Cation trapping by cellular acidic compartments: beyond the concept of lysosomotropic drugs

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Abstract

"Lysosomotropic" cationic drugs are known to concentrate in acidic cell compartments due to low retro-diffusion of the protonated molecule (ion trapping); they draw water by an osmotic mechanism, leading to a vacuolar response. Several aspects of this phenomenon were recently reexamined. **(1)** The proton pump vacuolar (V)-ATPase is the driving force of cationic drug uptake and ensuing vacuolization. In quantitative transport experiments, V-ATPase inhibitors, such as bafilomycin A1, greatly reduced the uptake of cationic drugs and released them in preloaded cells. **(2)** Pigmented or fluorescent amines are effectively present in a concentrated form in the large vacuoles. **(3)** Consistent with V-ATPase expression in trans-Golgi, lysosomes and endosomes, a fraction of the vacuoles is consistently labelled with trans-Golgi markers and protein secretion and endocytosis are often inhibited in vacuolar cells. **(4)** Macroautophagic signalling (accumulation of lipidated and membrane-bound LC3 II) and labelling of the large vacuoles by the autophagy effector LC3 were consistently observed in cells, precisely at incubation periods and amine concentrations that cause vacuolization. Vacuoles also exhibit late endosome/lysosome markers, because they may originate from such organelles or because macroautophagosomes fuse with lysosomes. Autophagosome persistence is likely due to the lack of resolution of autophagy, rather than to nutritional deprivation. **(5)** Increased lipophilicity decreases the threshold concentration for the vacuolar and autophagic cytopathology, because simple diffusion into cells is limiting. **(6)** A still unexplained mitotic arrest is consistently observed in cells loaded with amines. An extended recognition of relevant clinical situations is proposed for local or systemic drug administration.

Keywords: lysosomotropic drugs; ion trapping; vacuolar ATPase; cell vacuolization; autophagy; phospholipidosis

Contents

Foreword

Drugs that are protonable and possess a cationic form in equilibrium with an uncharged one, essentially primary, secondary and tertiary amines, are numerous and dispersed in many therapeutic classes. They have many different molecular targets for their specific actions, yet share several predictable cellular effects of clinical interest. We are aiming to summarize work conducted in many laboratories about such "lysosomotropic drugs", and illustrate the concept with data from ours. If we attempt to generalize concepts and find commonalities in their cellular actions, it is all too natural that resistance is to be expected and clinical relevance, questioned. Fragmentary supportive evidence for a general model of cell response to cation trapping abounds in numerous experimental and clinical reports; we hope to fill the gap via the present review paper. An extended recognition of clinical situations where this is relevant is proposed. When addressing this phenomenon, one has to keep in mind two principles: (1) the cell responses transcend conventional drug classes because the physicochemical properties of the studied drugs determine the effects. (2) As we shall see, the responses are largely species and cell type-independent, down to one of the most basic eukaryotic cells, the yeast. However, when considering the medical consequences of ion trapping, some tissue specificity consideration may emerge, such as the importance of phagocytic leukocytes conferred by their prominent acidic organelles.

1. Lysosomotropic drugs: initial model and predictions; physicochemical properties of susceptible drugs

Numerous cationic drugs (weak bases with a pK_a ~8-10) are known to concentrate in acidic cell compartments (low retro-diffusion rate under a protonated form at low pH = ion trapping) and draw water probably by an osmotic mechanism (inhibited by cell dehydratation with mannitol, Morissette et al., 2008b and literature cited herein). This leads to a vacuolar response in cultured cells (Fig. 1, pathway *1*). This is one of the most spectacular cellular changes that a microscopist can observe in response to chemicals in a manner largely independent from the animal species and cell type (Figs. 2, 3). A classical review paper on the subject by the Nobelist De Duve included morphological studies of vacuolar cells (light and electron microscopy), drug transport measurements and important predictions, such as the dependence of the cationic drug accumulation on the active pumping of protons into acidic organelles (De Duve et al., 1974). Indeed, the concentration ratio for the cationic drug was calculated to be equal to the ratio of hydrogen ion concentration in the acidic organelles to that in the extracellular fluid and simple diffusion at the plasma membrane was predicted to be the limiting factor for this pseudotransport (De Duve et al., 1974). Agents subjected to this form of distribution were said to be lysosomotropic, a questionable category because there are other acidic compartments in the cells (see below). The vacuolar response applies to amine drugs in a manner that transcends conventional therapeutic classes. For instance, the nonmedicinal tertiary amine triethylamine (Et_3N) induces this cellular response as well as many therapeutically used drugs that may be considered substituted Et_3N (Fig. 2, Supplementary table 1) (De Duve et al., 1974; Morissette et al., 2004). Another chemical series of drugs active in this respect may be based on 2,2-dimethylaminoethanol (DMAE) and higher homologues (Supplementary table 2).

2. The proton pump V-ATPase is the driving force of cationic drug concentration, retention and ensuing vacuolization

Moriyama (1996) further reviewed this form of transport of cationic drugs, which operates against a concentration gradient, is energy-dependent but transporter-independent and described early use of bafilomycin A1, concanamycins and other tools, such as proton conductors (nigericin, etc.) used to analyse the phenomenon. V-ATPase-dependent ion trapping was recorded for radiolabeled chlorpromazine, haloperidol or propranolol into isolated chromaffin granule membranes in the presence of ATP (Moriyama, 1996). This transport participates to the retention of monoamine neurotransmitters and drugs in synaptic vesicles of presynaptic nerve terminals (Moriyama, 1996). As for the drug transport aspect of the phenomenon, Kaufmann and Krise (2007) have discussed elsewhere precise quantitative predictions of the model, and observed deviations from it in experimental data sets, for parameters such as the drug pK_a. It was notably found that the extent of cation concentration in acidic organelles is positively correlated with the pK_a, as predicted, but that the expected delayed equilibrium period for higher pK_a values (greater than 8) did not occur. The reader is referred to previous texts for algebraic models of drug concentrations in the extracellular fluids and concerned cellular compartments (De Duve et al., 1974; Kaufmann and Krise, 2007).

Since 2004, we reexamined many aspects of this phenomenon. The proton pump vacuolar (V)-ATPase is the driving force of cationic drug uptake by cells and of ensuing vacuolization, as shown by the inhibitory effect of its specific inhibitors, bafilomycin A1 or FR 167356 (Morissette et al., 2008b; 2009). V-ATPase is a rotary enzyme complex analogous to mitochondrial ATP synthase, but functioning in reverse mode relative to the latter: it consumes ATP to pump protons (Fig. 1). It is distributed to the trans-Golgi and derived organelles (the endosomes, lysosomes, secretory granules) and, in specialized cell types, to the plasma membrane (e.g., H^+-secreting osteoclasts) (Forgac, 2007). Thus, in our fully quantitative or semi-

quantitative experiments dealing with the transport of 3 substituted Et_3N molecules (procainamide, amiodarone or quinacrine) (Morissette et al., 2008b; 2009; Marceau et al., 2009) or with the primary amine methoxamine (Supplementary Fig. 1), V-ATPase inhibitors greatly reduced the cellular drug uptake and released the drugs in preloaded cells, in a manner as least as effective to drug washout. The cellular uptake of such drugs is also largely reduced when cells are incubated on ice, supporting a metabolic requirement. Therefore, cationic drug concentration into acid vesicles is the steady state of a very dynamic process that is rapidly reversible if the motor force for proton transport is acutely inhibited or if the extracellular concentration of the drug falls below a certain threshold.

Our recent results support the original model in which the concerned amine drugs cross the plasma membrane by simple diffusion. Thus, there is an inverse correlation of the threshold concentration that causes vacuolization with solubility in lipids [logP] in series of compounds (Supplementary Fig. 2, Supplementary tables 1 and 2) and uptake inhibition by a low extracellular pH that decreases the proportion of the uncharged form (Fig. 1; Morissette et al., 2008b), although the contribution of transport or extrusion mechanism is difficult to exclude at the plasma membrane. Our quantitative cation transport studies in smooth muscle cells indicated that the cellular uptake and concentration of procainamide or quinacrine were unaltered by pharmacological blockers of several organic cation transporters (OCTs) and of P-glycoprotein (Morissette et al., 2008b; Marceau et al., 2009). Tetraethyl-ammonium (Et_4N^+), unlike Et_3N, does not vacuolize cells up to 2.5 mM (Morissette et al., 2004), a finding consistent with the simple diffusion model if the charged state of this quaternary amine at physiological pH is considered.

In studies involving pigmented or fluorescent amines, the drugs are effectively present in a concentrated form in the large vacuoles (acridine orange and anthracyclines, Moriyama, 1996; neutral red, Morissette et al., 2004; chloroquine, Morissette et al., 2008b; BODIPY FL histamine, Morissette et al., 2008a; quinacrine, Marceau et al., 2009; amiodarone, Morissette et al., 2009). Thus, in murine dermal fibroblasts, quinacrine (green fluorescence) and amiodarone (violet fluorescence) are both concentrated in perinuclear granules in a V-ATPase-mediated manner (Fig. 2).

While long term inhibition or gene knockout of V-ATPase subunits are incompatible with animal life (inactivating mutations are embryonically lethal in *Drosophila melanogaster* and *Mus musculus*, Dow et al., 1997; Inoue et al., 1999), yeast cells are more tolerant when grown in controlled laboratory conditions. Sertraline, an antidepressant drug with a secondary amine structure, is toxic for yeast cells and a fraction of mutant cells selected for resistance to the drug exhibited inactivating mutations for V-ATPase subunits (Rainey et al., 2010). Wild type yeast cells respond by a vacuolization to sertraline and this morphological effect is lost in cells treated with bafilomycin A1 or in V-ATPase-null mutants (Rainey et al., 2010), reinforcing the ion trapping model.

