Amiodarone and Desethylamiodarone Toxicity in Isolated Hepatocytes in Culture[1](42844)

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Abstract. **Amiodarone, a class III antiarrhythmic drug, has been found to be effective in the management of patients with life-threatening ventricular arrhythmias. Recent reports describe the presence of myelinoid inclusion bodies following amiodarone therapy in liver, myocardium, white blood cells, lung, cornea, skin, and lymph nodes; their relationship to toxicity is unclear. The exact role of desethylamiodarone, the major metabolite, of amiodarone in systemic toxicity of the parent drug is not known. Concentration-response relationships for amiodarone and desethylamiodarone were investigated by adding 1–50 μg/ml of the compounds of dimethyl sulfoxide (controls) to hepatocytes isolated from Sprague-Dawley rats and cultured in Leibovitz L-15 medium. Using lactate dehydrogenase release into the medium to quantitate cell death, both drugs were found to cause cell death in a concentration-dependent manner within 24 hr of incubation; this data showed desethylamiodarone to be significantly more toxic than amiodarone. In experiments with 50-μg/ml concentrations of amiodarone or desethylamiodarone, we found desethylamiodarone to produce a significantly greater release of lactate dehydrogenase as compared with amiodarone within 2–4 hr. Electron microscopic studies indicated the presence of myelinoid inclusion bodies at early culture stages followed by progressive swelling of mitochondria and rough endoplasmic reticula, disruption of membranes, aggregation of subcellular structures, and ultimately cell death. Ultrastructural changes occurred sooner in the hepatocytes treated with desethylamiodarone than with amiodarone. These data demonstrate that (i) desethylamiodarone is more toxic than amiodarone; (ii) acute toxicity of desethylamiodarone and amiodarone can be quantitated by lactate dehydrogenase release; (iii) both desethylamiodarone and amiodarone can induce myelinoid inclusion bodies in cultured hepatocytes; and (iv) toxicity is characterized by progressive subcellular changes leading to cell death.**

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The clinical usefulness of amiodarone, a class III antiarrhythmic agent, is limited due to its association with systemic toxicity. Recent clinical reports suggest an association between drug-induced toxicity with chronic amiodarone therapy and ultrastructural changes characterized by intracellular myelinoid inclusion bodies in ocular tissue, peripheral nerve, skeletal muscle, lung, heart, skin, and liver (1). Amiodarone is metabolized mainly in the liver. Asymptomatic increases in liver enzymes have been found in patients on chronic drug therapy (2) and necrosis, cirrhosis, and hepatitis have been described in livers from patients receiving amiodarone (3). The reported cases indicate that amiodarone-induced hepatotoxicity is more common than previously thought and that controlled experimental studies to carefully examine the dose, duration of therapy, serum, and tissue drug levels are necessary.

Studies in our laboratory have shown that myelinoid inclusion bodies are prevalent in circulating neutrophils (4) and myocardial tissue (5, 6) at an early stage of amiodarone therapy and before the onset of clinical signs and symptoms of toxicity. An important question that remains to be answered is whether these lesions progress with continued drug therapy, eventually leading to clinical toxicity.

Although the multiple organ toxicity of amiodarone is well known, correlation between the dose, du-

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ration of therapy, tissue drug levels, and systemic toxicity is unclear. Amiodarone has many unusual pharmacologic properties, including a long biologic half-life, a long latency of onset of action, and delayed toxicity (7), which make it difficult to investigate in animal models. In view of the large number of vertebrate animals required to conduct further studies, an *in vitro* model of cultured rat hepatocytes has been developed since isolated hepatocytes retain most of the functional properties of intact cells (8). Desethylamiodarone, a major metabolite (9–11), is found soon after amiodarone therapy is started, but its role in systemic toxicity has not been studied. The present study was designed to evaluate the acute effects of high doses of amiodarone and its major metabolite, desethylamiodarone, on hepatocytes in primary culture to determine: (i) whether the parent drug or the metabolite is more toxic to the cells; (ii) whether the toxicity, as evidenced by release of lactate dehydrogenase (LDH) or cell death, can be correlated with ultrastructural changes; and (iii) whether myelinoid inclusion bodies can be produced in this preparation and, if so, do they occur prior to development of cell toxicity.

