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Amiodarone impairs trafficking through late endosomes inducing a Niemann-Pick C-like phenotype

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ABSTRACT

Patients treated with amiodarone accumulate lysobisphosphatidic acid (LBPA), also known as bis(monoacylglycero)phosphate, in airway secretions and develop in different tissues vacuoles and inclusion bodies thought to originate from endosomes. To clarify the origin of these changes, we studied *in vitro* the effects of amiodarone on endosomal activities like transferrin recycling, Shiga toxin processing, ESCRT-dependent lentivirus budding, fluid phase endocytosis, proteolysis and exosome secretion. Furthermore, since the accumulation of LBPA might point to a broader disturbance in lipid homeostasis, we studied the effect of amiodarone on the distribution of LBPA, unesterified cholesterol, sphingomyelin and glycosphyngolipids. Amiodarone analogues were also studied, including the recently developed derivative dronedarone. We found that amiodarone does not affect early endosomal activities, like transferrin recycling, Shiga toxin processing and lentivirus budding. Amiodarone, instead, interferes with late compartments of the endocytic pathway, blocking the progression of fluid phase endocytosis and causing fusion of organelles, collapse of lumenal structures, accumulation of undegraded substrates and amassing of different types of lipids. Not all late endocytic compartments are affected, since exosome secretion is spared. These changes recall the Niemann-Pick type-C phenotype (NPC), but originate by a different mechanism, since, differently from NPC, they are not alleviated by cholesterol removal. Studies with analogues indicate that basic pKa and high water-solubility at acidic pH are crucial requirements for the interference with late endosomes/lysosomes and that, in this respect, dronedarone is at least as potent as amiodarone. These findings may have relevance in fields unrelated to rhythm control.
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1. Introduction

Amiodarone is a cationic antiarrhythmic drug extensively used for the control of supraventricular and ventricular arrhythmias. Its action mechanism includes blockade of K- and Na channels and interference with β-adrenoreceptors and Ca currents [1,2]. Amiodarone has a large volume of distribution (66 l/kg), steady state serum levels between 0.7 and 3.7 μM, an elimination half-life of several weeks and a propension to accumulate in different tissues, like adipose tissue, skeletal muscle and the liver, but not in the brain [3–5]. Recent evidence suggests that accumulation in tissues might happen by at least two mechanisms, that is the association with cell membranes, due to the high lypophilicity of amiodarone, and trapping in the lumen of acidic organelles after protonation of the amine function present in the lateral group diethylamino-β-ethoxy [6,7]. Amiodarone catabolism includes stepwise dealkylation and deamination of the lateral group diethylamino-ethoxy, major metabolite being N-desethylamiodarone (MDEA) whose activity and serum levels are similar to those of

Abbreviations: LBPA, lysobisphosphatidic acid; ESCRT, endosomal sorting complex required for transport; VLP, virus like particles.

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the parent drug [8]. Chronic exposure to amiodarone induces the formation of vacuoles and inclusion bodies in blood leukocytes, cells of the corneal epithelium, skin cells, alveolar macrophages, liver cells and cardiomyocytes [7]. Although there is agreement that these structures derive from interference of amiodarone with the endocytic pathway, there is uncertainty about the set of endocytic organelles involved, the mechanism of formation of vacuoles and the origin of materials, both amorphous and membrane-like, accumulating in the lumen of inclusion bodies.

Regarding the section of the endocytic pathway involved, it has been claimed that amiodarone does not interfere with early compartments of the endocytic pathway, since the drug does not modify the distribution of the early endosomal marker EEA1 [6]. However this issue remains undecided, since some found no interference of amiodarone with the activity of diphtheria toxin, whose active moiety enters the cytoplasm from early endosomes [9], while others found that amiodarone inhibits the toxin [10]. On the other side several lines of evidence suggest that amiodarone interferes with late compartments of the endocytic pathway. In fact, (i) vacuoles and inclusion bodies bear the markers of late endosomes/lysosomes, like Rab7 and CD63 [6,7], (ii) patients treated with amiodarone accumulate in the airways lysobisphosphatidic acid (LBPA), also known as bis(monoacylglycero)phosphate [11], which is a component of late endosome lumenal membranes [12], (iii) amiodarone inhibits *in vitro* the activity of the anthrax toxin, whose subunits (edema factor and lethal factor) enter the cytoplasm from late endosomes/multivesicular bodies [10,13]. Thus far it is unknown whether amiodarone interferes with recycling endosomes or with the pathway connecting directly early endosomes with the Golgi. Nor is it known if amiodarone interferes with the ESCRT complex, a highly conserved set of proteins associated with a specialized portion of the early endosomes, necessary for the formation of lumenal vesicles and the eventual maturation of this compartment into multivesicular bodies [14]. The ESCRT system is also involved in membrane deformations topologically equivalent to endovesiculation, like cytokinesis and the budding of membrane bound viruses [14]. Interestingly mutations of the ESCRT system lead to phenotypes characterized by the presence of vacuoles and inclusion bodies bearing markers of both early and late endosomes [14].