3. Other sites of cationic drug sequestration and interactions with the V-ATPase-mediated uptake

Cationic drugs frequently exhibit large apparent volumes of distribution, consistent with various forms of sequestration by cells, and concentration in acidic cell compartments is only one of them. A few cationic drugs bind to DNA by different modes (intercalation such as the

anthracyclines, minor groove binding such as the dye Hoechst 33258) and label cell nuclei (pathway *5* in Fig. 1) (Moriyama et al., 1996; Morissette et al., 2008b). The mitochondrion is another known site of trapping for some cationic drugs (Modica-Napolitano et al., 2001) (pathway *4* in Fig. 1) in which the negative membrane potential of the inner membrane ($\Delta\Psi M$) created by ATP synthesis and ion translocation drives the accumulation of lipophilic cations in the matrix. Drugs that are efficiently concentrated in this manner exhibit a high lipophilicity and a preferentially delocalized positive charge, for example an amine function in a resonant system of unsaturated bonds (Modica-Napolitano et al., 2001). Examples of agents selectively concentrated by this mechanism in mitochondria include the neurotoxin N-methyl-4-phenylpyridium (Moriyama, 1996) and several mitochondrial dyes used by cell biologists (e.g., rhodamine 6G, Mitotracker dyes; Morissette et al., 2008b; Marceau et al., 2009). The uptake of such drugs, which can be quantified using their fluorescence in cell extracts, is selectively inhibited by mitochondrial toxins that abolish $\Delta\Psi M$, such as the combination oligomycin+antimycin, while the transport of cationic agents that are trapped into acidic vesicles, sensitive to bafilomycin A1, is unaffected by oligomycin+antimycin (Morissette et al., 2008b; Marceau et al., 2009; Supplementary Fig. 1C). The concentration of a purely DNA-binding chemical, such as Hoechst 33258, into intact cells is not inhibited by either class of metabolic inhibitors (Morissette et al., 2008b) and may be compared to equilibrium dialysis across the plasma and nuclear membranes.

The concentration of cationic drugs into the mitochondrial matrix often leads to inhibition of one or more of the enzyme complex of the oxidative phosphorylation in steps that are seldom well characterized, but that can be ultimately recognized as the rapid loss of $\Delta\Psi M$ and secondary events, such as cytochrome c release and cell apoptosis or necrosis (Modica-Napolitano et al.,

2001). Agents that are concentrated only via the V-ATPase mechanism usually exhibit much less toxicity, but elicit other characteristic responses such as alterations of vesicular trafficking, mitotic arrest and autophagic signaling (see below). However, a number of amine drugs, especially the most lipophilic ones, exhibit a mixed behavior that determines a particularly complex toxicological profile. Thus, the prototype "lysosomotropic" drugs, chloroquine and hydroxychloroquine, concentrate into cells and induce vacuolization at concentration levels (< 50 μM) that are essentially similar to those that dissipate mitochondrial potential (Boya et al., 2003b; Jiang et al., 2008; Morissette et al., 2008b). This justifies the study of more hydrophilic substituted Et_3N derivatives (Figs. 2, 3, Supplementary table 1), such as procainamide, as model drugs to isolate the V-ATPase-mediated component of the cytopathology from other responses. Another example is found among decongestant α-adrenoceptor agonists: phenylephrine induces the vacuolar cytopathology at concentrations that do not interfere with mitochondrial $\Delta\Psi M$, while the more lipophilic agonist xylometazoline rapidly deenergizes mitochondria and has a reduced capacity to induce V-ATPase-mediated vacuolization, presumably due to the toxic effects of the first kind of effect on cells (Morissette et al., 2007a). The same duality applies to the local anesthetics lidocaine and bupivacaine: the concentration-effect curves for vacuolar concentration of the amines and for mitochondrial inactivation overlap for the latter, but not the former drug; consequently, lidocaine induces a strong vacuolar response whereas bupivacaine elicits only a modest vacuolar response that blends with cytotoxicity (Morissette et al., 2011). Further, adding mitochondrial toxins to lidocaine reproduces the profile of bupivacaine: decreased vacuolization, but retained macroautophagic accumulation were observed (Morissette et al., 2011).

Finally, other cationic drugs have affinity for both DNA and the V-ATPase-mediated transport: the anthracycline daunorubicin is an example. While its intrinsic reddish fluorescence labels cell nuclei, the site of its anti-mitotic action, the drug is also concentrated in vacuoles in a bafilomycin A1-sensitive manner (Ouar et al., 2003). Some tumor-derived cells selected for the resistance to the drug had an increased capacity to sequester daunorubicin into acidic vesicles, thus protecting nuclei from drug accumulation, an interesting example of cationic drug redistribution in a cell and one of several potential mechanisms of drug multiresistance in oncology (Ouar et al., 2003). This is further illustrated by the substituted Et_3N derivative, quinacrine, which has some affinity for DNA but does not strongly label smooth muscle cell or fibroblast nuclei and is efficiently concentrated by ion trapping (Fig. 2; Marceau et al., 2009). We recently observed that the green fluorescence of quinacrine is found in both vesicles and nuclei in a specific tumor-derived cell type, and that bafilomycin A1 co-treatment nearly abolished the vacuolar concentration of the drug, but significantly increased the nuclear drug content as judged from the green fluorescence intensity (Supplementary Fig. 3). Chloroquine has affinity for all 3 uptake mechanisms (see below, section 6).

4. Origin and fate of amine-induced vacuoles

Kaufmann and Krise (2007) have noted that lysosomes isolated from cells are fragile and cannot physically expand. On the other hand, procainamide-induced vacuoles are highly spherical and harder than the nucleus where they are imprinted if large enough (three-dimensional reconstitution of cells from confocal sections; Morissette et al., 2005). The bafilomycin-sensitive vacuolization of HeLa cells induced by hydroxychloroquine (a substituted Et_3N) was attributed to the swelling of lysosomes (Boya et al., 2003b) without strong experimental proof. The subcellular origin of the giant vacuoles induced by amines is an unresolved issue, and may have

been obscured by the fact that these vacuoles evolve rapidly and uniformly to become autophagosomes (see below). Several observations support that they originate at least in part from the trans-Golgi (vacuoles induced by TRIS: Peterlik et al., 1979; chloroquine: Bäck and Soinila, 1996), although the swelling and reorganization of the organelles that derive from the trans-Golgi, such as the lysosomes and endosomes, is certainly dominant (see below). Both Golgi p230 and golgin-97 are peripheral membrane proteins associated with the cytosolic leaflet of the trans-Golgi membrane; these proteins are present at the surface of within a rather small subset of contiguous giant vacuoles in amine-treated smooth muscle cells (Morissette et al., 2005; 2008b; Fig. 4A). This may be caused by the vacuolization of other organelles that express V-ATPase further down the secretory pathway. On the other hand, Chen et al. (2011) and Piccoli et al. (2011) failed to observe changes in the distribution of trans-Golgi markers in ARPE-19 or BHK cell made vacuolar with lipophilic amines (light microscopy).

There are important functional effects of ion trapping on vesicular transport, while some discrepant conclusions may dependent on the studied drug and cell type. Consistent with the alteration of the secretory pathway, 3 different cell types subjected to vacuolization by concentrated amines failed to secrete a green fluorescent protein (GFP) variant possessing the prepro-insulin N-terminal sequence (Bawolak et al., 2010), a membrane protein (the bradykinin B_1 receptor) or α-fetoprotein (Morissette et al., 2005). In the latter case, the rat hepatoma cell line was still capable of synthesizing and processing the glycoprotein; however, the secretion process was inhibited. Procainamide-induced inhibition of secretion was relieved by bafilomycin A1, suggesting that the vacuolar morphology interferes with the process (more than vacuolar pH). In another case, several "lysosomotropic" drugs (chloroquine, hydroxychloroquine, amodiaquine, azithromycin) and bafilomycin A1 reduced the secretion of transforming growth factor-β in cells

or live mice by preventing the cleavage of the pro-form (Basque et al., 2008), showing that the secretory pathway of the cell is very much accessible to cationic drugs. Peterlik et al. (1979) report other results supportive of this idea. On the other hand, the highly lipophilic drug amiodarone has comparatively little effects of exosome release from K562 cells (Piccoli et al., 2011).

While it has been consistently observed that early endosome markers such as Rab5 do not label vacuoles induced by cationic drugs (Morissette et al., 2008b; Piccoli et et, 2011), whether a functional defect in the early endocytic function exists is controversial. Thus, amiodarone has not influenced transferrin recycling and Shiga toxin processing in cellular models (Piccoli et al., 2011). However, vacuolar smooth muscle cells exhibited defective endocytosis of BSA-Alexa fluor 594 (Morissette et al., 2008b) and transferrin (Supplementary Fig. 4). Similarly, ARPE-19 cells made vacuolar with chloroquine have a decreased capacity for the uptake of rhodamine-dextran (< 20% of control; Chen et al., 2011). Tamoxifen is a micromolar-potency "lysosomotropic" drug, independently of its estrogen receptor-modulating activity (Supplementary table 2, Supplementary Fig. 6), with endomembrane pH neutralization and depression of various forms of vesicular transport (Altan et al., 1999). Bafilomycin treatment also causes these functional defects, as V-ATPase has been recently shown to be essential for the early to late endosome transition via its intrinsic pH sensing function and interaction with ARNO/ARF6 signaling (Hurtado-Lorenzo et al., 2006) and to the exocytic and endocytic pathways in general (Marshansky and Futai, 2008). Thus, pH buffering by cationic drug retention has the potential to alter in a general manner the intracellular vesicular traffic and provides limited insight into the origin of the vacuoles, while supporting the dramatic consequences of the

vacuolization-associated cytopathology. It is not currently known whether the trafficking of V-ATPase itself is altered by "lysosomotropic" drugs.