Materials and Methods

Animals. Male Sprague-Dawley rats, 4–6 weeks old, were purchased from Charles River Breeding Co. (Portage, MA). Rats were housed one per polycarbonate cage and fed Purina Laboratory Chow Blox (Ralston Purina Co., St. Louis, MO) and water *ad libitum.*

Hepatocyte Isolation and Culture. Rat hepatocytes were isolated by a modified two-stage enzyme perfusion technique (12, 13) and cultured in Leibovitz's L-15 medium supplemented with glucose (1 mg/ml), dexamethasone (1 μM), gentamicin (50 μg/ml), and 10% fetal bovine serum as described previously (12, 13). Preliminary studies using various concentrations of either amiodarone or desethylamiodarone added to cultured hepatocytes obtained from five different rats incubated in triplicate were performed to determine the concentration-response relationship for LDH release (toxicity) with each compound. The concentration which produced almost 100% toxicity at 24 hr (50 μg/ml) was chosen for the electron microscopic portion of the study from 0 to 6 hr. Isolated hepatocytes (1×10^6) were placed into 25-mm^2 flasks (run in triplicate) and allowed to attach for 4 hr; the media wash changed and replaced with fresh L-15 medium containing 50 μg/ml of either amiodarone or desethylamiodarone in dimethyl sulfoxide (DMSO). Controls consisted of solvent control (DMSO, 0.2% final concentration) and untreated cultures.

Hepatocyte toxicity was evaluated by measuring LDH leakage into the medium. Medium was sampled from each flask, filtered to remove any cells, and assayed for LDH on a Beckman Multistat Analyzer (Beckman Instruments Corp., Palo Alto, CA). Total LDH per culture was determined from 0.01% Triton X-100 cell lysate. LDH measured in the Triton X-100-treated cells reflects the total amount of available LDH present in the culture. LDH activity also was determined in fresh cell-free culture medium (L-15 plus 10% fetal bovine serum). Cytotoxicity was expressed as a percentage of total LDH released using the following equation: [LDH in medium of treated clutures − LDH in cell-free medium]-[LDH in Triton-X-treated cultures − LDH in cell-free medium] X 100. Neither amiodarone nor desethylamiodarone interfered with LDH measurement by the Beckman Multistat Analyzer. The percentage of total LDH released was determined in triplicate cultures for each concentration of each compound and the means ± SEM were determined. Treatments were statistically compared with one-way analysis of variance.

Electron Microscopic Studies. Cells cultured for 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hr were obtained from three different culture dishes from six rats and were processed for electron microscopy. Immediately prior to fixation, the culture medium was removed from the petri dish, replaced by the primary fixative solution (3.0% glutaraldehyde in 0.1 N Na-cacodylate buffer, pH 7.4), and allowed to sit for 2–3 days at room temperature. The cells were then scraped from the petri dish and centrifuged with new fixative to form a pellet. All subsequent processing was performed in 0.5-ml Eppendorf centrifuge tubes. Cells were rinsed with 0.1 N Na-cacodylate, postfixed in 2% OsO_4 in 0.1 N Na-cacodylate buffer, and dehydrated in a graded series of ethanol. Samples were cut on a Sorvall MT2B Ultramicrotome, placed on 200-mesh copper grids, and stained with 2% uranyl acetate and lead citrate (0.03 g of lead citrate in 10 ml of 0.1 N NaOH). Sections were examined with a Philips 300 transmission electron microscope at 60 kV and photographed using Kodak 4489 film. Control electron micrographs were obtained at corresponding intervals from tissue cultures to which 0.2% DMSO (the solvent for amiodarone or desethylamiodarone) was added.

Results

Release of LDH. Using LDH release into the medium to quantitate cell death (12, 13), the concentration-response relationship (Fig. 1) showed desethylamiodarone to be more toxic than amiodarone since the entire dose-response curve for the metabolite was to the left of the parent drug (14). Also, desethylamiodarone, in a 50-μg/ml concentration, was found to be lethal to most cells in 4–6 hr whereas amiodarone killed most cells between 6 and 24 hr in this concentration. Thus, hepatocytes cultured for various time periods and treated with lethal concentrations (50 μg/ml) resulted in greater LDH releases with desethylamiodarone

than with amiodarone at corresponding time intervals (Fig. 2); for example, there was a total LDH release of 86.4% at 4 hr of culture in the case of desethylamiodarone vs 24.2% at 4 hr of culture in the case of amiodarone (Fig. 2).

