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REVIEW

Pharmacological regulators of autophagy and their link with modulators of lupus disease

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Autophagy is a central regulator of cell survival. It displays both anti- and pro-death roles that are decisive in the maintenance of cell homeostasis. Initially described in several eukaryotic cellular models as being induced under nutrient stress favouring survival by energy supply, autophagy was found later to display other decisive physiological roles, especially in the immune system. Thus, it is involved in antigen presentation and lymphocyte differentiation as well as in the balance regulating survival/death and activation of lymphocytes. Autophagy therefore appears to be central in the regulation of inflammation. The observation that autophagy is deregulated in systemic lupus erythematosus is recent. This discovery revives the programme dealing with the design and development of pharmacological autophagy regulators in the therapeutic context of lupus, a debilitating autoimmune disease that affects several million people in the world. A large number of molecules that positively and negatively regulate autophagy have been described, most of them with therapeutic indications in cancer and infection. Only a few, however, are effectively potent activators or inhibitors endowed with experimentally demonstrated selective properties. In this review article, we highlight the most relevant ones and summarize what we know regarding their mechanism of action. We emphasize the link between pharmacological regulators of autophagy and inducers or inhibitors of lupus disease and discuss the fundamental and pharmacological/therapeutic interest of this functional interplay.

Abbreviations

CMA, chaperone-mediated autophagy; CQ/HCQ, chloroquine/hydroxychloroquine; HSPA8/HSC70, heat shock cognate protein of 70 kDa; LAP, LC3-associated phagocytosis; LC3, microtubule-associated protein light chain 3; MHC-I/II, MHC class I or MHC class II; mTOR, mammalian target of rapamycin; PRR, pattern recognition receptors; SLE, systemic lupus erythematosus

Table of Links

TARGETS	LIGANDS	
AKT	Amiodarone	Rottlerin (mallotoxin)
AMPK	Azithromycin	Taxol (paclitaxel)
ATG1/ULK1	Chloroquine	Valproate
HSPA8/HSC70	Dexamethasone	Vinblastine
HSP90	Hydroxychloroquine	Wortmannin
Imidazoline receptors	Loperamide	
IP_3R, IP_3 receptors	Metformin	
mTOR	Nitrendipine	
PI3K	PP-242	
PRR, pattern recognition receptors		
TLR 9	Rapamycin	

This Table lists the protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, b, c, d).

Autophagy and its implication in human diseases

Autophagy is a catabolic process related to lysosomal degradation. Opposed to heterophagy, which implies degradation of substrates from outside the cell, autophagy catabolizes the cytoplasmic content. There are three main pathways involved in autophagy. The first one, called microautophagy, involves the direct engulfment of cytosolic material into the lysosome. Its core molecular machinery and regulation as well as its possible implication in human diseases are still poorly understood. In this review, we will focus the first part of our comments on the two other types of autophagy, namely, macroautophagy and chaperone-mediated autophagy (CMA). Historically, the term 'autophagy' was proposed by Christian De Duve in the 1950s (Yang and Klionsky, 2010). The term referred to the identification of single- or double-membrane vesicles containing organelles or cytoplasm in partial state of degradation. The double-membrane structures were called autophagosomes and were shown to ultimately fuse with lysosomes to form single-membrane autolysosomes (Figure 1). The process that is currently named macroautophagy (often simply referred as 'autophagy', introducing sometimes some confusion) is active at basal levels in all cell types. Macroautophagic activity is increased under energy stress caused by amino acid starvation. This degradative process catabolizes cytoplasmic contents, abnormal protein aggregates and excess or damaged organelles. The first molecular actors of the complex network regulating macroautophagy were discovered only 30 years later by screening mutant yeasts with survival defects under nitrogen starvation (Tsukada and Ohsumi, 1993). Several orthologous genes were found in mammals (Feng *et al.*, 2014). They are called *ATG*, autophagy-related genes, as their function is sometimes not only related to macroautophagy. These genes can be classified into four main groups involved in different steps along macroautophagy progression (Figure 1). The complex I containing ATG1/ULK1 is implicated in the initiation of the macroautophagy process and is able to indirectly sense energy stress, as described later, and initiate autophagy (Russell *et al.*, 2014). The complex II involves ATG9a and allows recruitment of membrane to the so-called phagophore, at the origin of the autophagic vesicle. The complex III contains Beclin-1 (mammalian orthologue of *Atg6*) and Vps34, a class III PI3K (PI3KCIII), and allows docking of (phosphatidylinositol 3-phosphate)-binding proteins, necessary for further steps. Finally, two ubiquitin-like systems characterize the complex IV, necessary for phagophore elongation. The covalently conjugated proteins ATG5–ATG12 transiently locate to the phagophore until autophagosome completion, where they recruit ATG16L1. This complex is thought to facilitate, with the help of ATG7 protein, ATG8/microtubule-associated protein light chain 3 (MAP1LC3) lipidation. The latter protein, most generally named LC3, exists in two main forms. The first one, LC3-I, which is soluble and cytosolic, covalently links to phosphatidylethanolamine to generate the second, autophagosome membrane-bound form, LC3-II. In contrast to the ATG5–ATG12/ATG16L1 complex, LC3-II is integrated into the elongating phagophore and remains associated during the whole macroautophagy process. Measurement of the LC3-II marker is therefore an excellent indicator of the autophagosomal load (Klionsky *et al.*, 2012). The autophagosome then fuses with a lysosome leading to the autolysosome where the material is degraded (Figure 1). It is noticeable that suppression of any one of the core genes of the macroautophagy machinery can prevent or at least alter the process.