Amiodarone-induced large vacuoles in alveolar macrophages were identified as late endosomes (CD63-positive), but not early endosomes (EEA1 negative; Stadler et al., 2008). Time lapse photography has shown that the large vacuoles have a generalized perinuclear origin before filling the whole cytosol (Morissette et al., 2004). These observations may be compatible with a late endosomal origin for most vacuoles, notably because vacuolization first appears in a distinctively perinuclear area where late endosomes are found. An elegant method to isolate the endosomes and lysosomes is based on the endocytosis of dextran-iron and the magnetic isolation of the iron-loaded organelles, after an appropriate chase time period (Kaufmann and Krise, 2007). While isolated lysosomes can concentrate such amines as doxorubicin and Lysotracker dye, it turns out that large amine-induced vacuoles are hybrid organelles, sharing lysosomal and late endosomal markers. We have recently found that giant vacuoles induced by procainamide or lidocaine in smooth muscle cells were labeled with fluorescent protein-conjugated Rab7, CD63 (LAMP-3, LimpI) and LAMP-1 and, in part, with the macro-autophagy effector GFP-LC3 (Fig. 4A). LC3 (Atg8) processing (from the cytosolic I to the active cleaved and lipidated II form; Figs. 4A, B) was also evidenced using immunoblots (Morissette et al., 2008b; Bawolak et al., 2010). Enough confirmatory work has been done using other cell types and drugs to state that the presence of autophagic signaling is a generalized response to cationic drugs that induce vacuolization, e.g. histamine receptor-1 ligands, quinacrine, local anesthetics, the β_2-adrenoceptor agonist salmeterol, tamoxifen, amiodarone and dronedarone (Morissette et al., 2008a; 2009; 2011; Marceau et al., 2009; Bawolak et al., 2010; Piccoli et al., 2011; Supplementary Figs. 5 and 6). The accumulation of the lipidated and membrane-bound form of LC3 (LC3 II) occurs

precisely at incubation periods and concentrations of procainamide, quinacrine and other drugs that cause vacuolization (pathway *2* in Fig. 1; Fig. 4B; Supplementary Fig. 6). The labeling of damaged cell components with LC3 is a secondary event to autophagosome formation, as may also be the presence of the late endosome/lysosome markers Rab7 and CD63 (Jäger et al., 2004). These elements disguise the origin of the large vacuoles; what appears certain now is that they are evolving towards lysosomes like all autophagosomes (step *3* in Fig. 1). Autophagolysosome formation may be responsible for the late presence of amorphous material in large vacuoles induced by amines, which are initially filled with clear fluid (electron microscopy, Fig. 3, 4-h treatment). Further analysis of the fate of giant vacuoles was made in lidocaine-treated cells (Bawolak et al., 2010): inhibiting macroautophagic envelopment with the phosphatidylinositol-3-kinase inhibitor 3-methyl-adenine (step *2* in Fig. 1) logically reduced lidocaine-induced LC3 II formation and GFP-LC3 labeling of giant vacuoles. However, most vacuoles were still positive for Rab7 under these circumstances, suggesting that the origin of the majority of vacuoles was from a Rab7-positive stock, such as the perinuclear late endosomes. A dominant negative (GDP-locked) Rab7 construction had the power to inhibit lysosome fusion to lidocaine-induced vacuoles (step *3* in Fig. 1), as estimated by CD63 absence in the vacuole membranes; this approach allowed dissociation of autophagy from lysosome fusion (Bawolak et al., 2010).

The most probable explanation for the accumulation of endogenous LC3 in cells, or of that of recombinant GFP-LC3 into perinuclear granules, in response to treatment with concentrated cationic drugs is the blockade of the "autophagic flux." This means that a basal rate of autophagy, with continuous synthesis and turnover of LC3 and other effectors of autophagy, is blocked due to lysosomal trapping of the weak bases with ensuing pH neutralization. Thus, cationic drugs such as chloroquine, desmethylclomipramine, lidocaine, imatinib, tamoxifen and a novel cationic

glycosylated lipid induce LC3 II accumulation in various cell types without evidence of increasing autophagy via metabolic deprivation signaling (Iwai-Kanai et al., 2008; Rossi et al., 2009; Jahreiss et al., 2009; Bawolak et al., 2010; Gupta et al., 2010; Fig. 4B; Supplementary Fig. 6E). Rapamycin, the inhibitor of mTor that mimics metabolic deprivation and initiates autophagy in cultured cells, has an additive effect on LC3 II concentration in cells co-treated with a "lysosomotropic" drug (Iwai-Kanai et al., 2008; Rossi et al., 2009). The accumulation of endogenous proteins normally cleared by autophagy, like p62/SQSTM1, is also an effect of desmethylclomipramine or chloroquine attributed to the inhibition of the autophagic flux (Rossi et al., 2009; Sheen et al., 2011). Logically, treatment of cells with bafilomycin A1, that inhibits vacuolar acidification more directly than amines, reproduces the inhibition of the autophagic flux with LC3 II accumulation (Fig. 4B). A second, more speculative, cause of macroautophagy in vacuolar cells that have accumulated weak bases is the membrane damage due to the rapid expansion of acidic organelles.

The cationic anti-neoplasic drug imatinib induces vacuolization and LC3 II accumulation at micromolar concentration levels in cultured cells (Supplementary table 1, Ertmer et al., 2007). It is of interest that evidence of feed-back lysosomal genesis has been obtained in this system under the form of LAMP-1 upregulation. Similarly, a 24-h treatment of ARPE-19 cells with chloroquine (19.4 µM) upregulated the lysosomal proteins LAMP-1 and -2 (Chen et al., 2011). A 24-h treatment of a tumor-derived cell line with the "lysosomotropic" drugs tamoxifen or DMAE or with bafilomycin A1 also upregulated LAMP-1 in a tumor-derived cell line (Supplementary Fig. 6E).

5. Phospholipidosis

The hallmark of drug-induced phospholipidosis is intracellular accumulation of phospholipids within lamellar bodies (diversely called myeloid, onionoid etc.), lysosomal structures that contain multiple concentric layers of undegraded lipids (Reasor and Kacew, 2001), better seen using electron microscopy but also evidenced with fluorescent dyes such as Nile red or LipidTox (Nioi et al., 2007). This affects various cell types in lung, liver, brain, kidney, cornea and other tissues. More than 50 drugs in various therapeutic classes have been identified to induce phospholipidosis upon chronic dosing, including antibiotics (gentamycin), antidepressants (fluoxetine, imipramine etc.), antipsychotics, antimalarial (chloroquine) and antiarrythmic (amiodarone) drugs. These drugs are essentially organic amines that are proven (e.g., amiodarone, imipramine, chloroquine) or suspected to induce V-ATPase-mediated vacuolization; this coincidence had been noted before (Reasor et al., 2006). Amiodarone, a substituted Et_3N (Supplementary table 1), is especially prone to this type of reaction that has been observed in peripheral leukocytes of patients taking the drug (Somani et al., 1986) and pulmonary alveolar macrophages in dosed animals. These changes have been observed in cultured cells exposed to representative cationic amphiphilic drugs. A phospholipidosis-like reaction to sertraline has been observed in yeast cells (Rainey et al., 2010). Chloroquine (50 μM) induced in 4 h a phospholipidosis-like reaction in MDCK cells, with multilamellar and multivesicular bodies in which the drug was present in a concentrated form; all these changes were prevented by bafilomycin A1 co-treatment (Zheng et al., 2011b).

The mechanism of drug-induced phospholipidosis is obscure, although the direct binding of amphiphilic amine drugs to phospholipids with ensuing inhibition of phospholipases has been repeatedly proposed (Reasor et al., 2006). While this condition is usually seen as harmless and slowly reversible once the drug treatment is terminated, the US FDA has established a phospholipidosis working group in 2004 due to definite concerns such as similarity with

hereditary lipid storage diseases (e.g., Niemann-Pick's; Vallance et al., 2004; Piccoli et al., 2011) and the uncertainty over the consequences of the reaction on cell and tissue functions (Nioi et al., 2006). Procainamide-induced cell vacuolization at a supra-therapeutic concentration (2.5 mM) in cultured cells may evolve slowly (≥ 48 h) towards a phospholipidosis-like cell phenotype, with many lipid-filled vacuoles (Nile red-positive; Morissette et al., 2008b; concentric organization of some large vacuoles, with some material seen in negative, is seen in some vacuoles after 48 h of treatment, electron microscopy, Fig. 3, panel c). The progressive "filling" of amiodarone-induced vacuoles by lipids and LC3 has also been observed in cultured cells (Morissette et al., 2009). These studies support a novel hypothesis about the sequence of events leading to this type of outcome: repeated cycles of autophagy may be the source of the multilamellar bodies (Morissette et al., 2008b), each cycle of macro-autophagy adding 2 membrane layers derived from the endoplasmic reticulum via a phosphatidylinositol-3-kinase-mediated envelopment process (Codogno et al., 2005). Signs of early double envelopment of spherical structures can be seen in section of smooth muscle cells submitted to a procainamide treatment (Fig. 3, panels a, b) and in an older electron microscopy study of cutaneous histiocytes in an amiodarone-treated patient (Delage et al., 1975). Thus, the above-presented hypothesis may account for the concentric accumulation of membranes. The slow accumulation of amorphous material in vacuoles (as in Fig. 3, panel b) is a usual feature of autophagy resulting from lysosomal fusion, probably reinforced by the accumulation of undigested material due to lysosomal incompetence. Interestingly, recent results concerning cells treated with amiodarone and related drugs show that there is a qualitative change of lipid composition in vacuoles, with the accumulation of lysobisphosphatidic acid being the most important alteration noted (Piccoli et al., 2011). These observations make stronger the analogy of the cation-induced cytopathology with some aspects of the Niemann-Pick storage disease. However, the reversibility of the cellular vacuolar

phenotype upon drug washout should correspond to the progressive functional restoration of hydrolases in autophagosomes and the resolution of the latter.

