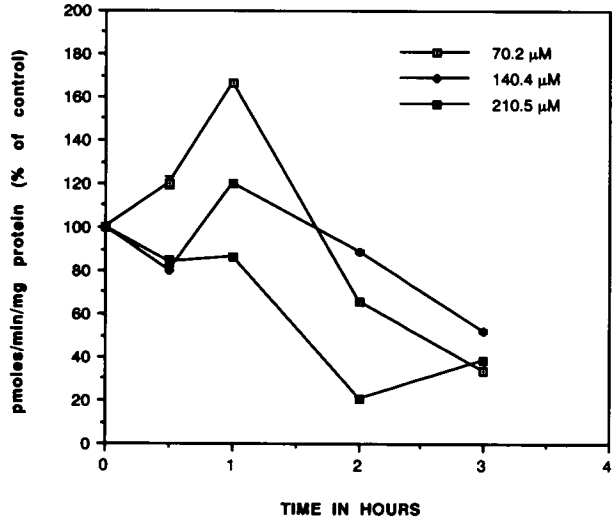

Figure 6. Dose effect of pMC540 on the *in vitro* activity of succinate dehydrogenase in MCF-7 human breast cancer cells. Data shown is percent of control values calculated from mean ± SD of three separate experiments each performed in triplicate.

Discussion

The purpose of this study was to investigate the possible mechanisms involving the intracellular site(s) or organelles involved in the breast tumor cells cytotoxicity induced by pMC540 and merodantoin. In previous studies, we have reported that photoproducts in preactivated MC540 (pMC540) are cytotoxic to cultured tumor cells including human breast cancer cells *in vitro* as well as *in vivo*, but very sparing of normal cells and tissues.[13] One mechanism of cytotoxicity of these compounds appears to involve an initial interaction with topoisomerase II leading to apoptosis.[16] These enzymes are known for their effects on chromatin topology during transcription and replication.[22,23] Based on these observations it was hypothesized that involvement of these enzymes as part of a suicide program might account for the observed morphological changes such as blebbing of the plasma membrane, chromatin condensation and compromised mitochondrial structure as well as the nuclear envelope.[24]

Most cellular oxidations and ATP production functions of a eukaryotic cell are carried out in the mitochondria, and as such they represent an important cellular target. Rhodamine 123, a fluorescent dye, is known to localize in the midochondria of living cells. The retention of this cationic dye is dependent on the maintenance of the mitochondrial integrity and membrane potential. Alterations in membrane potential can induce changes in dye binding and concomitant alterations in fluorescence emanating from these organneles. These properties of rhodamine 123 have been exploited in studies of the mitochondrial morphology and function.

In this paper, we have shown that even a brief period (10 min) of treatment of MCF-7 cells with pMC540 or merodantoin caused a significant reduction in the fluorescence intensity of the mitochondria as determined by the fluorescence microscopy. A marked loss of fluorescence from mitochondria was easily visible after 30 min of drug treatment, suggesting that pMC540 and merodantoin may have induced rapid alterations in mitochondrial morphology and functions. These visual observations were further confirmed by measuring the release of rhodamine 123 from prestained cells, which has been shown to be caused by compounds that act as respiratory poisons or ionophores.[21] By using this method, it was determined that pMC540 caused the release of rhodamine 123 from prestained MCF-7 cells in a time and drug dose dependent manner.

Next the ultrastructures of the drug treated and control cells were examined by electron microscopy. Data from these experiments clearly demonstrated that treatment of cells with pMC540 or merodantoin caused a significant change in the mitochondrial morphology. In the treated cells mitochondrial changes were more pronounced with altered cristae structure, more dilute matrices and changes in organelle shape. Such morphological changes have been associated with alterations in

cellular bioenergetics. Depending on cell lines analyzed, a reduced capacity for glycolysis may or may not be associated with impaired oxidative phosphorylation or it may be a component of a more global alteration in metabolic activity that remains to be determined. The structural and functional dynamics of mitochondria are complex. Originally, Hackenbrock[25] linked ultrastructural transformations in mitochondria,[25,26] to changes in energy metabolism. Assessing changes within a given cell type, he demonstrated that smaller, more electron-dense (condensed) mitochondria displayed higher levels of oxidative phosphorylation when compared with larger, sparsely staining (orthodox) mitochondria.[26] However, it is not clear that these morphologies can be used to infer specific bioenergetic differences when comparing two different cell types. Further structural alterations associated with functional changes have been demonstrated by Knoll and Brdiczka,[27] who demonstrated that actively phosphorylating mitochondria have an increased frequency of inner–outer mitochondrial membrane contact sites and this regulation may be disrupted in tumor cells of varying glycolytic capacity.[28]

An analysis of the ATP content, succinate dehydrogenase and oxygen consumption of MCF-7 cells treated with pMC540 and merodantoin revealed that significant dose dependent decreases in ATP levels, succinate dehydrogenase activity and oxygen consumption were induced as compared to untreated controls, suggesting an altered energy metabolism. It is likely that reduced levels of mitochondrial enzyme succinate dehydrogenase activity may influence the decreased ATP production which is the end product of oxidative phosphorylation. However, whether decreased cellular ATP content would correlate to a reduction in cell viability is an important question, particularly when one considers the premise that mitochondrial function, i.e. ATP production, may be a critical component in neoplastic cell metabolism and growth.[28] Thus, average cellular ATP levels and cell viability of MCF-7 cells treated with pMC540 and merodantoin were compared (data not shown). Data from these experiments did not produce any significant correlation between these two parameters. Whether such a relationship holds for each cell remains to be shown. Pertinent to the relationship observed here is the report by Garewal *et al.*,[29] who proposed that ATP levels could be used to quantitate viable cells determined by agar colony-forming assays. It cannot be stated with certainty that a cause–effect relationship exists between cellular ATP and viability, but reduction in the availability of ATP for metabolic activities would logically offer a desirable goal of therapeutic intervention of neoplastic growth.

Our *in vitro* studies of cytotoxicity have shown mitochondria to be a sensitive target for pMC540 and merodantoin mediated damage, and such damage could result from either or both of the altered structural and metabolic states. Whether or not the observed changes in mitochondrial morphology and cellular bioenergetics are central to the expression of pMC540 and merodantoin sensitivity has yet to be determined. Precisely how these findings may relate to other changes in response to pMC540 and merodantoin are currently under investigation.

Conclusion

Disruption of mitochondrial morphology and function in intact MCF-7 human breast cancer cells occurs by their treatment with pMC540 and merodantoin. These data suggest that mitochondria may be an important intracellular target of cytotoxic action mediated via disruption of the energy balance by these compounds.

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