The first signalling pathway described, leading to macroautophagy ignition driven by starvation, is the inhibition of the mammalian target of rapamycin (mTOR; Shimobayashi and Hall, 2014; Figures 1 and 2). The latter protein can asso-

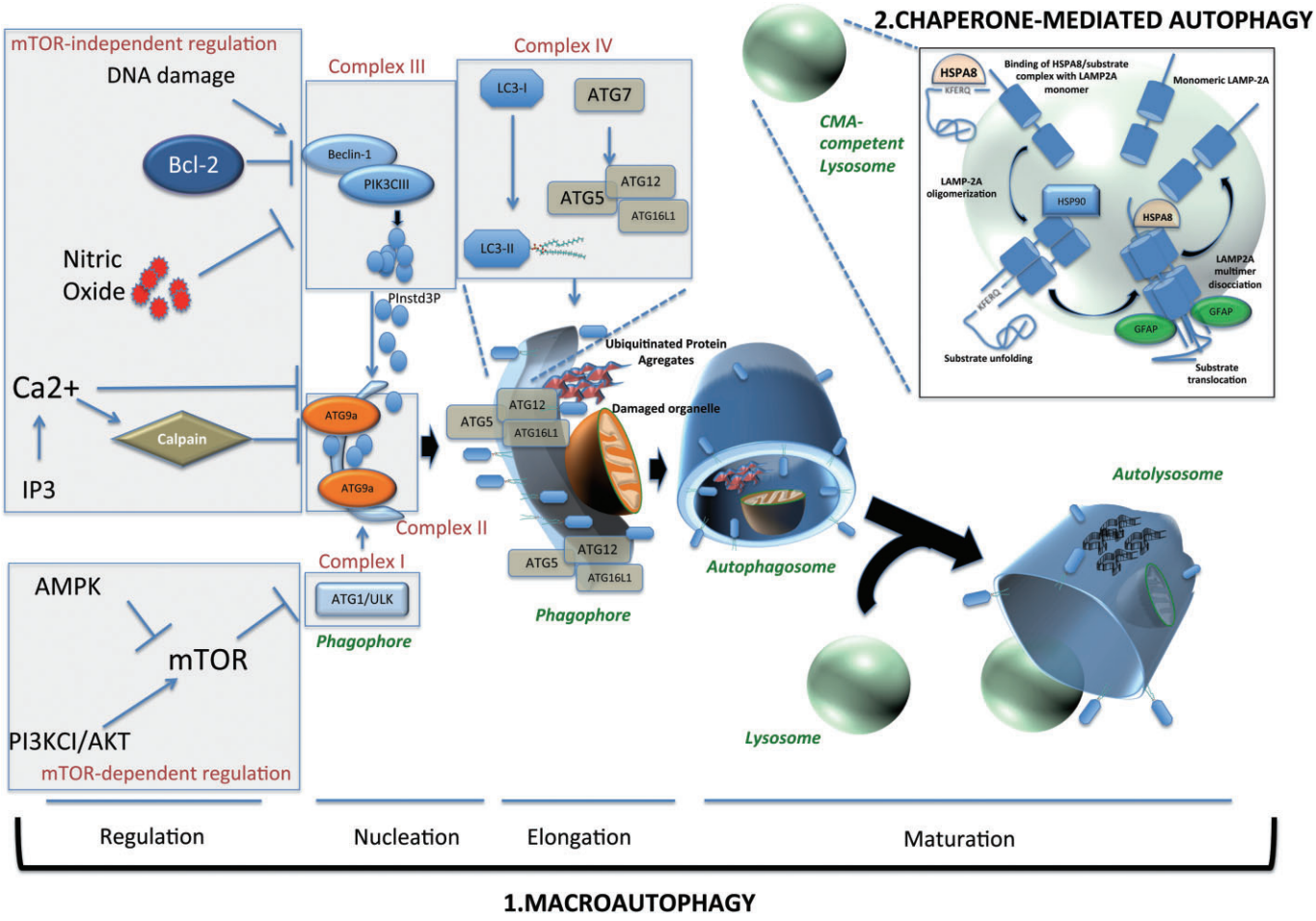


Figure 1

Overview of macroautophagy and CMA processes. (1) Macroautophagy can be induced by mTOR-dependent processes: mTOR indirect inactivation by AMPK or by decrease of PI3KCI/AKT axis activity. mTOR-independent processes can also regulate macroautophagy: macroautophagy is induced by DNA damage, inhibited by association of Beclin-1 with Bcl-2 and by high intracellular levels of NO, IP_3 and calcium (the latter influencing macroautophagy directly or indirectly via calpain activation). Activation of macroautophagy is allowed by activation of complex I composed of ATG1/ULK1 and complex II consisting of Beclin-1/PI3KCIII generating PIn3P at the phagophore membrane containing the transmembrane ATG9a. The latter structure elongates via incorporation of the ATG5-ATG12/ATG16L1 complex thanks to ATG7 ubiquitin ligase activity, and via the incorporation of LC3-I modified with a covalent linkage to a phosphatydylethanolamine, then labelled LC3-II PE. The closure of the phagophore generates an autophagosome. LC3 remains associated with the membrane whereas the ATG5–ATG12/ATG16L1 complex is released from the vesicle. The autophagosome then fuses with lysosomes leading to the degradation of its content in a so-called autolysosome. The internal lipid bilayer is degraded and LC3-II still decorates the remaining membrane. (2) CMA is active in lysosomes expressing LAMP-2A. Protein substrates for CMA possess a KFERQ or KFERQ-like motif allowing their recruitment by HSPA8. The complex that is formed between this latter chaperone and the substrate permits dimerization of LAMP-2A at the surface of the lysosomal membrane. The dimer association is maintained by intralysosomal HSP90. LAMP-2A tetramers are then formed and associate with GFAP. The substrate can then translocate inside the lysosomal lumen and is degraded by the low pH and enzyme activity. LAMP-2A dimers need to dissociate before entering a new cycle of CMA substrate internalization. It is feasible in the presence of HSPA8 molecule pool, present in the lysosomal lumen.