6. Cytopathology

In addition to (and perhaps as a consequence of) rather generalized perturbation of vesicular traffic, other observations support a consistent vacuolar cytopathology induced by amines in cultured cells: a still unexplained mitotic arrest not relieved, but in fact mimicked by the V-ATPase inhibitor (Morissette et al., 2004; 2005; 2007b; Bawolak et al., 2010), a decreased cell motility (Morissette et al., 2004) and a generally mild cytotoxicity over 24 h relieved by bafilomycin (Morissette et al., 2007a; 2008b). Thus, the V-ATPase inhibitor bafilomycin A1 inhibits amine-induced vacuolization, drug uptake and, in part, the secretory impairment of vacuolar cells, but rather reproduces other effects of amines: mitosis arrest, endocytosis inhibition and autophagic accumulation.

Mitotic arrest. A still unexplained mitotic arrest is always observed in vacuolar cells loaded with amines (Et_3N and procainamide: Morissette et al., 2004; 2005; lidocaine: Bawolak et al., 2010; DMAE: Morissette et al., 2007b; tamoxifen and DMAE in estrogen-receptor negative tumor cells, Supplementary Fig. 6C). Amine-induced vacuolization does not trigger NF-κB nor p38 nor ERK1/2 MAP kinase signaling in 2 cell types (Morissette et al., 2005; Bawolak et al., 2010) or the "unfolded protein response" of endoplasmic reticulum origin, despite the intracellular accumulation of membranous and secretory proteins (Morissette et al., 2008b). The effects of amines on ploidy are mild and most cells end up in G_1/G_0, reminiscent of the mitotic arrest induced by bafilomycin A1 in fibroblasts (Saurin et al., 1996) or serum starvation in general. In estrogen receptor-negative HT1080 fibrosarcoma cells, tamoxifen is cytostatic at 5-10 µM

(Supplementary Fig. 6). It remains to be seen whether the stress-controlled mitotic inhibitors p53, p21 or p27 are stabilized and show up in cell lysates following treatment with amines that cause vacuolization, a plausible explanation for mitotic arrest via inactivation of specific cyclins. Some of the cell cycle checkpoint proteins, p21 and more constantly p27, were increased in systems where tamoxifen is cytostatic (Lee et al., 1999; Mercier et al., 2003), a likely explanation. In transformed B lymphocytes, chloroquine-induced vacuoles evolved toward a persistent form of autophagy with lysosome fusion and p53-dependent cytotoxicity (Maclean et al., 2008). The mechanism of p53 recruitment by chloroquine can be particularly complex, because the drug binds to and affects the packing of DNA, therefore triggering the ataxia-telangiectasia mutated (ATM) sensor of DNA damage (Kitagawa et al., 2004). However, in the B cells, it turned out that the inhibition of protein degradation in the ineffective autophagosomes induced by the continuous presence of chloroquine triggered a form of starvation that determined p53 expression (Maclean et al., 2008). To further complicate things, chloroquine inactivates mitochondrial potential at concentrations that are close to the range associated with vacuolization (Jiang et al., 2008) and this alone leads to apoptosis. Thus, this drug recapitulates all 3 pathways of cationic drug uptake by cells (Fig. 1) at rather similar concentrations. There is clearly a need to isolate the effect of V-ATPase-mediated amine uptake in mitotic arrest and cell toxicity, which is achievable using a drug more hydrophilic than chloroquine, and investigate the relationship between frustrated macroautophagy and p53-mediated effects. There are alternate plausible reasons for mitotic arrest in vacuolar cells. For instance, transferrin uptake is disrupted in vacuolar cells (Supplementary Fig. 4) and this is theoretically sufficient to stop cell proliferation via iron deprivation (Trowbridge et al., 1982).

7. Pharmacological considerations

7.1. Pharmacokinetic considerations

We have reviewed at least 3 mechanisms of cell concentration for cationic drugs (Fig. 1), which can all lead to high apparent volumes of distribution for the concerned drugs due to accelerated clearance from extracellular fluid. Isolating the role of V-ATPase-mediated pseudotransport is difficult, but may be suspected from the occurrence of high volume of distribution for drugs known to be substrates for this form of concentration, such as astemizole (Morissette et al., 2008a), a formerly clinically used antihistamine. Indeed, astemizole has one of the highest apparent volume of distribution (290 l/kg) of all antihistamines, supporting a very high degree of sequestration; the drug had also a long residual pharmacological effect after cessation (weeks) that may derive from a slow release from intracellular reservoir (Simons and Simons, 1999).

It can be said that the hypothetical V-ATPase-mediated sequestration of cationic drugs away from molecular sites of actions is implicitly accounted for in their clinically used dosage and frequency of administration. However, nobody has ever verified this in vivo. In rats, the substituted Et_3N amiodarone (Supplementary table 1, 10-50 mg/kg i.v.; a known substrate for V-ATPase-mediated transport into acidic cell compartments; Stadler et al., 2008; Morissette et al., 2009) follows a complex and multicompartmental pharmacokinetics (Wyss et al., 1990). A very large proportion of the drug disappears from plasma in the first minutes, with distribution to the liver and lungs (15-60 min; lesser quantities and slower kinetics in other organs; surprisingly little in fat despite the high lipophilicity of the drug) (Wyss et al., 1990). This rapid fall of concentration and preferential trapping of the most lipophilic molecule in histiocyte-rich organs may be the signature of V-ATPase-mediated sequestration. However, V-ATPase inhibitors are poorly tolerated in vivo and the gene knockout is not compatible with animal life, making these conjectures difficult to verify. Extensive extraction and retention of amiodarone (30 µM) in perfused isolated rat lungs (Camus et

al., 1990) is an example of an experimental system more amenable to the verification of the role of V-ATPase and of specific cell types.

7.2. Deliberate addressing of anti-infectious agents in acidic organelles of phagocytes

Classical antimalarial drugs, like chloroquine, concentrate in the parasite digestive organelle, a lysosomal equivalent, thus stopping the nutriment supply by buffering the pH. Two Et_3N groups were purposefully introduced in the novel anti-malarial drug with a heme target, T3.5, in order to direct it to this digestive organelle (Kelly et al., 2009). Similarly, the diamine anti-tuberculosis drug ethambutol is concentrated in macrophage phagosomes, where replicating mycobacteria reside, and its more lipophilic analog SQ109 is even more efficiently concentrated at this location (Jia et al., 2005). It is also remarkable that the macrolide antibiotic azithromycin, possessing two tertiary amine functions but only a moderate lipophilicity (logP 2.08), has a well-known tropism for phagocytic leukocytes (McDonald and Pruul, 1991). Imiquimod and other immunostimulant purine analogs are agonists of Toll-like receptor-7, a receptor for microbial nucleic acids exclusively located in late endosomes; these drugs are concentrated in a bafilomycin A1-sensitive manner into vesicles of human dendritic cells that are relevant for antigen processing and presentation (positive for LAMP-1, CD63, HLA-DR and Toll-like receptor-7; Russo et al., 2011).

7.3. Modulatory effect of cell surface transporters

The classical cation trapping model discussed by De Duve et al. (1974) is based on the simple diffusion of the uncharged form of the amines (usually secondary or tertiary amines). Polyspecific organic cation transporters (OCTs) are members of a large group of homologous solute carriers (SLC) expressed at the plasma membrane and form the SLC22A subgroup that

includes electrogenic transporters (OCT1-3) and electroneutral transporters (OCTN1-3) (Ciarimboli, 2008). Since many of the transporters are expressed in intestine, liver, kidney and special interfaces (e.g., blood-brain, testicular and placental barriers), the transporters of the SLC22A family play important roles in drug absorption, excretion and distribution. The expression of transporters like the OCTs, except perhaps for OCT3, OCTN1 and OCTN2, tends to be rare outside these organs (Ciarimboli, 2008). For instance, OCTN3 is found exclusively in the testis. On the other hand, OCT3, the mediator of the extraneuronal monoamine transport (= catecholamine uptake-2), is widely expressed and mediate the cell uptake of the cationic drug formoterol in smooth muscle cells (Horvath et al., 2007). OCTs are rather selective for the charged form, work in a reversible manner and not against a gradient of concentration (Ciarimboli, 2008); their function is best understood when considering the passage of solutes though a polarized cell layer. Transporters at the plasma membrane level would complicate the ion trapping model, but not invalidate it, because intracellular V-ATPase would mediate the high-capacity concentration and retention of the amines in the acidic vesicles in conjunction with the plasma membrane transport to the cytosol (Fig. 1). Moriyama (1996) has discussed the case of monoamine neurotransmitters at the presynaptic membrane: their V-ATPase-mediated concentration and retention in vesicles is preceded by their recapture by specific transporters. Extrusion mechanisms, such as the multidrug ABC transporter P-glycoprotein, occasionally confuse study of cation transport, as for quinacrine in mouse brain endothelial cells (Dohgu et al., 2004). Knockout/mutant mouse strains for several OCT genes and P-glycoprotein are an interesting resource for future investigations of these issues, especially since mouse cells behave just like their human counterparts relative to the concentration of a series of substituted Et_3N (Fig. 2).

