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Cytotoxic interaction between amiodarone and desethylamiodarone in human peripheral lung epithelial cells

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ABSTRACT

The potent and efficacious anti-dysrhythmic agent amiodarone (AM) can cause potentially life-threatening lung damage (amiodarone-induced pulmonary toxicity; AIPT), which is characterized by cell death in the lungs, followed by inflammation and fibrosis. AM's major metabolite, desethylamiodarone (DEA), has a greater toxic potency than AM and it has been suggested that DEA may act synergistically with AM to cause lung toxicity. The objective of this study was to determine the type of cytotoxic interaction between AM and DEA in HPL1A human peripheral lung epithelial cells. Cytotoxicity was measured by lactate dehydrogenase release. AM and DEA caused concentration-dependent cytotoxicity in HPL1A cells. The concentration of drug causing 50% cell death (LC_{50}) and the Hill slope factor, which represents steepness of the concentration–cell death curve, were significantly different between AM and DEA (12.4 μM and 1.98; 5.07 μM and 5.43, for AM and DEA, respectively) indicating that they may induce cytotoxicity through different mechanisms. A combined concentration of 7.13 μM AM plus DEA, equivalent to 41% of each compound's individual LC_{50} value, resulted in 50% cell death. Isobolographic analysis revealed this effect to be additive, although the combined concentrations were only slightly higher than the concentrations that defined the threshold of synergy (threshold of synergy = 4.21 ± 1.98 μM AM plus 1.73 ± 1.05 μM DEA; experimental data point = 5.06 ± 0.47 μM AM plus 2.07 ± 0.47 μM DEA). The cytotoxic interaction between AM and DEA may be clinically relevant in the development of AIPT.

1. Introduction

Amiodarone (AM; Fig. 1), an iodinated benzofuran derivative, is a commonly prescribed and highly efficacious class III antidysrhythmic agent [1,2]. AM has an extremely long elimination half-life and accumulates in adipose tissue, thyroid, liver, and lungs [3]. AM delays myocardial repolarization mainly by blocking potassium channels and is approved for use in serious dysrhythmias such as ventricular and supraventricular tachycardia, as well as for treatment of atrial fibrillation [4,5].

Although it is considered the "gold standard" drug of its class, AM use is limited by its adverse effects throughout the body [6]. Most effects are minor and reversible; however, the toxicity of greatest concern is the potentially fatal amiodarone-induced pulmonary toxicity (AIPT). This adverse effect is usually associated with chronic AM use and occurs in up to 5–13% of patients in a dose-dependent manner, resulting in death in 10–23% of cases [7]. The etiology of AIPT is not fully understood, but is believed to be initiated by cell death of pulmonary epithelial cells, followed by inflammation, fibroblast proliferation and excessive collagen deposition in the lungs (i.e. fibrosis) [8].

In the liver, AM is metabolized to its primary metabolite, N-desethylamiodarone (DEA), by cytochrome P450 enzymes (Fig. 1). DEA exhibits anti-dysrhythmic activity and accumulates to a greater extent than AM in pulmonary tissues [9]. In addition, DEA has greater cytotoxic potency *in vitro* compared to AM and likely plays a critical role in the initiation and development of AIPT [10,11].

It has been suggested that the presence of AM and DEA together in pulmonary tissues may result in synergistic cytotoxicity, or a toxic effect greater than that expected from the added effects of the two drugs individually [3,11]. Individually, AM and DEA cause cytotoxicity through different mechanisms. In HPL1A cells, immortalized human-derived lung epithelial cells that retain morphological and biochemical features associated with healthy adult pulmonary tissues [12], AM was found to activate mainly necrotic cell death pathways, whereas DEA also activated apoptotic path-

Abbreviations: AM, amiodarone; DEA, desethylamiodarone; AIPT, amiodarone-induced pulmonary toxicity; LDH, lactate dehydrogenase; LC_{50}, concentration causing 50% cell death.

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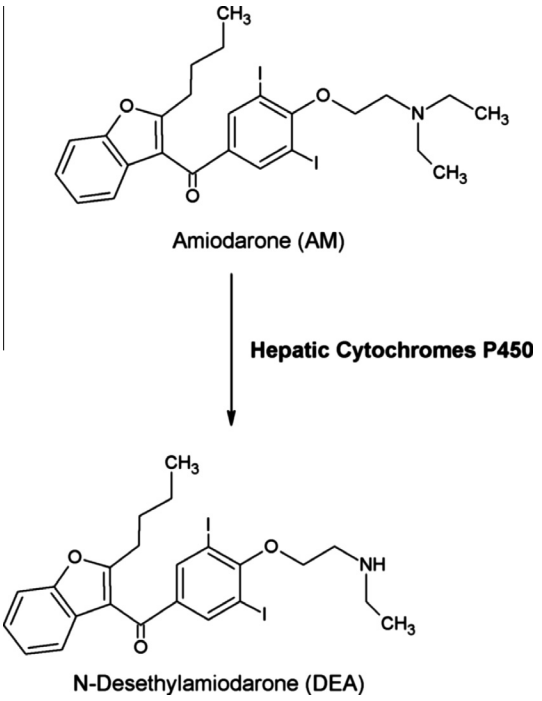

Fig. 1. Structures of AM and its major metabolite, DEA.

ways [13], supporting the possibility that AM plus DEA might cause synergistic cytotoxicity. Appropriate evaluation of drug-drug interactions requires systematic analysis, with the use of an isobologram curve most commonly recommended [14,15]. The present investigation employed such an analysis to determine whether AM plus DEA causes synergistic cytotoxicity in HPL1A cells. Concentration–cytotoxicity curves for AM and DEA were established using lactate dehydrogenase (LDH) release to assess loss of viability, and the cytotoxicity of AM plus DEA in combination was measured to evaluate their interaction.

