INTRODUCTION

Amiodarone (2-butyl-3-(3’,5’-diiodo-4’α-diethylamino ethoxybenzoyl)- benzofuran) is a potent antiarrhythmic agent frequently used in the treatment of supraventricular (1) and ventricular arrhythmias (2). Both types of arrhythmias are commonly observed; atrial fibrillation (by far the most common supraventricular arrhythmia) affects approximately 1.5% of the general population, the prevalence rising to 5% beyond 65 years and 10% beyond 75 years of age (3). Similarly, ventricular arrhythmias are frequently encountered in patients with heart failure and/or a history of myocardial infarction and amiodarone reduces the rate of arrhythmic and total mortality in certain subgroups of such patients (2). Due to the delayed onset of action, a 2-3 g loading regime precedes maintenance dosages (usually 200 mg daily), with treatment duration ranging from few weeks to several years. Although the role of amiodarone in clinical practice is well established, its use is limited by extra-cardiac adverse effects (4). Despite some uncertainty from earlier reports, recent evidence indicates that it may be an under-recognized side-effect, with an incidence at the range of 5% and mortality rates 5-10% in affected patients (5). Amiodarone-induced pulmonary toxicity is characterized initially by alveolitis and interstitial inflammation and subsequently by pulmonary fibrosis (6, 7). These changes are caused by amiodarone itself, or by its active metabolite desethylamiodarone, which also exhibits cytotoxicity and tends to accumulate in the lung more intensively than amiodarone (8). Risk factors include high daily and cumulative dosages, increased patient age, pre-existing lung disease and thoracic or non-thoracic surgery. Of these, dosage appears to be the most important, as the risk decreases by approximately two thirds in patients receiving daily doses less than 400 mg (9).

Although considerable progress has been noted during the last decade, the underlying pathophysiology of amiodarone-induced pulmonary toxicity remains unclear. Several mechanisms have been implicated, including immunological reactions, alterations of membrane properties, phospholipidosis and apoptosis (6, 7, 10). Apoptosis plays a prominent role in tissue homeostasis (13); apoptotic signalling can be divided into the intrinsic pathway, which depends mainly on mitochondrial changes, and the extrinsic pathway, which is activated by extracellular signals. Amiodarone interferes with both pathways, either by altering mitochondrial membrane potential, thereby causing the release of apoptotic factors, or by activating the extrinsic pathway (12,

AMIODARONE ATTENUATES APOPTOSIS, BUT INDUCES PHOSPHOLIPIDOSIS IN RAT ALVEOLAR EPITHELIAL CELLS

Amiodarone-induced pulmonary toxicity is a serious side-effect, but the underlying molecular mechanisms remain unclear. We examined phospholipidosis and apoptosis in rat alveolar epithelial cells after medium-term oral amiodarone treatment. Amiodarone (30 mg/kg daily, a dosage corresponding to that used clinically) or vehicle was administered by gavage in 33 Wistar rats for two weeks. Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) and the expression of apoptosis- and phospholipidosis-related proteins was measured by immunohistochemistry. Amiodarone decreased phospholipase-C-γ1 and increased phosphatidylinositol-(4,5)-bisphosphate, resulting in phospholipidosis, evidenced by the appearance of intracellular inclusion bodies with a multi-lamellated interior. Amiodarone exerted two opposite effects on apoptosis; compared to controls, the expression of activated-caspase-8 was higher in treated rats, while the expression of apoptosis inhibitors survivin, Bcl-2 and c-Flip was lower. On the other hand, the expression of activated-caspase-3 was lower after treatment. Overall, amiodarone attenuated apoptosis, evidenced by fewer TUNEL-positive cells. Medium-term oral amiodarone administration induced phospholipidosis in rat alveolar epithelial cells. Although such treatment decreased anti-apoptotic proteins, apoptosis was attenuated via a decrease in the caspase-3 pathway. These findings improve current understanding on the mechanisms underlying amiodarone-induced pulmonary toxicity.

Key words: amiodarone, apoptosis, phospholipidosis, pulmonary toxicity, alveolar epithelial cells, phosphatidylinositol-(4,5)-bisphosphate
14). In vitro studies have indicated that amiodarone induces apoptosis in rat and human alveolar epithelial cells (15, 16), but data from in vivo experiments are scarce.