7.4. The cargo makes the cationic drug toxic, not its vacuolar sequestration per se

Boya and Kroemer (2008) have reviewed the mechanisms and chemical agents that induce cell death via lysosomal membrane permeabilization, a process that releases toxic hydrolases, like the cathepsins, into the cytosol and eventually leads to apoptosis or necrosis. The most detergent "lysosomotropic drugs" will trigger these responses (sphingosine, hydroxychloroquine, some fluoroquinolone antibiotics, etc.; Boya and Kroemer, 2008; Boya et al., 2003a; 2003b) but it seems that the more hydrophilic amines are rather better tolerated (low toxicity of millimolar procainamide, phenylephrine in 24 h despite massive cell vacuolization; Morissette et al., 2005; 2007a). Jahreiss et al. (2009) have studied an experimental anti-cancer drug that is a primary amine ether-linked with a long aliphatic chain [1-O-hexadecyl-2-O-methyl-3-O-(2'-acetamido-2'-deoxy-D-glucopyranosyl)-sn-glycerol] and shown its V-ATPase concentration in cells along with inhibition of the autophagic flux (LC3 II accumulation). However, the lipophilic agent also released cathepsins in the cytosol and induced cytotoxicity independently of autophagy. In support of this general mechanism, it could be convincing to show that the variable acute toxicity of detergent "lysosomotropic" drugs can be prevented by a V-ATPase inhibitor, which has been done for procainamide and phenylephrine in the above-cited studies and also for fluoroquinolones (Boya et al., 2003a). Whether such secondary cytotoxic effects are exploitable, e.g., in oncology or parasitology, will depend on the selectivity of the concentration mechanism in pathogenic cells and on their relative resistance to the triggered events.

7.5. Pharmacodynamic distortion occurring below the threshold concentration for vacuolization

V-ATPase-mediated intracellular sequestration of cationic drugs that bind cell surface receptors may remove the drugs from the vicinity of receptors and distorts their pharmacology. This has been illustrated using rabbit aorta contractility assays. Bafilomycin pretreatment modestly influenced the

kinetics (accelerated contraction and relaxation) and the concentration-effect relationship (2-fold potentiation in the nM range of agonist) in the rabbit isolated aorta only for the most lipophilic agent of the α-adrenoceptor agonist series, xylometazoline (logP 3.5), as if the V-ATPase-dependent reservoir was slow to successively fill and empty in control tissues (Morissette et al., 2007a). Still using the rabbit aorta contractility, it was easy to find a drug class where at least some agents would surpass the logP value of xylometazoline: the antihistamines (H_1 receptor antagonists) (Morissette et al., 2008a). Histamine itself (logP -0.97) did not cause cell vacuolization up to 10 mM and its concentration-effect relationship for contractility was unaffected by bafilomycin A1; the H_1 receptor agonist/H_3 receptor antagonist betahistine (logP 0.68) induced vacuolization at 2.5 mM in cultured cells. However, H_1 receptor antagonists caused smooth muscle cell vacuolization at 5-500 µM (vacuoles labeled with Rab7, CD63 and LC3). Antihistamines shift the histamine curves to the right in a concentration-dependent manner. The distorting role of V-ATPase-mediated drug sequestration is thus illustrated: the most lipophilic antagonists (logP for astemizole 5.2, for terfenadine 6.5) are potentiated 3.6-13-fold by bafilomycin A1 co-treatment of tissues, but this is not the case for less lipophilic (logP≤3.4) antagonists, diphenhydramine and pyrilamine (Morissette et al., 2008a). This kind of pharmacodynamic distortion is unrelated to potency at H_1 receptor but, interestingly, may be related to off-target side effects: both astemizole and terfenadine have been withdrawn from the market as arrhythmogenic drugs, whereas the hydrosoluble metabolite of terfenadine, fexofenadine (logP 2.6), is safe and now marketed. We observed that V-ATPase-mediated intracellular sequestration of the drugs involves a pharmacological distortion (underestimation) of the potency in a compact tissue, the isolated rabbit aorta (Morissette et al., 2007a; 2008a), implying that the diffusion rate from the bathing fluid to the receptors in the tissue does not fully compensate for the drug sequestration from the cell surface into the intracellular acidic vacuoles.

8. Clinical applications

8.1. Application of concentrated amines in a topical form or to an anatomically confined area

Since only concentrated amines are subjected to ion trapping and cause cell vacuolization, this response could be dismissed as of toxicological interest only. For instance, the therapeutic concentrations of one of the model drugs in our studies, procainamide, do not reach in vivo the mM levels necessary for the vacuolar/autophagic responses (Figs. 2, 3). However, some locally or topically applied cationic drugs reach such concentrations. Solutions of lidocaine ($\geq$ 42 mM) and other tertiary amine local anesthetics are injected into confined anatomical areas and cause V-ATPase-mediated vacuolization ($\geq$1 mM) in cultured cells (Fig. 2; Bawolak et al., 2010). Without referring to the " lysosomotropic" drug concept, several groups of investigators have described cell vacuolization, with more or less inflammation and toxicity, in tissues infiltrated with local anesthetics. Thus, lidocaine 1% (42 mM) applied into the anterior chamber of the eye caused vacuolization of the locally represented cell types and evidence for extensive drug uptake was obtained (Anderson et al., 1999; Atilla et al., 2003). The topical formulation EMLA (2.5% lidocaine, 2.5% prilocaine) applied to the skin occasionally causes a histopathologic reaction that includes focal vacuolization in the epidermal and dermal (histiocytes, endothelial cells, fibroblasts, pericytes) cells (Hoss et al., 1999; Vallance et al., 2004; Cazes et al., 2007). Local anesthetics are rarely used for more than a few hours and the self-resolving vacuolar cytopathology may not be of great clinical significance for the most hydrophilic of these molecules, whereas cytotoxicity originating from other "off target" causes may be dominant for more lipophilic agents (discussed by Bawolak et al., 2010; Morissette et al., 2011).

The "cosmeceutical" agents DMAE (Supplementary table 2) and triethanolamine (Supplementary table 1) are examples of tertiary amines found in high concentrations in numerous anti-wrinkle topical preparations (Morissette et al., 2007b). By analogy with the local anesthetic cream EMLA that induces both cell vacuolization and skin thickening (Hoss et al., 1999), the massive vacuolization of epidermal cells may be the cellular basis of the rapid action of the "cosmeceutical" agents; this is a concern due to collateral effects on cell functions (mitotic arrest, etc.). We have mentioned above the cell concentration of imiquimod via the V-ATPase-mediated ion trapping; this agent is applied to the skin as a cream to locally stimulate the immunity in specific viral infections and skin cancers (Russo et al., 2011).

Concentrated α-adrenoceptor agonists are often topically applied to mucosae (vasoconstrictor decongestants, mydriatics). Synthetic α-adrenoceptor agonists produced V-ATPase-dependent cell vacuolization (prevented by bafilomycin A1; imidazoline drugs such as naphazoline, xylometazoline; also phenylephrine at 2.5 mM; Morissette et al., 2007a). It is of interest that these drugs cause considerable mucosal injury, with inflammation and vacuolization (Suh et al., 1995), and that corneal application of phenylephrine caused recognizable cell vacuolization in this tissue (Edelhauser et al., 1997). Again, these studies were descriptive, without reference for an underlying cause for morphologic changes.

There is also a potential for cell vacuolization and reservoir formation by the inhaled β-adrenoceptor agonists used as bronchodilators, especially in the long acting β_2-AR agonist (LABA) subclass, that are lipophilic secondary amines (logP for formoterol 2.1; for salmeterol 5.0). Following the general relationship that we are currently establishing between logP and the threshold for vacuolization (Supplementary table 1, Supplementary Fig. 2), we have confirmed that salmeterol ($\geq$ 10 μM) and

formoterol ($\geq$ 250 μM) produce this morphological response by a V-ATPase-dependent mechanism in human smooth muscle cells (effect prevented by bafilomycin cotreatment, Supplementary Fig. 5).

8.2. Systemically administered amines

As illustrated in Supplementary Fig. 2, the threshold concentration for vacuolization (phase contrast microscopy) of cultured smooth muscle cells regularly decreases with increasing lipophilicity (quantified as the logP) for a rather wide range of pK_a values (7.8-10.1) in a series of 15 amines. Amiodarone is one of the most effective and prescribed anti-arrhythmic drugs (Vassalo and Trohman, 2007), mainly used in patients with atrial fibrillation and left ventricular dysfunction. It has a large volume of distribution (66 L/kg), consistent with extensive uptake by tissues, a delayed onset of action and a long elimination half-life (up to 6 months). The major hepatic metabolite of amiodarone, N-desethylamiodarone, is a secondary amine nearly equivalent to the parent compound, if its properties as a weak base are considered, and only slightly less lipophilic. The therapeutic plasma range for amiodarone and its metabolite is 0.7-3.7 μM (Vassalo and Trohman, 2007), but it is widely acknowledged that the tissues contain more drug during chronic dosing. Amiodarone, a highly lipophilic (logP > 7) and systemically administered substituted Et_3N (Supplementary table 1), produces a constellation of side effects (Somani et al, 1986; Vassalo and Trohman, 2007), many of which may be related to drug-induced phospholipidosis/vacuolization driven by V-ATPase-dependent ion trapping: corneal microdeposits (>90% incidence), blue-gray skin discoloration with photosensitivity (4-9%), nonalcoholic steatohepatitis that can lead to cirrhosis (<3%) and a serious pulmonary toxicity that includes interstitial pneumonia, fibrosis, and virtually always "foamy macrophages" that are vacuolar cells containing the typical multilamelar bodies (Myers, 1987). This lung pathology occasionally takes nodular necrotizing or consolidating forms with high densities of