Electron Microscopy. Hepatocytes treated with DMSO, amiodarone, or desethylamiodarone were examined following culture for 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 hr. As shown earlier (14) DMSO caused no structural changes in the hepatocytes. Cells cultured for 0.5 hr following treatment with amiodarone resembled normal hepatocytes, having a nucleus containing a peripheral chromatin pattern, numerous mitochondria and glycogen granules, and abundant endoplasmic reticulum. An unusual feature, however, was a small number (three to four per cell) of round intracytoplasmic onionoid inclusion bodies. They were membrane bound and contained densities and concentric membranous whorls (Fig. 3). By 1 hr in culture, the inclusion bodies were increased in both size and number and many contained multiple membranous whorls

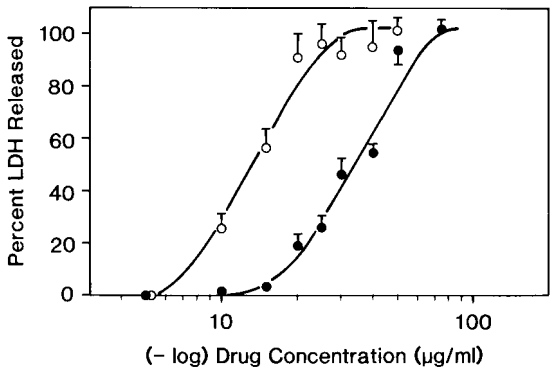

Figure 1. Log concentration-LDH release relationship for amiodarone (●) or desethylamiodarone (○) in the isolated cultured hepatocytes after 24 hr of drug exposure. Data at each point represent mean ± SEM obtained from five rat hepatocytes in triplicate.

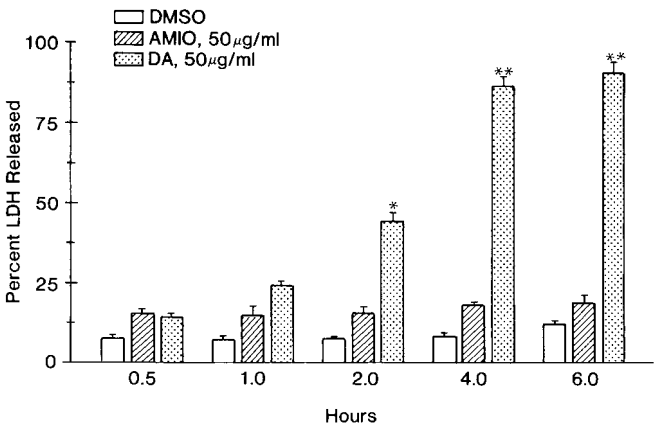

Figure 2. Bar graph showing the time course of LDH released from 0 to 6 hr in culture medium ($N = 6$) of control hepatocytes or those treated with desethylamiodarone or amiodarone. * = $P < 0.05$; ** = $P < 0.01$ as compared with either DMSO- or amiodarone-treated group.