2. Materials and methods

2.1. Reagents

Chemicals and reagents were obtained as follows: AM HCl, bovine insulin, fetal bovine serum, hydrocortisone, HEPES, and trypan blue from Sigma–Aldrich (Oakville, ON, Canada); and antibiotic–antimycotic, L-glutamine, Ham's F-12 nutrient mixture, human transferrin, and trypsin–EDTA from Invitrogen Canada Inc. (Burlington, ON, Canada). DEA HCl (99.9% purity) was synthesized by Dr. Manlio Alessi (Department of Chemistry at Queen's University, Kingston, ON).

All other reagents were of analytical grade and were purchased from standard commercial suppliers. For the objective of examining the interaction between AM and DEA, stock solutions of 4.0 mM AM and 1.0 mM DEA were prepared fresh by dissolution at 65 °C in reverse osmosis purified water.

2.2. Cell culture

HPL1A cells [12] were cultured in Ham's F-12 nutrient mixture medium, pH 7.2, supplemented with 1.18 g/L sodium bicarbonate, 1% fetal bovine serum, 15 mM HEPES buffer, 1x antibiotic–antimycotic, 100 nM hydrocortisone, 0.13 ng/ml triiodothyronine, 5.0 μg/ml human transferrin, and 5.0 μg/ml bovine insulin (HPL1A medium) [12]. Cells were grown in T-75 flasks (Corning Inc., Corning, NY). Culture medium was replaced every 3 to 4 days, and cells were subcultured approximately every 7 days between passages 8 and 12. Cells were incubated at 37 °C under 95% air, 5% CO_2. After reaching 80–90% confluence, cells were removed from tissue culture flasks by washing twice with 10 ml of phosphate-buffered saline (0.2 g/L KH_2PO_4, 0.8 g/L NaCl, 2.16 g/L $Na_2HPO_4 \bullet 7H_2O$, pH 7.3), and then treated with 0.1% trypsin and 1.06 mM EDTA in phosphate-buffered saline. The cells were then incubated at 37 °C for 5–10 min and re-suspended in HPL1A medium. Cells were seeded at a density of 1.04×10^5 cells/well in 24-well plates and incubated for approximately 72 h (~75% confluence) before drug treatment.

2.3. Lactate dehydrogenase release assay

Cells were treated for 24 h with AM, DEA or vehicle (reverse osmosis purified water) and cell viability was measured by a kinetic LDH activity microassay [16] using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). LDH is a cytoplasmic enzyme that, when found extracellularly, indicates loss of plasma membrane integrity. Briefly, cell culture medium was taken 24 h after treatment, centrifuged at room temperature for 1 min at 12,400*g* to remove cellular debris and combined with sodium pyruvate and β-NADH. The slope of the decrease in absorbance at 340 nm was recorded over 6 min. LDH content from the viable cells remaining in the 24-well plate was also measured (to determine total amount of cells) by lysing the cells using Triton-X-100.

2.4. Determination of drug–metabolite interaction

To determine the interaction between AM and DEA, different concentrations of AM plus DEA were tested in combination. Total drug-plus-metabolite concentrations of 2.5, 5.0, 7.5, and 10.0 μM, containing concentrations of AM and DEA that would be expected to produce equivalent cytotoxicity individually, were tested on HPL1A cells, and cell viability was assessed using the LDH release assay (Section 2.3). From these data, the combination AM plus DEA concentration that would cause 50% cell death was determined. This combination concentration was then plotted on a 50% cell death isobologram curve for AM and DEA and the interaction between AM and DEA was determined [15].

2.5. Data analysis

Each experiment was conducted five times ($N = 5$), with triplicate determinations of the dependent variable for each experiment. For initial AM and DEA LDH release assay experiments, data were fitted to a sigmoidal dose–response curve (variable slope) and the concentration of drug or metabolite producing 50% cell viability loss (LC_{50}) was interpolated using nonlinear regression analysis, which takes into account baseline or control cytotoxicity. The Hill slope factor, a coefficient representing steepness of the curve, and LC_{50} value were statistically compared for AM and DEA using the Sum-of-squares *F* test. To compare cytotoxicity of different total concentrations of AM plus DEA in combination, a 1-way ANOVA was performed. For isobolographic analysis, the experimental combination concentration of AM plus DEA that produced 50% cell death was compared to the synergistic or antagonistic combination concentration of AM plus DEA, as defined by Tallarida [15]. All analyses were performed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA), and statistical significance was defined as $p < 0.05$.