Phospholipidosis is a cellular process caused by excessive formation and accumulation of intracellular phospholipids (11, 17). Lipid accumulation leads to the formation of abnormal lamellated inclusion bodies, which are considered characteristic features of this process (11). Cationic amphiphilic drugs, such as amiodarone, have been shown to cause phospholipidosis in animal and human cell cultures (17) by inactivating phospholipase C-γ1 (PLC-γ1), but hitherto data are controversial. PLC-γ1, a tyrosine-kinase widely distributed in bronchiolar epithelial cells and fibroblasts, regulates cell proliferation and differentiation (18). Activated PLC-γ1 hydrolyses phosphatidylinositol (4,5)-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate and diacylglycerol; inositol 1,4,5-trisphosphate regulates intracellular calcium levels, while diacylglycerol activates protein kinase C, which is involved in the control of apoptosis (18). These findings support the hypothesis previously put forward, suggesting a functional relation between phospholipidosis and apoptosis, likely mediated by PLC-γ1 levels (19).

The purpose of the present study was to further explore the effects of amiodarone on alveolar epithelial cells. Specifically, the aim of this work was threefold: first, to investigate the effect of amiodarone on the intrinsic and extrinsic apoptotic pathways; second, to study the effects of amiodarone on phospholipidosis. Lastly, to examine the possible relation between phospholipidosis and apoptosis after amiodarone treatment.

MATERIAL AND METHODS

Animal population

The study was conducted in 33 female Wistar rats, of similar age (20±1 weeks) and weight (200–250 g). The animals received humane care and all procedures conform to the National Research Council’s guide for the care and use of laboratory animals. The study protocol was approved by the responsible state authority (Department of Veterinary Medicine and Animal Welfare, Prefecture of Ioannina, Greece). All rats were housed in individual plexiglas chambers and were given water and standard rat chow ad libitum. Laboratory conditions were kept optimal in a controlled environment, in terms of humidity (65–70%), temperature (21±1°C) and (12:12-hourly) light-dark cycles.

Drug administration

Twenty rats (22±4 g) were randomly assigned to amiodarone treatment, while 13 rats (224±6 g) served as controls. Amiodarone was kindly provided by Sanofi-Aventis, Montpellier, France. The drug was administered orally by gavage, once daily for 2 weeks at a dose of 30 mg/kg, as previously reported by our group (20). We used 5-French (1.65 mm) silicone feeding tubes (model 4165039, Nutri-Cath, Utah Medical Products Inc., Midvale, UT, USA) gently advanced through a custom-made mouth-piece. This method minimizes animal distress and avoids perforation of the gut. Before each administration, a fresh solution was prepared in 0.6% methylcellulose to obtain the necessary drug concentration in 3 ml. Prior work from our group (20, 21) indicates adequate absorption of amiodarone using this regimen. In the control group, 0.6% methylcellulose alone was given for the same time period. In clinical practice, amiodarone is usually administered at a loading dose of 2-3 g, followed by a maintenance weekly dosage of 1000-1400 mg (2). Thus, the daily dosage of 30 mg/kg administered for 14 days (resulting in a cumulative dosage of 420 mg/kg) used in the present and previous studies (20, 21) corresponds to a medium-term clinical administration.

Histological analysis

After two weeks of treatment, the animals were anaesthetised with isoflurane and were sacrificed by intracardiac injection of potassium chloride. To preserve alveolar architecture, the lungs were carefully removed and instilled with 4% paraformaldehyde in phosphate-buffered saline at 20 cm H2O constant pressure, as previously described (22), and were then immersion-fixed in 10% paraformaldehyde for 24 hours. Subsequently, lung tissues were embedded in paraffin and were cut in 5-6 µm sections.

Histological evaluation was performed in a blinded fashion independently by two of the authors (E.K. and A.S.) and was comprehensively reviewed by an experienced pathologist (V.M.-M.). In general, samples considered technically inadequate were excluded from the analysis. Initial histological evaluation was performed after haematoxylin-eosin staining, followed by the assessment of apoptosis and phospholipidosis.

Apoptosis

Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) assay, as previously described (23, 24). DNA fragments were detected in tissue sections (5-6 µm), using a commercially available apoptosis kit (Apoptag kit, Oncor, Gaithersburg, MD, USA), according to the manufacturer’s instructions. This method utilizes the free 3-OH DNA ends generated by DNA fragmentation in apoptotic cells.