ciate into two different complexes, mTOR complex (mTORC) 1 and 2. mTORC1 is the complex involved in macroautophagy regulation and is composed of mTOR, a so called regulatory-associated protein of mTOR (Raptor), proline-rich AKT (also known as PKB) substrate of 40 kDa (PRAS40), mammalian lethal with SEC13 protein 8 (mLST8) and the so-called dishevelled, EGL-10 and pleckstrin domain containing mTOR-interacting protein or Deptor (Jewell *et al.*, 2013). Intracellular amino acid levels can be sensed by a complex consisting of Ras-related GTP-binding protein (Rag), v-ATPase and Ragulator present at the surface of lysosomes. Under amino acid starvation, this complex remains inactive and does not sequester Raptor, compromising therefore its interaction with the so-called Ras homologue enriched in brain (RHEB). RHEB cannot activate mTORC1 any more and macroautophagy can be initiated through the activation of ATG1/ULK1 (Figure 2). In addition to high intracellular amino acid levels, insulin and several growth factors inhibit macroautophagy by triggering the AKT and class I PI3K (PI3KCI) pathway (Lum *et al.*, 2005; Vander Haar *et al.*, 2007).

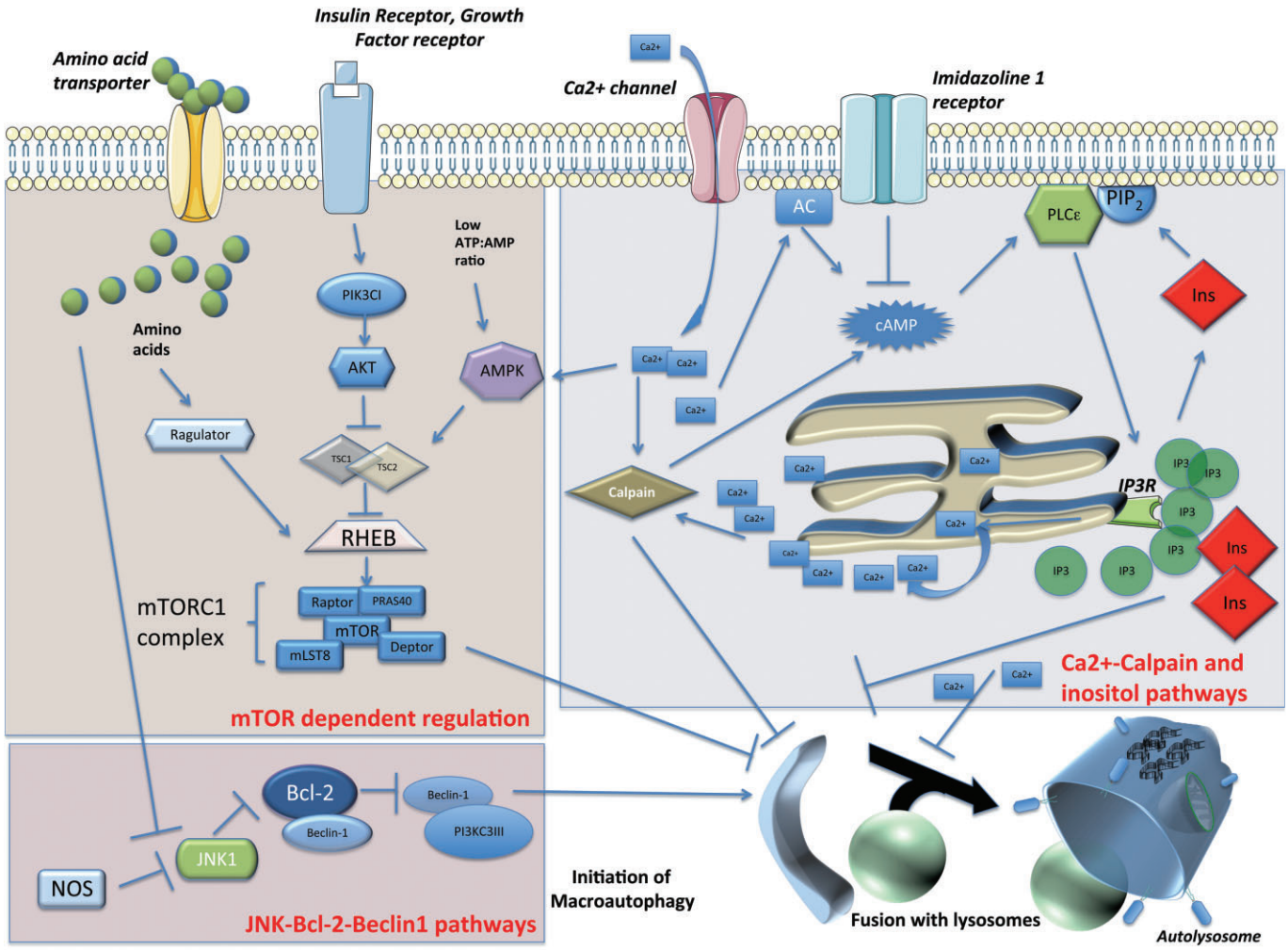


Figure 2

Macroautophagy is regulated by several signalling pathways. Macroautophagy is regulated by the mTOR pathway. The protein Ragulator in complex with v-ATPase and Rag can sense the level of intracellular amino acids. Under nutrient-rich condition, Ragulator activates RHEB, which activates mTORC1 composed of mTOR, PRAS40, mLST8 and Deptor. mTORC1 activation prevents macroautophagy initiation. mTOR can also be indirectly stimulated by insulin and other growth factors. The interaction of the latter with their receptors activates the PI3KCI/AKT axis relieving RHEB inhibition by TSC1/TSC2. RHEB can then activate mTORC1 and inhibit macroautophagy activation. The energetic level of the cell can be sensed by AMPK complex activated upon low ATP/AMP ratio. AMPK then activates TSC1/TSC2 and inhibits RHEB. In consequence, mTORC1 is inhibited thus favouring macroautophagy initiation. Macroautophagy is activated through the JNK1/Bcl-2/Beclin-1 pathway. Upon nutrient starvation, NOS activity, the kinase JNK1 is activated. The activation of JNK1 allows the dissociation of Bcl-2 and Beclin-1 and then permits the association of Beclin-1 with PI3KCIII and initiation of