vacuolated histiocytes. The skin discoloration is parallel to the actual presence of amiodarone in enlarged vacuoles in various cell types of the upper dermis (fibroblasts, macrophages, endothelial cells etc., electron microscopy) and is a storage disease secondary to drug deposition (Ammoury et al., 2008). In paraffin sections of patients' skin, tissue macrophages are often a dominant site of vacuolar changes (Delage et al., 1975). Paraffin tissue sections from the face of a patient with amiodarone-induced blue-gray skin discoloration (Ammoury et al., 2008) exhibited vacuoles positive for LC3 accumulation in superficial dermis macrophages (CD68 positive cells; Morissette et al., 2009). These recent results confirm our prediction of macroautophagic accumulation in vivo and support the relative cell type specificity of the vacuolar cytopathology, possibly related to the abundance of acidic organelles in tissue macrophages (histiocytes) of target organs. A close but noniodinated analog recently approved for atrial flutter/fibrillation, dronedarone, has been recently introduced (Supplementary table 1); however it remains a tertiary amine with high logP (7.1) that will also be subjected to ion trapping and may reproduce the side effects caused by tissue deposits of drugs. We and others have observed that amiodarone induced the vacuolization of cultured cells at micromolar concentrations (Baritussio et al., 2001; Morissette et al., 2009); dronedarone was slightly more potent in this respect in rabbit alveolar macrophages (Quaglino et al., 2004) and these agents inhibited the degradation of surfactant protein A and of a viral protein in these cells (Baritussio et al., 2001; Quaglino et al., 2004; Stadler et al., 2008), consistent with a defective lysosomal function possibly caused by endomembrane acidity buffering. Multilamellar structures were observed in cells after 24 h of drug treatments (Baritussio et al., 2001). The cell uptake of amiodarone (^{125}I form, Stadler et al., 2008, or evaluated using its violet fluorescence, Morissette et al., 2009) was found to be largely but not completely reduced by V-ATPase inhibitors, and the remaining drug distribution was diffuse (as in Fig. 2), consistent with a partition in lipids for this particularly lipophilic molecule. However, the proportion of V-ATPase-dependent amiodarone uptake was larger for lower µM

concentrations (Morissette et al., 2009); it was also noted that macrophages derived from peripheral blood monocytes had a greater avidity for amiodarone (lower threshold concentration, higher uptake) than other cultured cell types. Thus, at the high end of the lipophilicity scale in the Et_3N (Supplementary table 1), amiodarone may be the prototype agent with a systemic toxicology dictated by V-ATPase sequestration, with a tropism for phagocytic leukocytes, the production of phospholipidosis, skin discoloration and huge intracellular reservoirs. Other cationic drugs that induced cytoplasmic inclusions in many tissues have been discussed by Hruban (1984).

There is a vast literature about the responses of transformed cells to "lysosomotropic" drugs. If a given cell type is particularly dependent on autophagy for its nutritional requirements, it can be predicted that the block of autophagic flux with such a drug will exert a relatively selective cytotoxic effect, as recently shown with human melanoma treated with chloroquine (Sheen et al., 2011). Glioma cells appear to be sensitive to a range of "lysosomotropic" agents that induce autophagic vacuole accumulation (Geng et al., 2010), possibly indicating a special dependency of these particular cells on the autophagic flux. In other cases, inhibition of the terminal stage of autophagy with chloroquine alleviates chemoresistance to other agents (Notte et al., 2011). The cytostatic effect of tamoxifen may already be at work in estrogen-receptor independent cancers. A large survey has just shown that European women who receive anti-estrogens (mainly tamoxifen) for breast cancer over a long period are protected against the mortality, but not the incidence, of a second form of neoplasm, lung cancer (Bouchardy et al., 2011).

9. Conclusions: towards a new model

Although there is a certain risk to overinterpret the convergence of responses to cationic drugs that are so diverse, and despite the relative paucity of in vivo and clinical supporting data, a

working model emerges. The V-ATPase mediated concentration of cationic drugs is a widespread phenomenon that may contribute to explain pharmacokinetic properties of the agents, some pharmacodynamic distortions and, at a given concentration threshold that is a function of lipophilicity, an autophagic vacuolar cytopathology with lysosomal incompetence followed by feed-back lysosomal genesis. Cytotoxicity from the lysis of vacuoles may occur for molecules that have detergent properties. The vacuolar origin may be multiple, and the consequences on cellular vesicular traffic are profound. An extended recognition of clinical situations where this is relevant needs to be pursued, with a clinical spectrum of toxicological effects that may encompass drug-induced phospholipidosis, the accumulation of persistent autophagic bodies in tissues, and lysosomal incompetence, especially in phagocytic leukocytes. The accumulation of autophagic effector proteins (Morissette et al., 2009b; 2009) and of "signature lipids" such as lysobisphosphatidic acid (Piccoli et al., 2011), are objective cell responses that could experimentally and clinically predict phospholipidosis.

In this line of investigation, progress is needed on many fronts: (1) confirm with many relevant drug examples that V-ATPase has a role in the particular pharmacodynamics profile of cationic molecules (high apparent volume of distribution, relative specificity in the delivery to some organs and cell types); (2) improve the characterization of cell organelles where the agents are concentrated, notably with the application of more extensive cell fractionation methods and subcellular transport studies (Zheng et al., 2011a); (3) exploit gene knockout models to address such questions as the role of facilitating or inhibiting concurrent transport mechanisms in specific cell types and the effectors of death by lysosomal membrane permeabilization; an early application was the use of embryonic mouse fibroblasts in which the macroautophagy effector Atg5 gene is deleted to dissociate the vacuolization and toxicity induced by an amine from

autophagy (Jahreiss et al., 2009); (4) study the effects of accumulating lipids and the accumulation of specific lipids (Piccoli et al., 2011) on organelle biogenesis and cell trafficking pathways; (5) integrate new concepts about the possible feed-back lysosome genesis in cells loaded with cationic drugs, such as a central role of the transcription factor TF-EB that controls the synthesis of LAMP-1, of V-ATPase subunits and of hundreds of other genes expressed in lysosomes (Peña-Lopis et al., 2011); (6) establish the contribution of the cytopathology to the side effects of clinically used drugs, but also perhaps to their desirable effects in oncology.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Schematic representation of the cellular sites and mechanisms of organic cation trapping (modified after Morissette et al., 2008b, Bawolak et al., 2010). $\Delta\Psi_M$, negative membrane potential of the inner mitochondrial membrane. Numbered pathways refer to translocation events discussed in the text.

Fig. 2. V-ATPase-dependent concentration of cationic drugs of the triethylamine series (log P values indicated for individual drugs). The evidence of cell uptake for the all drugs is cell vacuolization and, for the 2 most lipophilic drugs, the presence of their intrinsic fluorescence in vacuoles. Primary C57BL/6 mouse fibroblasts were cultured from subcutaneous conjunctive tissue explants in DMEM supplemented with 10% fetal bovine serum and antibiotics and passaged in this medium. All drug treatments: 4 h. Original magnification 400×. Unpublished material from our laboratory.

Fig. 3. Preliminary EM observation in human smooth muscle cells treated with procainamide (PA, 2.5 mM, various time periods). The perinuclear area is shown (nuclei at the right or lower right in the images of the left column, 2500 ×). The right column close-up views are described in the text. Unpublished material from our laboratory.

Fig. 4. Effect of lidocaine on cultured smooth muscle cells. A. Lidocaine-induced giant vacuoles in rabbit aortic smooth muscle cells: labeling with relevant fusion proteins. Modified from Bawolak et al., (2010), with permission from Springer. In control cells, GFP-golgin-97 labels

perinuclear ribbon-like structures. In lidocaine-treated cells, many structures that contain condensed GFP-golgin-97 are enclosed in spherical membranes positive for Rab7 (see colocalization in Bawolak et al., 2010). B. Immunoblot for LC3 in extracts of human vascular smooth muscle cells submitted to lidocaine (0.25-2.5 mM, 4 h) or other treatments (unpublished material from our laboratory).

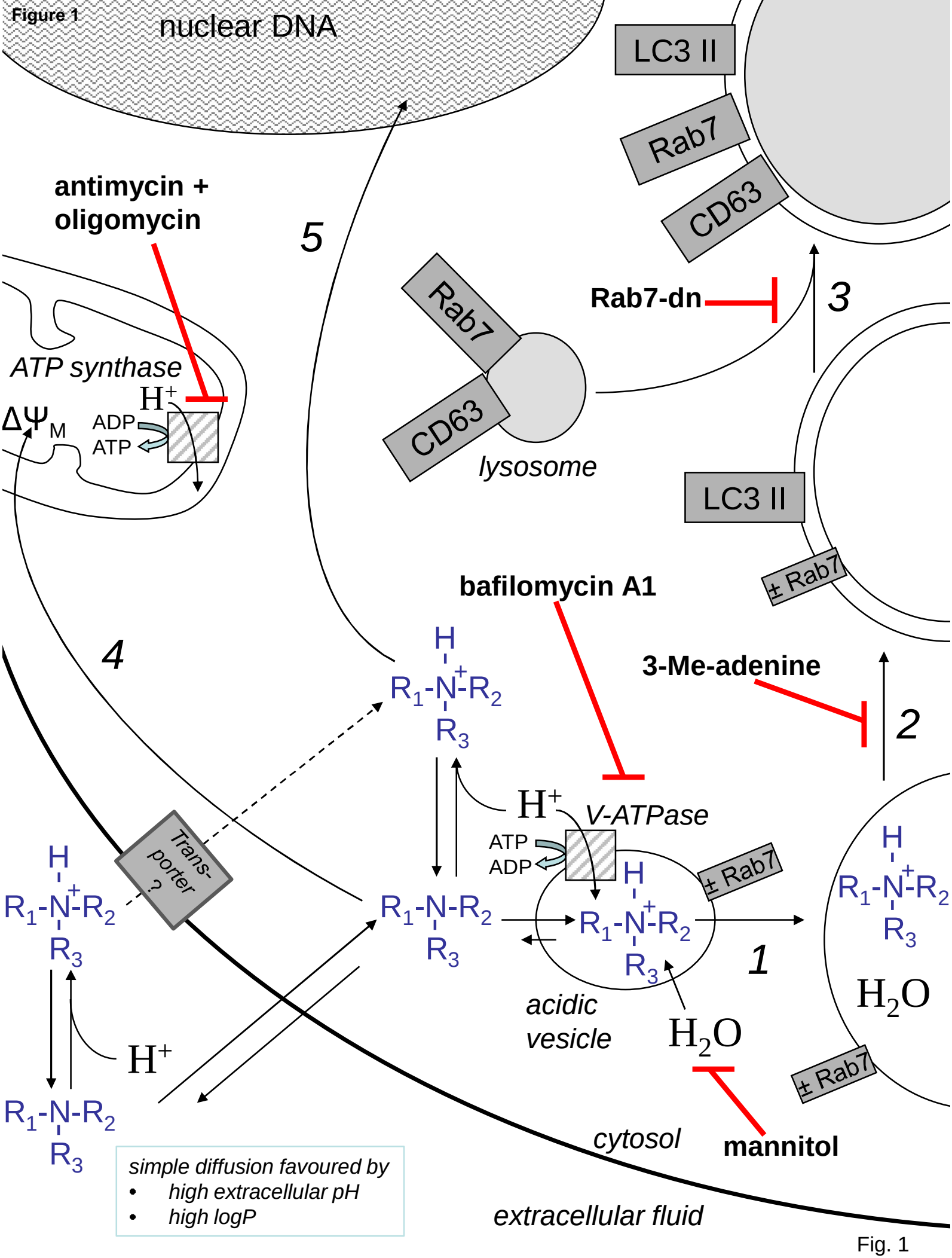
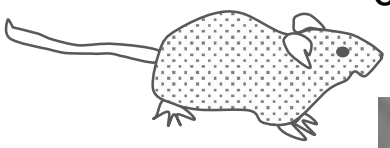