Tissue sections were de-paraffinized in xylene and rehydrated through decreasing concentrations of ethanol, followed by washing in phosphate-buffered saline. Sections were incubated with 20 µg/mL proteinase K, followed by quenching of endogenous peroxidase with 3% H2O2. Subsequently, the enzyme terminal deoxynucleotidyl transferase (TdT) was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragments. Apoptotic signals were detected by the addition of an anti-digoxigenin antibody conjugated with peroxidase after chromogen development with 3,3-diaminobenzidine. Finally, sections were counterstained with haematoxylin, dehydrated in ethanol, cleared with xylene, and mounted with coverslips in permanent medium. Cells in the visible epithelial layer were examined, while interstitial cells were excluded from the analysis. Morphologically intact cells in haematoxylin-eosin-stained slides, displaying condensed, hyperchromatic, ring-like, crescentic or beaded chromatin, surrounded by a clear halo, were considered TUNEL-positive and were classified as apoptotic cells. Special care was taken to identify inflammatory cells; these cells were extensively TUNEL-positive, but were omitted during counting.

The number of apoptotic cells was recorded by assessment of 10 randomly selected fields (using the X40 objective lens), totalling approximately 100 cells per animal. The apoptotic index was determined as the percentage of apoptotic cells in the total number of counted alveolar cells. Reactive lymph nodes served as positive controls; for negative controls, the TUNEL method was repeated, only omitting the TdT reaction step.

Apoptosis-related proteins

The following apoptosis-related proteins were measured by immunohistochemistry: activated-caspase-3, activated caspase-
8, apoptosis inducing factor, Bcl-2, survivin and c-FLIP. Specifically, immunohistochemical reactivity was tested with antibodies against Bcl-2 (1:80 dilution, 1-hour incubation; SantaCruz), apoptosis inducing factor (1:80 dilution, 1-hour incubation; SantaCruz), active-caspase-3 (1:300 dilution, 1-hour incubation, BD Bioscience), survivin (1:80 dilution, 1-hour incubation, SantaCruz), c-Flip (1:100 dilution, 1-hour incubation; SantaCruz).

Phospholipidosis

Phospholipidosis was assessed by histological evaluation, aiming at identification of abnormal unicentric or multicentric inclusion bodies and by quantification of the expression of PLC-γ1 and PIP2 by immunohistochemistry, as follows: PLC-γ1 (1/500 dilution, 1-hour incubation, SantaCruz) and PIP2 (1/600 dilution, 1-hour incubation, A-21327 Molecular Probes). Staining was performed in a bond automated immunohistochemistry system (Vison Biosystems), using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate method (Bond Polymer Define Detection System, DS9713, Vision Biosystems).

Correlation between phospholipidosis and apoptosis

The interrelation between amiodarone-induced phospholipidosis and apoptosis was examined by correlating PLC-γ1 with apoptotic index, caspase-3 and caspase-8.

Statistical analysis

All values are given as mean±standard error of the mean. Normal distribution of continuous variables was assessed with the use of the Kolmogorov-Smirnov test for normality. Accordingly, differences in continuous variables between the two groups were examined with Student’s unpaired t-test or with Mann-Whitney U-test, when a normal distribution was present or absent, respectively. Linear correlation was performed between continuous variables and Spearman’s correlation coefficient R was calculated. Statistical significance was defined at an alpha level of 0.05. The statistical software program SPSS for Windows (Release 14.0) was used for statistical analysis.

RESULTS

Aptosis

The apoptotic index was lower (p=0.0004) in the amiodarone group (0.75±0.20%) than in controls (2.22±0.33%). Nuclear expression of activated caspase-3 was evident in 1.57±0.24% of cells in the amiodarone group, lower (p<0.0001) than in controls (4.23±0.60%). A representative example of activated caspase-3 from each group is shown in Fig. 2. As expected, the apoptotic index correlated (R=0.608, p=0.0004) with caspase-3 expression (Fig. 3). In contrast to caspase-3, cytoplasmic expression of activated caspase-8 was higher (p=0.0332) in amiodarone-treated rats (17.47±1.72%) than in controls (11.25±1.52%). A representative example of activated caspase-8 from each group is shown in Fig 4.

Immunohistochemistry staining for apoptosis inducible factor was comparable (p=0.42) in the two groups (2.62±0.46% in amiodarone-treated rats, versus 2.62±0.46% in controls). In contrast, the expression of Bcl-2 (6.26±1.14% vs. 17.58±2.08%, p<0.0001), survivin (7.38±0.82% vs. 17.45±2.14%, p<0.0001)
and c-Flip (3.93±0.92% vs. 8.18±1.57%, p=0.0203) was lower in the amiodarone group than in controls. All values are depicted in Fig. 5.