macroautophagy. IP3 and intracellular calcium regulate macroautophagy activity. Activation of AC, for example, downstream GPCR signalling, leads to the production of cAMP that will activate PLCε and then to the production of IP3 from phosphatidyl inositol diphosphate PIP_2. IP_3 can further be transformed into inositol and then back to PIP_2. cAMP level increase can also be induced after inhibition of imidazoline 1 receptors. Binding of IP_3 to its receptor expressed at the surface of ER leads to the release of Ca^{2+} stores. Both intracellular Ca^{2+} and Ca^{2+} imported from the extracellular milieu can activate calpain that will further lead to the production of cAMP. Calpain activity, high IP_3 and inositol intracellular levels inhibit macroautophagy initiation. High Ca^{2+} levels have been shown to block phagosome–lysosome fusion, thereby preventing macroautophagy flux.

Activation of the PI3KCI/AKT axis inhibits the tuberous sclerosis complex (TSC) 1 and 2. This inhibition alleviates the inactivation of RHEB, which becomes able to stimulate mTORC1, finally inhibiting macroautophagy (Manning and Cantley, 2003). mTORC1 can also be negatively regulated by 5′AMP-activated protein (serine/threonine) kinase (AMPK) activity (Mihaylova and Shaw, 2011). AMPK is activated by low ATP/AMP ratios, indicative of energy stress. Activation of AMPK triggers TSC1 and TSC2 leading to the inhibition of RHEB and mTORC1. AMPK can also directly activate ATG1/ULK1 through phosphorylation.

A second set of macroautophagy activation pathways that are mTORC1 independent have been described. One of these pathways involves activation of AC leading to increased cAMP levels, which in turn allows inositol 1,4,5-trisphosphate (IP_3) production through activation of phos-

pholipase Cε (Sarkar *et al.*, 2009; Figures 1 and 2). IP_3 by itself could inhibit macroautophagy initiation but can also stimulate IP_3 receptors (IP_3R) at the surface of endoplasmic reticulum (ER), allowing the release of Ca^{2+} in the cytosol. Intracytosolic Ca^{2+} not only released from the ER but also imported from the extracellular milieu has been shown to inhibit autophagosome/lysosome fusion (Gordon *et al.*, 1993; Williams *et al.*, 2008; Ganley *et al.*, 2011). Moreover, intracellular calcium can activate calpain, described as a macroautophagy inhibitor (Williams *et al.*, 2008).