The observation that patients treated with amiodarone accumulate LBPA, which is only detected in late endosomes and is intimately connected with unesterified cholesterol [12,15], suggests that the drug might alter the distribution of other lipids too, as found in Niemann-Pick type C disease, where mutations of NPC1 or NPC2 proteins cause the accumulation of free cholesterol, glycosphyngolipids, sphingosine, LBPA and sphingomyelin in late endosomes/lysosomes [16–20]. Interestingly in Niemann-Pick type C cells accumulating lipids not only are a manifestation of disturbed traffic, but also play a pathogenetic role, since alleviation of the phenotype can be obtained by decreasing cholesterol levels [21], by inhibiting glycosphingolipid synthesis [22] or by increasing lipid degradation [23]. Very recently it has been shown that Niemann-Pick type C cells try to combat cholesterol accumulation by increasing the secretion of exosomes[24], which are vesicles located in the lumen of late endosomes/multivesicular bodies destined to be secreted rather than to be destroyed [25]. We speculated that, as it happens in Niemann-Pick type C disease, increased LBPA levels observed in patients treated with amiodarone might point to a more complex disturbance in the homeostasis of cell lipids.

In order to clarify some of these issues, we examined the effects of amiodarone on different endosomal activities. These included transferrin recycling, ESCRT functionality (by evaluation of lentiviruses budding) and the pathway connecting early endosomes with the Golgi complex probed with Shiga toxin 1, which after processing within early endosomes, moves to the Golgi through vesicular intermediates [26]. Furthermore we analyzed the effect of amiodarone on the secretion of exosomes and on the distribution of lipids trafficking through late endosomes/lysosomes, like LBPA, cholesterol, sphingomyelin and glycosphyngolipids. Besides this, since vacuoles may reflect a disorder in fluid phase endocytosis, we studied the effect of amiodarone on the traffic of a non-degradable sugar. Finally we correlated the physico-chemical properties of amiodarone analogues with effects on the distribution of LBPA and on the ability to degrade imported proteins. In particular the analogues studied included dronedarone, an amiodarone derivative recently introduced in clinical practice [27].

We found that amiodarone, at concentrations close to patient serum levels, interferes with late compartments of the endocytic pathway blocking the progression of fluid phase endocytosis and causing the inappropriate fusion of organelles, the collapse of lumenal structures, the accumulation of undegraded substrates and the amassing of lipids. These changes recall the Niemann-Pick type C (NPC) phenotype, but originate through a different mechanism, since they are not alleviated by cholesterol removal, while cells treated with NPC-phenotype inducer U18666A revert to the normal after reduction of the cholesterol load. Studies with analogues indicate that high water-solubility at acidic pH is a crucial requirement for the interference with late endosomes/lysosomes and that, in this respect, dronedarone is at least as potent as amiodarone.

2. Materials and methods

2.1. Cells

BHK and Vero cells, obtained from the European Collection of Cell Cultures (ECACC) and human fibroblasts obtained from normal skin biopsies, were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Sigma–Aldrich, Saint Louis, MO). K562 cells, obtained from ECACC, were grown in RPMI medium, 10% fetal calf serum, 50 μg/ml streptomycin, 50 U/ml penicillin(all from Sigma–Aldrich). Rabbit alveolar macrophages obtained by bronchoalveolar lavage and circulating monocytes obtained from healthy blood donors were cultured in Ringer buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM Na_2HPO_4, 10 mM glucose, 10 mM HEPES, pH 7.4) plus 0.1% bovine serum albumin (Sigma–Aldrich) as previously reported [28]. Human embryonic kidney cells stably transduced to express the simian virus 40 T antigen (293T), were kindly provided by Prof. D. Baltimore (Rockefeller University, New York, NY, USA). Cell viability was tested with trypan blue exclusion or with an assay based on the reduction of a tetrazolium salt (Cell Proliferation Kit 1[MTT], Roche Diagnostics, Mannheim, Germany). Data presented are from cells >95% viable with respect to control cells.

2.2. Plasmids

pEGFP-LC3, source Dr Taamotsu Yoshimori, was from Addgene (Cambridge, MA). eEGF-Rab7 was a kind gift from Dr Robert Lodge(Laval University, Quebec City, Canada). eGFP-Rab5 was from Addgene. pΔenv1 plasmid contains Gag/Pol and Rev encoding sequences of the feline immunodeficiency virus (FIV), p34TF10 strain (GenBank accession no. NC_001482) [29]. pSVC21 construct contains a complete HIV-1 provirus, molecular clone HXBc2 [30]. Transfections were done using Attractene Transfection Reagent from Qiagen (Milan, Italy) according to a protocol provided by the supplier or the calcium phosphate procedure.

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