**Phospholipidosis related proteins**

The expression of PLC-γ1 was lower (p<0.0001) in amiodarone-treated rats (28.75±4.83%) than in controls (63.07±4.82%), while the expression of PIP2 was higher (p=0.0002) in amiodarone-treated rats (20.93±1.87%) than in controls (10.69±1.35%). Cytoplasmic localization of PIP2 as an aggregated protein complex formed round bead-like inclusion bodies. Representative examples of immunohistochemistry staining for PLC-γ1 and PIP2 are shown in Figs. 6 and 7, respectively.

**Apoptosis- and phospholipidosis-related proteins**

Apoptosis- and phospholipidosis-related proteins showed a significant correlation. PLC-γ1 correlated with apoptotic index (R=0.37, p=0.0474) and caspase-3 (R=0.59, p=0.0003). In contrast, there was a weak negative correlation between PLC-γ1 and caspase-8, which did not reach statistical significance (R=-0.30, p=0.094).

**DISCUSSION**

The present study investigated phospholipidosis, apoptosis and their interrelation in rat alveolar epithelial cells after medium-term oral amiodarone administration. We report increased phospholipidosis in treated animals, evidenced by accumulation of PIP2 in multi-lamellated inclusion bodies. This effect appeared to be secondary to a decrease in PLC-γ1 by amiodarone, thereby reducing the hydrolysis of PIP2. Phospholipidosis enhanced the intrinsic apoptotic pathway, mediated by a decrease in anti-apoptotic proteins.
Phospholipidosis

Although the inhibitory effect of amiodarone on alveolar cell phospholipase activity, leading to intracellular accumulation of phospholipid-rich material, was suggested as early as in 1978 (25), subsequent studies have produced conflicting results (14, 19, 26-30). The reasons for the discrepancy in previous reports are unclear and are likely multi-factorial. First, the variable results may be due to different experimental settings (6). Indeed, most available information originates from in vitro studies (14, 19, 26, 27), while data from in vivo experiments are relatively limited. Since phospholipidosis may be actually caused by desethylamiodarone, the major metabolite of amiodarone (8), this effect may have been overlooked in some prior in vitro studies. Second, even in in vivo studies, the route of drug delivery may be an important confounding factor, since different responses were observed after intra-tracheal versus oral administration (29); in contrast to oral administration, intra-tracheal instillations in hamsters produced fibrosis, characterized by elevated lung hydroxyproline content and variable increases in lavage macrophage, neutrophil, and eosinophil number, raising the possibility of pulmonary eosinophilia and/or physical damage to the lung as additional mechanisms (29). Lastly, the pharmacologic effects of amiodarone are strongly dose-dependent (31, 32); thus, the variety in dosing regimens and in the duration of drug administration in previous studies may account for the observed differences.

Fig. 5. Apoptosis-related proteins. In contrast to apoptosis inducing factor (AIF), the expression of anti-apoptotic proteins was lower (asterisk, p<0.0001 for Bcl-2 and survivin, p=0.0203 for c-Flip) in the amiodarone group (red bars) than in controls (green bars).

Fig. 6. Example of immunohistological staining for phospholipase C-γ1 from a control sample (A) and from the amiodarone group (B) (microscopic magnification X40).

Fig. 7. Example of immunohistological staining for PIP2 from a control sample (A) and from the amiodarone group (B) (microscopic magnification X100).
In the present study, we report lower expression of PLC-γ1 in rat alveolar epithelial cells after medium-term oral amiodarone treatment, resulting in cytoplasmic PIP2 accumulation. We chose to examine the dosage of 30 mg/kg/day, which is closest to that used in the clinical setting, while the two-week administration scheme in rats is translated into a medium-term administration in patients (2, 21). Our group has previously shown that this dosing regimen exerts a potent antiarrhythmic effect, evidenced by a marked decrease in ventricular tachycardia and ventricular fibrillation episodes, during a 24-hour observation period after coronary ligation in rats (20). Our findings concur with an earlier *in vivo* study in rats (28), in which amiodarone was administered orally at a dose of 100 mg/kg/day. Such treatment inhibited PLC-γ1 activity and increased phospholipid levels in lavaged lung, alveolar lavage cells and surfactant material. Thus, our data confirm previous work and indicate that oral amiodarone treatment activates phospholipidosis, even when administered at dosages relevant to clinical use.