The third well-described mTOR-independent, regulation pathway of macroautophagy is the JNK1/Beclin-1/PI3KCIII axis (Figure 2). JNK1-mediated autophagy activation is observed upon starvation, under apoptotic condition or by increased cytosolic levels of NO (Marino *et al.*, 2014). Activated JNK1 leads to the dissociation of Beclin-1/Bcl-2 complexes allowing the interaction between Beclin-1 and PI3KCIII and subsequent macroautophagy initiation. Macroautophagy can also be activated via Beclin-1 through JNK1-independent mechanisms (Kang *et al.*, 2011; Salminen *et al.*, 2013). The Ca^{2+}-modulated death-associated PK can directly activate Beclin-1. PKD can activate Beclin-1 through JNK1 activation but can also directly elicit Vps34 activity. Cytosolic high-mobility group box 1 protein has also been shown to favour dissociation of Beclin-1 and Bcl-2, thus initiating macroautophagy. JNK1-independent down-regulation of Beclin-1/Vps34-induced macroautophagy has also been described by cyclin-dependent kinase 1 and 5 activities and by binding to tumour growth factor β-activated kinase 1 binding protein 2 and 3.

Non-canonical inductions of macroautophagy that are independent from Beclin-1 have also been described (Codogno *et al.*, 2012). Macroautophagy is observed upon treatment with pro-apoptotic compound in neurons or cell lines. This induction is dependent in most cases on ATG7 and ATG5-12. Another Beclin-1-independent initiation of macroautophagy was revealed outside the context of cell death. Interestingly, it was shown that resveratrol treatment induced the formation of PI3KC3/WD repeat domain phosphoinositide-interacting 1/ATG5/LC3 complexes at the level of ER and plasma membranes. The recent description of this non-canonical macroautophagy pathway underlines the necessity to further describe the relation between macroautophagy and endocytic routes. As discussed below, pharmacological modulators of macroautophagy can act via mTOR-dependent and mTOR-independent pathways, as well as by regulation of Beclin-1/Vps34 activity complex.

Macroautophagy was initially thought to be particularly up-regulated for the production of energy under stress conditions by recycling cytosolic constituents in a non-selective manner. Nowadays, we realize that selective forms of macroautophagy also exist (Hoyer-Hansen and Jaattela, 2007; Kirkin *et al.*, 2009; Jia and He, 2011; Jia *et al.*, 2011; Ashrafi and Schwarz, 2013; Birgisdottir *et al.*, 2013). They are committed to ensuring degradation of damaged organelles such as mitochondria (mitophagy), macromolecular complexes such as ribosomes (ribophagy), ER or lipid droplets (lipophagy), intracellular protein aggregates of misfolded proteins (aggrephagy) and intracellular pathogens such as viruses and intracellular bacteria (xenophagy). These specialized roles of macroautophagy definitively changed our initial vision that macroautophagy corresponds solely to a non-specific, in bulk, degradation system.