Figure 1
nuclear DNA
LC3 II
Rab7
CD63
antimycin +
oligomycin
5
Rab7
CD63
lysosome
Rab7-dn
3
ATP synthase
H+
ΔΨM
ADP
ATP
LC3 II
± Rab7
bafilomycin A1
4
3-Me-adenine
2
H
R1-N+-R2
R3
H+
V-ATPase
ATP
ADP
Trans-
porter
?
R1-N-R2
R3
± Rab7
1
acidic
vesicle
H2O
H2O
± Rab7
mannitol
cytosol
extracellular fluid
simple diffusion favoured by
• high extracellular pH
• high logP

Fig. 1

Figure 2

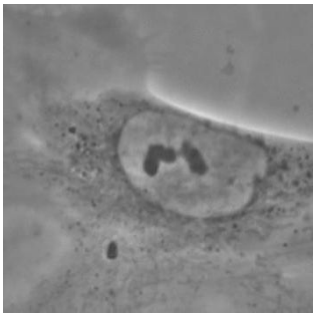


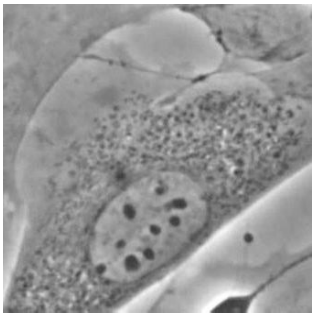
control (DMSO vehicle)

+ bafilomycin A1 100 nM

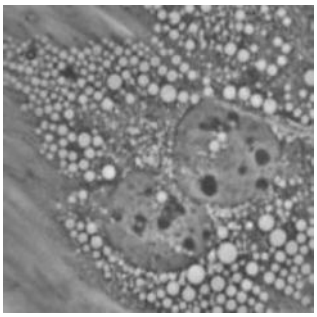
log P

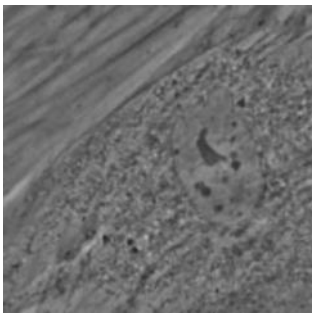
phase contrast control





procainamide 2.5 mM





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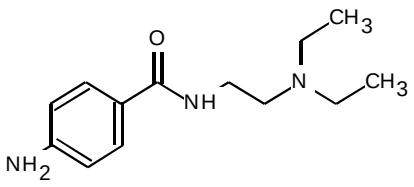
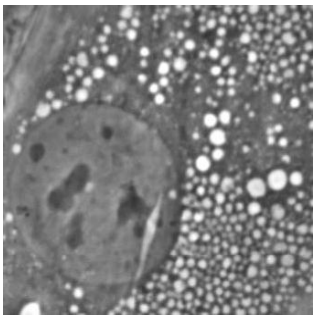
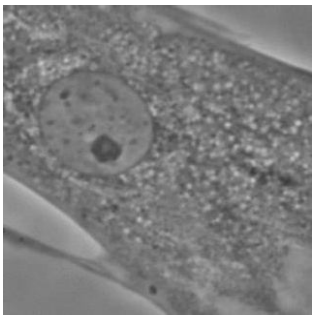


lidocaine 2.5 mM





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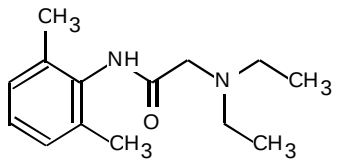
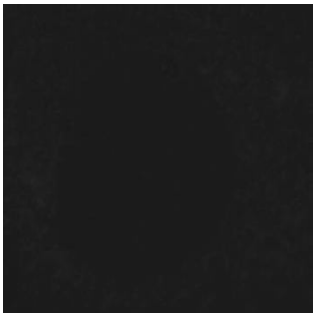
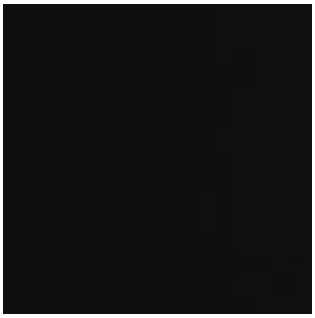
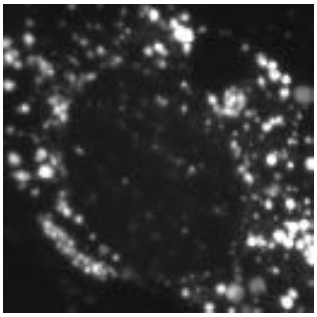


epifluorescence control





quinacrine 2.5 µM *(green fluorescence)*



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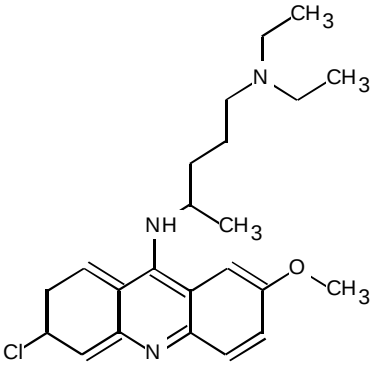
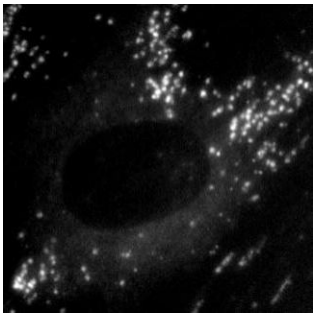
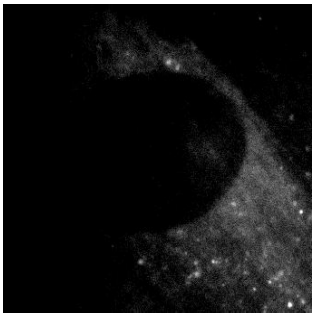