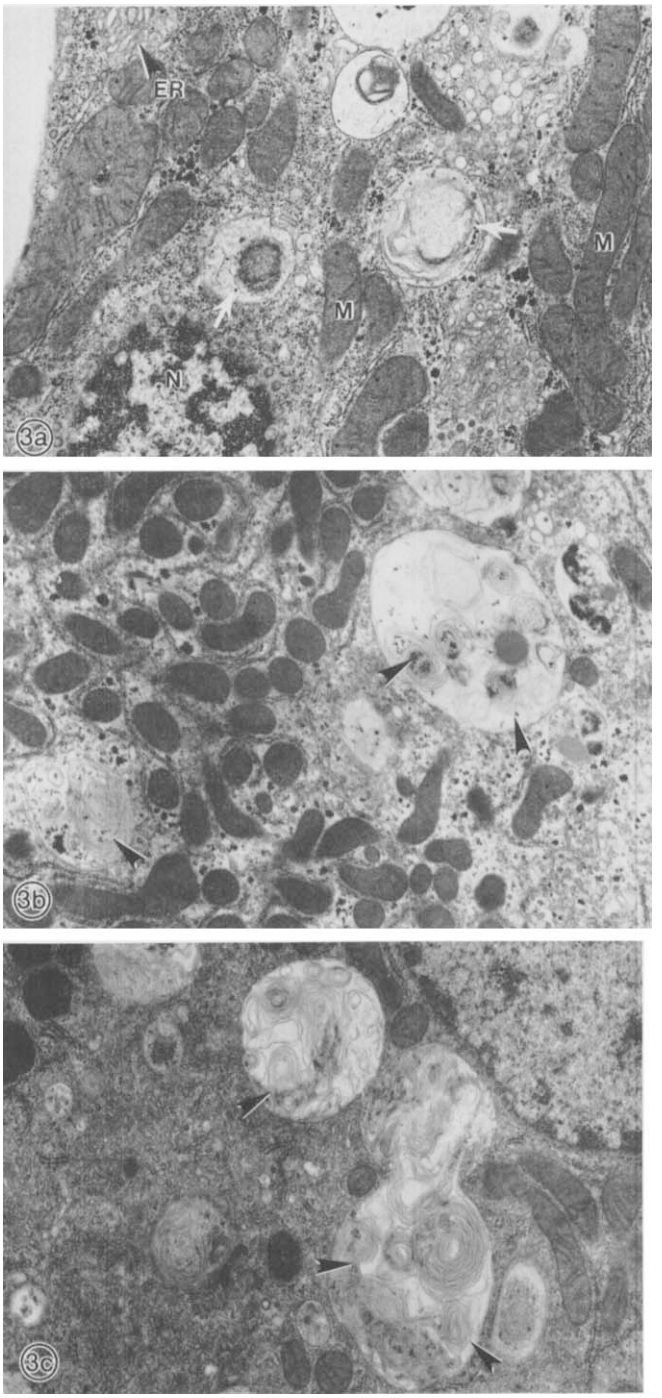

Figure 3. Electron micrographs of hepatocytes cultured and treated with amiodarone. (a) By 0.5 hr following amiodarone therapy hepatocytes appeared to be normal but often contained a small number of myelinoid inclusion bodies (arrows). M, mitochondria; N, nucleus; ER, endoplasmic reticulum (original magnification ×17,950). (b) By 1 hr in culture, hepatocytes contained normal mitochondria with no apparent swelling. Myelinoid inclusion bodies (arrowheads), (original magnification ×11,790). (c) Hepatocytes cultured for 2 hr retained normal mitochondria but had numerous large myelinoid inclusion bodies (arrowheads). Cellular membranes and nucleus appeared to be normal (original magnification ×17,950).

(Fig. 3). These changes were evident further in cells treated with amiodarone and cultured for 2 hr. The nuclei and mitochondria retained their normal appearance (Fig. 3). The most marked changes seen by 4 hr of drug exposure included swollen mitochondria within many of the hepatocytes (Fig. 4). The overall appearance of some hepatocytes, however, was that of normal untreated hepatocytes except for the presence of the myelinoid inclusion bodies. By 5 hr in culture, most cells treated with amiodarone contained markedly swollen mitochondria, areas of swollen endoplasmic reticulum, and nuclei containing aggregated chromatin (Fig. 4). Occasionally, the hepatocytes appeared to be unaffected, resembling those of earlier culture stages, containing normal subcellular structures and myelinoid inclusion bodies.

Hepatocytes in culture for 0.5 hr following treatment with desethylamiodarone contained normal subcellular structures and frequently contained myelinoid inclusion bodies (Fig. 5). By 1 hr in culture, many hepatocytes contained numerous slightly to moderately swollen mitochondria and large vacuolated structures containing membranous whorls (Fig. 5). Markedly swollen mitochondria were evident in most cells 2 hr following desethylamiodarone treatment (Fig. 5). Mitochondrial swelling, loss of membrane integrity, and aggregation of disrupted subcellular structures were evident by 4 hr (Fig. 5), and cellular disruption increased such that by 6 hr most cells were dead.