Since the early time of its discovery, in contrast, CMA has been considered as a rather specific autophagy process (Auteri *et al.*, 1983). In conditions of stress (e.g. nutrient deprivation, exposure to different toxins), CMA is activated and can degrade targeted cytosolic proteins in a variety of tissues such as liver, kidney, spleen and in many types of cultured cells. This process is selective in the sense that all targeted proteins harbour KFERQ-related sequences (Chiang and Dice, 1988; Kaushik and Cuervo, 2012). This degenerated motif, which has proved to be present in about 30% of cytosolic proteins, contains one or two positively charged residues, one or two hydrophobic residues, one or two negatively charged amino acid residues, and finally one glutamine residue at the N- or at the C-terminal end of the pentapeptide. Via this motif, protein substrates are recognized by the cytosolic heat shock cognate protein of 70 kDa, HSPA8/HSC70, and taken in charge to the lysosomal membrane where they interact with the short cytosolic tail of lysosomal-associated membrane protein type 2A (LAMP-2A; Cuervo and Dice, 1996). This binding will induce LAMP-2A multimerization, a process during which the stability of LAMP-2A is maintained by HSP90 located at the luminal side of the lysosomal membrane. The translocation complex thus formed (Bandyopadhyay *et al.*, 2008) allows the substrates to cross the lysosome membrane towards the lumen (Cuervo and Wong, 2014). LAMP-2A needs then to return to a monomeric state to be able to maintain CMA activity. A pool of HSPA8 molecules normally resident in the lysosome lumen is necessary for this mechanism. Its origin is poorly understood. Its stability in the lumen is strongly dependent on the lysosomal pH (Cuervo *et al.*, 1997).

CMA is induced, as macroautophagy, upon energy stress, providing cells with recycled amino acid residues and favouring ATP production. CMA is also involved in the quality control of proteins. Oxidative stress or protein denaturizing agents can induce CMA that will degrade damaged proteins. CMA can selectively degrade transcription factors or proteins involved in particular signalling pathways, thus modulating the intensity of transcriptional responses.

Macroautophagy or CMA deregulation is involved in several diseases (Nixon, 2013). Thus, in neurodegenerative diseases such as Huntington disease, Alzheimer disease and Parkinson disease, both macroautophagy and CMA have been shown to be down-regulated in pathological neurons, favouring accumulation of toxic aggregates or non-functional mitochondria, contributing to neurodegeneration (Orenstein *et al.*, 2013; Cuervo and Wong, 2014). Autophagy deregulation might also be tightly linked to metabolic diseases, such as obesity and diabetes (Choi *et al.*, 2013; Christian *et al.*, 2013). For example, macroautophagy inactivation promotes storage of lipids as triglycerides in the liver and obesity related to insulin-resistance is associated with a decrease of macroautophagic activity. Macroautophagy is also implicated in cardiovascular diseases (Xie *et al.*, 2011). Macroautophagy activity was shown to protect mice with genetically induced cardiomyopathy provoked by protein aggregation. Macroautophagy could thus be a mechanism triggered by stress to protect heart cells from death. Indeed, patients suffering from congenital cardiomyopathies as Danon's disease or patients

with heart failure show an accumulation of autophagosomes. Induction of macroautophagy in atherosclerotic plaques could also limit necrosis and inflammation by facilitating efferocytosis and enhancing macrophage survival.

Finally, autophagy deregulation could participate in cancer emergence or maintenance (Kimmelman, 2011; Choi *et al.*, 2013). Levine and colleagues showed that forced Beclin-1 expression in the MCF7 cancer cell line inhibits cell proliferation and that Beclin-1 expression was low in several carcinoma cell lines and tissues (Liang *et al.*, 1999). Macroautophagy is known to have anti-oncogenic properties *in vivo*, at steady state, as Beclin-1 haploinsufficiency in mice leads to spontaneous tumour development (Yue *et al.*, 2003). Macroautophagy could exert its protective role by limiting metabolic stress and reactive oxygen species (ROS) generated by damaged mitochondria. A protective role for CMA against cell transformation is also suspected but not yet proven.

In contrast, both macroautophagy and CMA are shown to favour survival of certain tumour cell types after its establishment. CMA facilitates glycolysis on which many tumour cells are dependent and, in most models studied, it protects tumour cells from stress (Kon and Cuervo, 2010; Ali *et al.*, 2011). A pro-survival role for macroautophagy in cancer cell survival has also been documented. Indeed, resistance of some tumour cell lines to chemotherapeutic treatment has been shown to be linked to macroautophagy activation. Moreover, some preclinical models suggest that inhibiting macroautophagy concomitantly to chemotherapeutic intervention could be beneficial, although the mechanism that provides selective advantage to tumour cells under treatment is not clear (Sui *et al.*, 2013). Hydroxychloroquine (HCQ) is, in this context, a candidate molecule leading to promising results. However, it remains to be clarified, from these preclinical models, if the beneficial effects that are observed are linked to macroautophagy inhibition.