**Phospholipidosis-induced apoptosis**

The present work further investigated the relation between phospholipidosis and apoptosis. We found a significant correlation between apoptosis- and phospholipidosis-related proteins. In agreement with previous work (18, 32), our findings indicate that phospholipidosis induces apoptosis in rat alveolar epithelial cells. The causal relation between these processes is complex and incompletely understood; since PIP2 is involved in calcium homeostasis, changes in intracellular calcium secondary to increased PIP2 may alter mitochondrial membrane potential, thereby affecting membrane permeability. This process is important in both necrosis and apoptosis; the increase in permeability of the inner mitochondrial membrane results in membrane depolarization, uncoupling of oxidative phosphorylation, mitochondrial swelling and release of intramitochondrial ions (14, 33). Our findings shed light on an additional mechanism, namely the decrease in intracellular anti-apoptotic proteins, secondary to phospholipidosis. In fact, it has been previously suggested that phospholipidosis may paradoxically reduce Bcl-2 phosphorylation, thereby enhancing apoptosis (34, 35). Nevertheless, more research on the interrelation between phospholipidosis and apoptosis is needed, before firm conclusions can be drawn.

**Apoptosis**

We found two opposing effects of amiodarone on apoptosis. On one hand, amiodarone tended to increase apoptosis, as shown by decreased expression of anti-apoptotic proteins and by increased expression of caspase-3. On the other hand, amiodarone decreased apoptosis mediated by the caspase-3 pathway. Overall, apoptosis was lower in amiodarone-treated rats than in controls, evidenced by fewer TUNEL-positive alveolar epithelial cells.

Our results contrast previously reported *in vitro* studies, indicating a pro-apoptotic effect of amiodarone; Choi *et al.* (15) demonstrated increased mRNA levels of bax and caspase-3 in a human lung epithelial cell line treated with amiodarone, with increased number of cells exhibiting apoptotic features. Similarly, Bargout *et al.* (16) found that amiodarone, and its metabolite desethylamiodarone, caused significant cell loss in cell cultures of another human derived alveolar epithelial cell line and on alveolar epithelial cells isolated from adult Wistar rats. This cell loss was attributed to apoptosis, because it was inhibited by the broad-spectrum caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone. Our findings also contrast those reported by Uhal *et al.* (22) in *in vivo* rat model of amiodarone-induced pulmonary toxicity, similar to that used in our experiments. These authors (22) reported enhanced apoptosis after six-month amiodarone oral administration, at a daily dosage of 50 mg/kg.

The explanation for the differing results between our experiments and previous work (15, 16, 22, 32) is difficult and may be due to differences in the experimental settings. Moreover, an important parameter may be the low dosage used in our *in vivo* experiments, as opposed to relatively high drug concentrations tested in previous *in vitro* studies. Thus, our present results, in context with previous studies (15, 16, 22, 32), raise the hypothesis that the effects of amiodarone on apoptosis of alveolar epithelial cells are dose-dependent (both in terms of daily and cumulative dosages). Specifically, the anti-apoptotic effects may prevail at relatively low dosages, while at high dosages, amiodarone may induce apoptosis in alveolar epithelial cells. This hypothesis may constitute subject for future research.

In conclusion, our study indicates complex actions of medium-term oral amiodarone administration, at dosages relevant to clinical use, in rat alveolar epithelial cells. Amiodarone decreased the expression of PLC-γ1, resulting in increased intracellular PIP2 and phospholipidosis. These actions decreased intracellular anti-apoptotic proteins and enhanced apoptosis, mediated by activation of caspase-8. However, amiodarone decreased caspase-3, resulting in an overall reduction of apoptosis. Our findings contrast previous reports implicating apoptosis as a primary mechanism of amiodarone-induced pulmonary toxicity and favour phospholipidosis as a more likely candidate. The importance of this mechanism deserves further examination in the future, using more sensitive methods, such as direct measurement of enzymatic activity. Better understanding of the mechanisms underlying amiodarone-induced pulmonary toxicity may set the stage for the development of strategies towards increased safety of this therapy.

**Acknowledgements:** We thank Sanofi-Aventis, Montpellier, France for providing amiodarone.

**Conflict of interests:** None declared.

**REFERENCES**


**Received:** July 8 2010  
**Accepted:** November 8, 2010

Author’s address: Dr. Theofilos M. Kolettis, Associate Professor in Cardiology, University of Ioannina, 1 Stavrou Niarxou Avenue, 45110 Ioannina, Greece; Phone:+30(265)1007227; Fax:+30(265)1007053; E-mail: thkolet@cc.uoi.gr