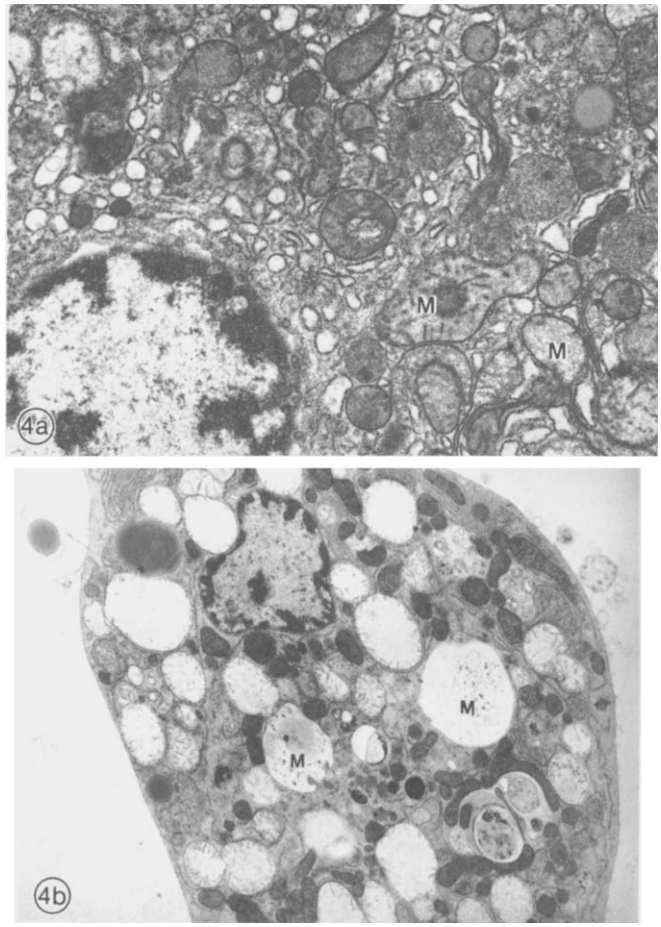

Figure 4. Electron micrographs of hepatocytes cultured for longer time periods following amiodarone therapy. (a) Mitochondria (M) within some hepatocytes cultured for 4 hr were swollen, but overall appearance of most cells resembled normal hepatocytes except for the presence of lamellar inclusion bodies (original magnification ×17,950). (b) By 5 hr in culture, most cells contained markedly swollen mitochondria (M). Other subcellular structures remained largely unaffected (original magnification ×8,440).

Discussion

Clinical reports have indicated an association between drug-induced toxicity with chronic amiodarone therapy, and ultrastructural changes characterized by multilamellar inclusion bodies have been found in a number of organs including cornea (15), lung (16), skin (17), lymph nodes (18), myocardium (5, 6), white blood cells (4, 18, 19), and liver (1). Although the multi-organ toxicity of amiodarone is well known, correlation between the dose, duration of therapy, tissue drug levels, and systemic toxicity is highly debatable. Early clinical studies failed to show any such correlation but it is generally found that systemic toxicity, especially the adverse effects on the lung, liver, and neuropathy, are noted in patients receiving large maintenance doses with plasma drug levels above 4 μg/ml. Recent data (20), however, suggest that patients with systemic toxicity frequently had plasma drug concentrations below 2.5 μg/ml. It is not clear, therefore, if drug levels can be related to toxic side effects.

Experimental data on the time for the onset of drug toxicity are also limited. Costa-Jussa *et al.* (21) treated rats or mice for 2–3 months with 200 or 400 mg/kg/day of oral amiodarone, respectively, and found lamellar inclusion bodies in the alveolar macrophages. However, no data on tissue drug concentration in the lung or other organs were provided, nor were data provided on the earliest time of onset of such inclusions either in the lung or other tissues. Goldman *et al.* (22) fed 5 mg/kg of amiodarone orally for 2 months and found that the drug had no significant effect on the number of the lamellar inclusion bodies in the amiodarone-treated rabbits as compared with that of the control group. However, these results were obtained in only three rabbits and the plasma (0.02–0.04 μg/ml) or tissue (2.5–20.8 μg/mg) levels of amiodarone were very low. In an experimental canine study (23) ultrastructural changes and myocardial tissue levels of amiodarone were compared after a single loading dose of 40 mg/kg iv or multiple doses (40 mg/kg iv loading dose plus 10 mg/kg/day iv × 7 days maintenance dose). Myelinoid inclusions were noted after only 7 days, although the tissue levels of amiodarone were high after a single iv dose (53.9 ± 30.3 μg/g vs 13.3 ± 6.8 μg/g after multiple doses) and yet no intracellular inclusion bodies were