amiodarone 5 µM *(violet fluorescence)*





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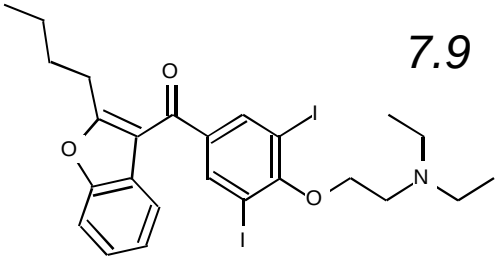
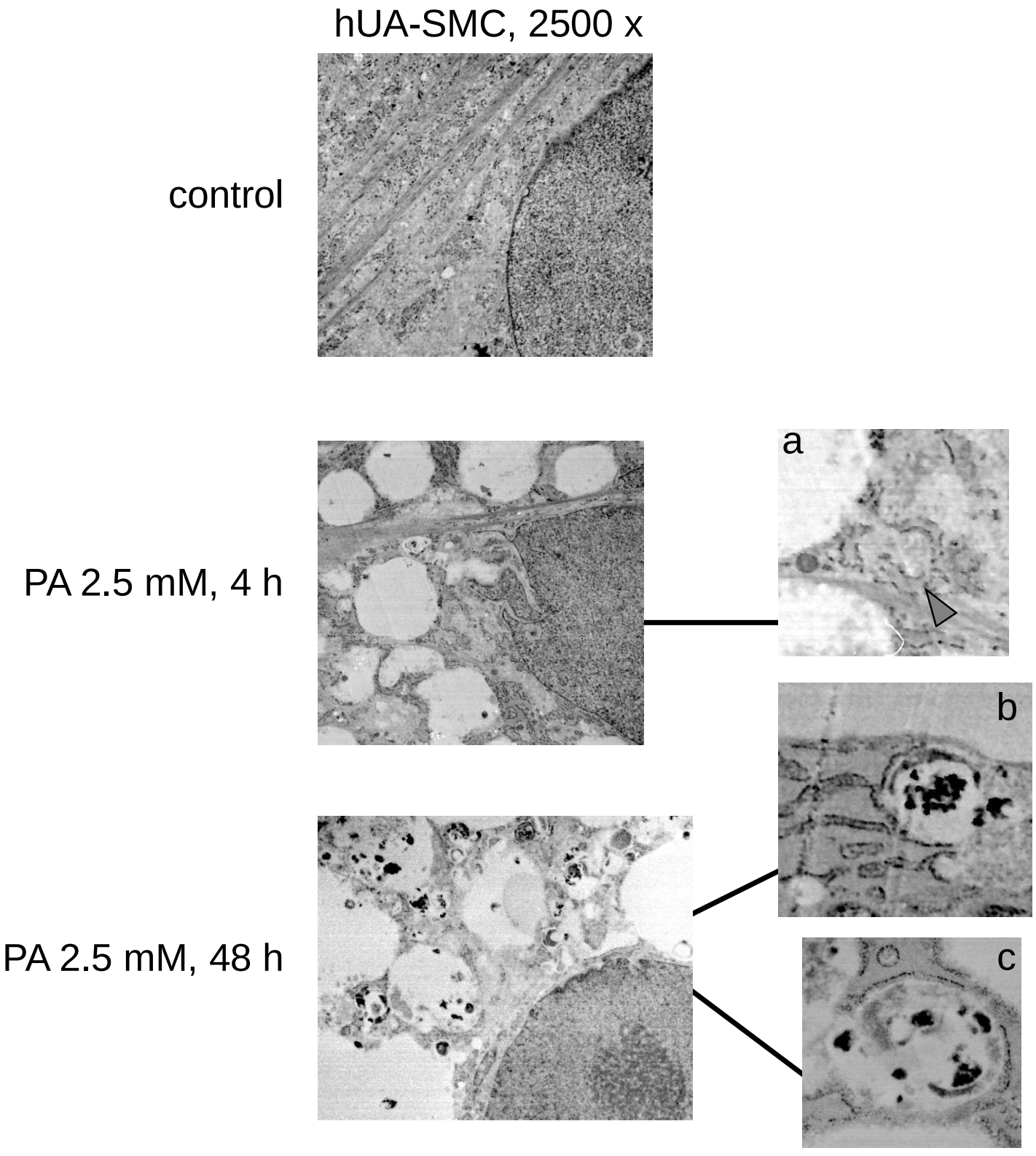
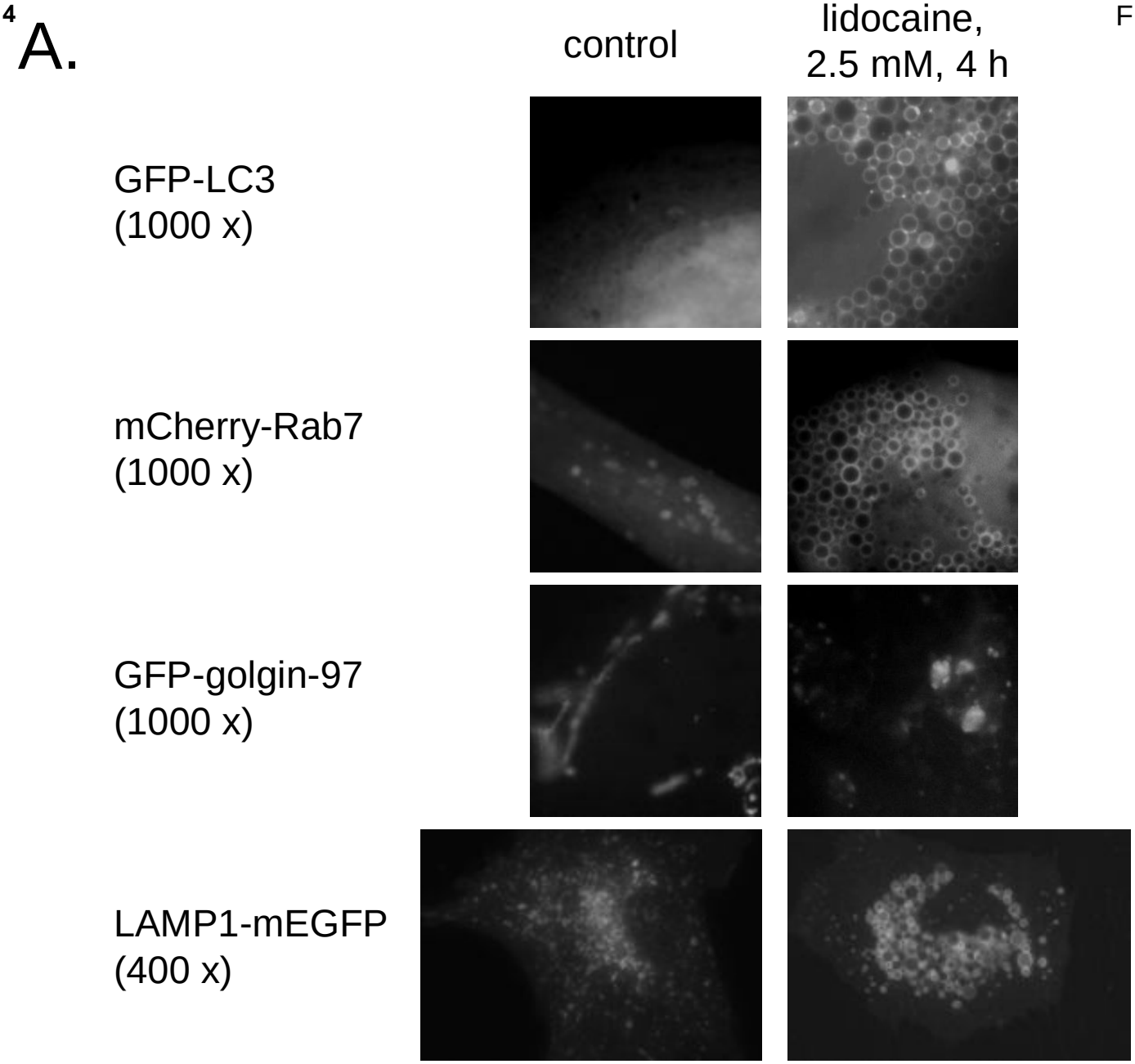


Figure 3

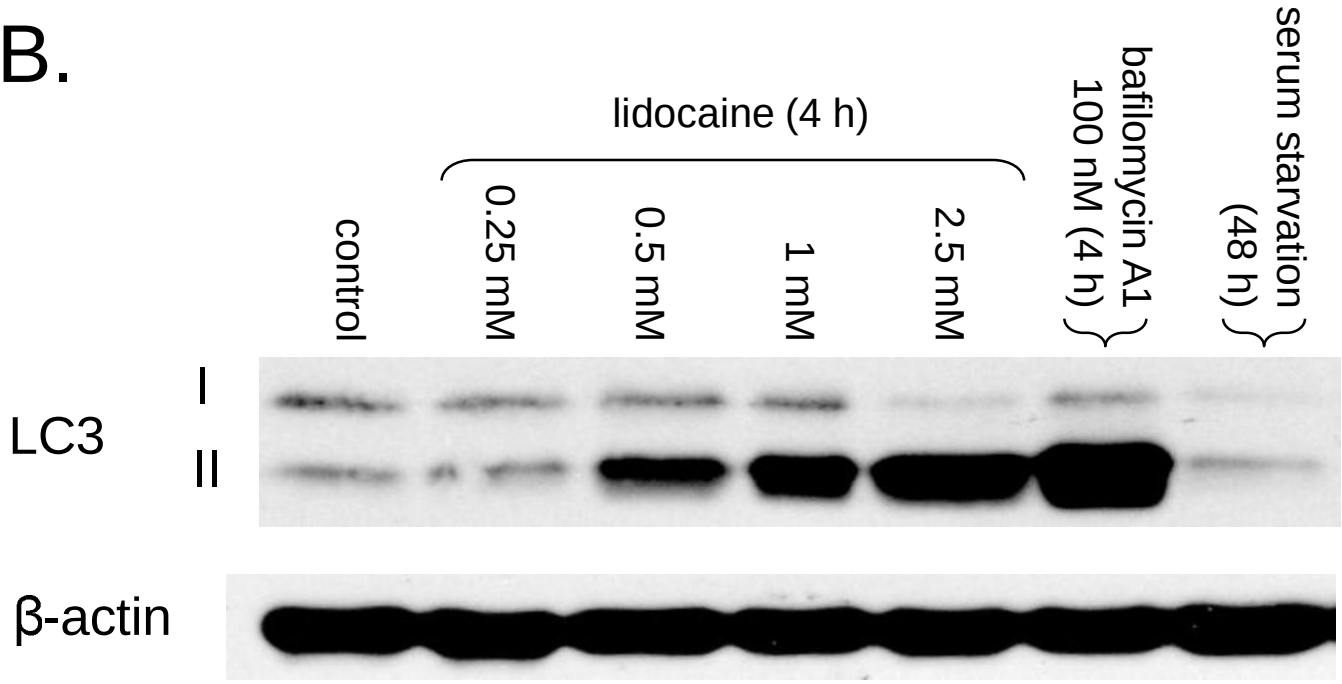
Fig. 3



A.


control
lidocaine, 2.5 mM, 4 h
GFP-LC3 (1000 x)
mCherry-Rab7 (1000 x)
GFP-golgin-97 (1000 x)
LAMP1-mEGFP (400 x)

B.


lidocaine (4 h)
control
0.25 mM
0.5 mM
1 mM
2.5 mM
bafilomycin A1 100 nM (4 h)
serum starvation (48 h)
LC3
I
II
β-actin

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Manuscript number (if applicable):

Article Title: Cation trapping by cellular acidic compartments: beyond the concept of lysosomotropic drugs

Author name: François Marceau, Marie-Thérèse Bawolak, Robert Lodge, Johanne Bouthillier, Angélique Gagné-Henley, René C.-Gaudreault, Guillaume Morissette

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Please state any competing interests

None.

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Signature (a scanned signature is acceptable, but each author must sign)	**Print name**
François Marceau	François Marceau
	Marie-Thérèse Bawolak
	Robert Lodge
Johanne Bouthillier	Johanne Bouthillier
Angélique Gagné-Henley	Angélique Gagné-Henley
	René C.-Gaudreault
Guillaume Morissette	Guillaume Morissette

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None.

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