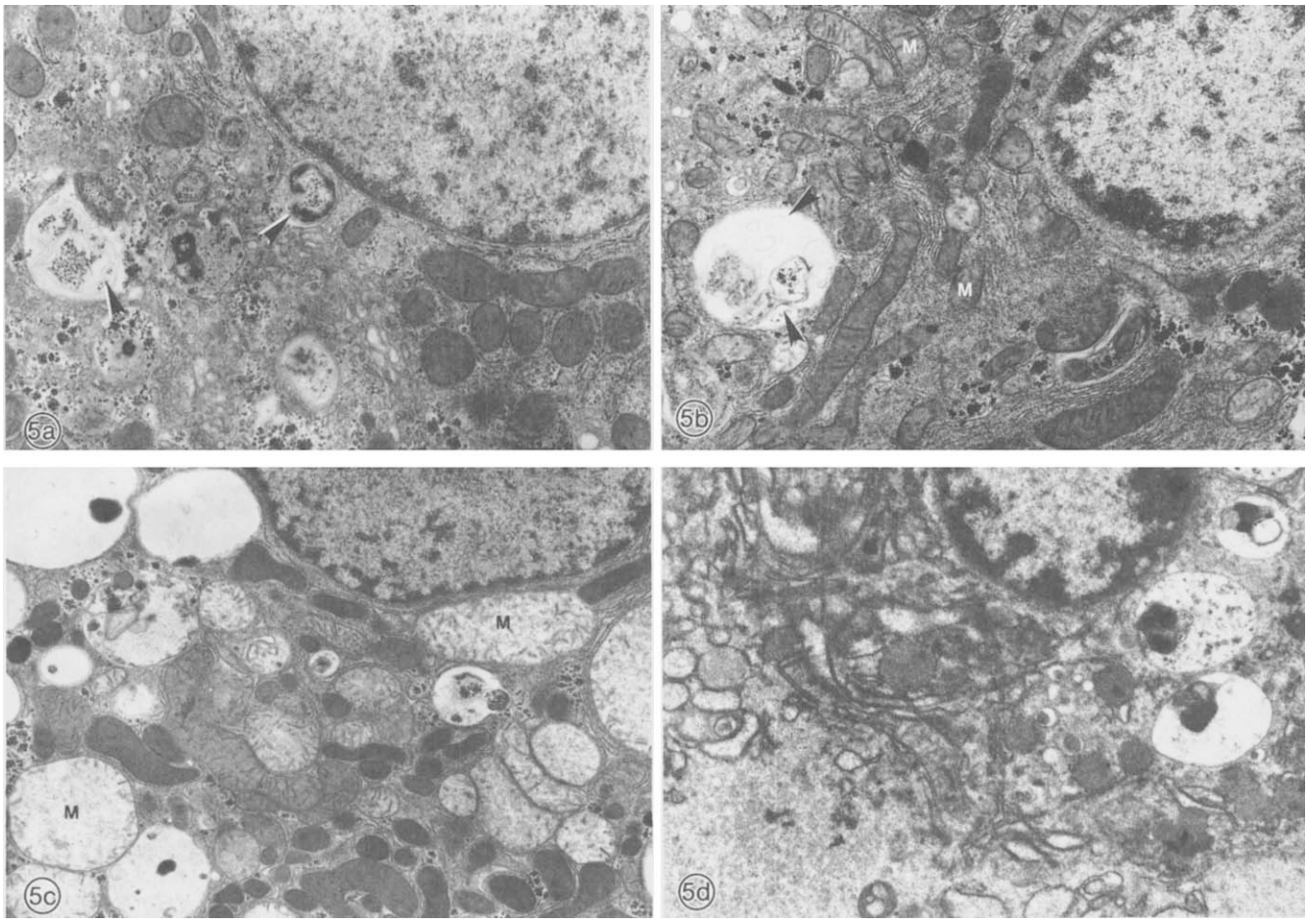

Figure 5. Electron micrographs of hepatocytes cultured and treated with desethylamiodarone. (a) Hepatocytes cultured and treated for 0.5 hr resembled normal hepatocytes. Membrane-bound inclusion bodies containing membranous whorls were often seen (arrowheads) (original magnification ×17,950). (b) Hepatocytes cultured for 1 hr began to demonstrate cellular damage evidenced by slightly to moderately swollen mitochondria (M). Lamellar inclusion bodies were also present (arrowheads) (original magnification ×17,950). (c) Hepatocytes cultured for 2 hr with desethylamiodarone often contained many swollen mitochondria (M) (original magnification ×11,970). (d) Hepatocytes cultured for 5 hr demonstrated lack of recognizable subcellular structures and aggregation of disrupted membranous material (original magnification ×14,160).

found after a single large dose of amiodarone. Experimental studies in whole animals are complicated because of possible differences in the animals species, variable bioavailability, drug metabolism, and differences in tissue drug or metabolite distribution. It would be desirable to develop an *in vitro* experimental model to study the direct effect of amiodarone or its major metabolite, desethylamiodarone.

Desethylamiodarone has been found to be the major metabolite of amiodarone and it can be identified in plasma and tissues within an hour of an intravenous dose (11). The exact role of the metabolite in toxicity has been unclear, especially whether amiodarone acts directly or if its toxic effects are mediated through desethylamiodarone. Preliminary results in rats showed that myelinoid inclusion bodies in liver cells were induced by either amiodarone (50 mg/kg ip × 14 days) or desethylamiodarone (25 mg/kg ip × 14 days) in Sprague-Dawley rats (24).

In the present study, we confirmed that desethylamiodarone itself was more toxic than amiodarone (14). Additionally, using lethal concentrations of 50 μg/ml, significant release of LDH occurred beginning at 2 hr and reaching almost maximum levels by 6 hr with desethylamiodarone but not with amiodarone. It is clear that desethylamiodarone is more toxic than amiodarone and their acute toxicities can be quantitated by LDH release. These data correlate well with electron microscopic findings, since in this series we found a rapid progression from initial formation of intracellular inclusion bodies, swelling of the mitochondria to their damage, and ultimately cell death at 6 hr with desethylamiodarone, but these changes occurred at a slower rate with amiodarone. The precise mechanism of toxic effects of the two drugs remains unclear.

It may be pointed out that although our *in vitro* data provide a rapid time course of toxic events with desethylamiodarone or amiodarone, the drug concentrations necessary to induce electron microscopic changes and cell death are 5 to 10 times as high as the

usual serum drug levels observed in patients (7, 9). If amiodarone is given by rapid intravenous infusion, serum drug levels may reach 2–4 μg/ml or higher (25), although recommended therapeutic drug levels are 1–4 μg/ml during long-term therapy. Thus, our data showing that minimal *in vitro* toxic concentration of amiodarone or desethylamiodarone is 10–15 μg/ml (Fig. 1) suggest that the drug has a narrow therapeutic index. Our data also show that desethylamiodarone is more toxic *in vitro* than the parent drug, which has recently been confirmed *in vivo* for liver (24) and pulmonary macrophages (26). Thus, it is possible that desethylamiodarone may play a significant role in clinical toxicity of amiodarone, especially because the hepatic levels of the metabolite are significantly higher than those of amiodarone (9). The elimination half-life ($t_{1/2}$ β) of desethylamiodarone also is longer than that of amiodarone (9, 27). Thus, it is possible, although unequivocal evidence is still lacking, that delayed amiodarone toxicity in our hepatocyte model or *in vivo* may be due to desethylamiodarone.

Data obtained in this and other series of experiments (14) demonstrate that myelinoid inclusion bodies can be clearly identified in the hepatocytes at time intervals of 0.5 and 1 hr when there is no significant release of LDH, and these data suggest that the presence of myelinoid inclusion bodies in itself does not appear to be evidence of cellular toxicity. These bodies are apparently unrelated to the cell death since they are found before morphologic evidence of cell injury, as shown in the present study, or with subtoxic drug concentrations when such inclusions are plentiful and yet no release of LDH occurred after 24- or 48-hr exposure to the drugs (14). These data in isolated hepatocytes confirm the findings in either whole animals (23, 24) or in man (4, 5, 6, 19) that intracellular inclusion bodies can be found soon after therapy with amiodarone is started and without any overt clinical toxicity. Whether or not continued presence or increasing size or number of these inclusions ultimately leads to cell death remains to be investigated.

It is concluded that cultured hepatocytes may prove to be a useful model for correlation of pharmacologic and morphologic changes since the culture system provides a controlled environment to study the time course of drug-induced changes. In addition, results from this study demonstrate changes similar to those found in studies using either intact animals or in patients treated with amiodarone.

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