Autophagy and immunity

The multiple roles of macroautophagy on innate immunity are well documented. An important immune role for macroautophagy, which probably emerged early along the evolution of eukaryotic organisms, consists of targeting pathogens for degradation. Pathogens can be sensed by 'pattern recognition receptors' (PRR) that are germ line-encoded proteins, expressed by cells of both the innate and the adaptive immune systems. PRR can trigger a signal in response to pathogens directly by recognition of 'microbial-associated molecular patterns' expressed by pathogens or indirectly by 'damage associated molecular pattern' released by the host under cellular stress. PRR signalling was shown to induce macroautophagy after stimulation in several experimental settings (Tang *et al.*, 2012). Pathogen-induced macroautophagy directly leading to the degradation of microorganisms in autolysosomes is called xenophagy (Figure 3, panel 1). This subtype of macroautophagy is particularly relevant for intracellular pathogens. Some virus components, for example, can be degraded directly by the autophagic machinery. Nevertheless, one has to bear in mind that autophagy can play dual roles in the overall viral response as, in some circumstances, macroautophagic membranes can also facilitate viral replication (Richetta and Faure, 2013). Intracellular bacteria such as *Mycobacterium tuberculosis*, *Salmonella*, *Listeria*, *Shigella*, group A *Streptococcus* are optimally degraded through macroautophagy (Huang and Brumell, 2014). IFN-γ, secreted in response to intracellular pathogens and facilitating degradation by macrophages, is widely recognized as an inducer of macroautophagy in phagocytes. Curiously, macroautophagy can also participate in the elimination of strictly extracellular microorganisms such as yeast or *Escherichia coli*. In this case, a process called LC3-associated phagocytosis (LAP) leads to phagosome decoration by LC3-II protein (Cemma and Brumell, 2012). LAP facilitates phagosome maturation and subsequent microorganism elimination (Figure 3, panel 2). Contrary to canonical macroautophagy, LAP is not characterized by the formation of double-membrane vesicles. Canonical autophagy and LAP can simultaneously contribute to the elimination of pathogens. LAP was also shown to be involved in the phagocytosis of dead cells (Martinez *et al.*, 2011) as described later.

Interestingly, macroautophagic activity is activated by inflammatory signals like the one delivered by TNF-α in macrophages, vascular smooth cells, in muscle and epithelial cells as well as in leukaemia cell lines (Harris, 2011). The precise signalling pathways vary according to cell types. In any case, this regulation may be particularly relevant for xenophagy of intracellular pathogens potentially known to induce TNF-α secretion.

Macroautophagy is also directly involved in the regulation of inflammation. Macrophages that are deficient for macroautophagy over-respond to inflammasome-activating signals. Excess of mitochondrial ROS and DNA, released in the cytosol in the absence of macroautophagy, not only can stimulate the inflammasome (Nakahira *et al.*, 2011; Zhou *et al.*, 2011a) but can also activate the calpain pathway (Castillo *et al.*, 2012; Watson *et al.*, 2012). These events lead to hyperproduction of IL-1α and β (Figure 3, panel 3). Macroautophagy is also involved in a particular form of cell death called NETosis. Under pro-inflammatory signals, neutrophils can excrete their genomic DNA complexed with antimicrobial peptides. The resulting structures called neutrophil extracellular traps (NETs) can immobilize and kill microorganisms. Inhibition of macroautophagy prevents intracellular chromatin decondensation, a critical step of NETosis and NET formation, and activates neutrophil cell death by apoptosis (Figure 3, panel 4; Remijsen *et al.*, 2011).

Macroautophagy can also regulate inflammation driven by T cells. Macroautophagy allows degradation of the adaptor protein Bcl-10 that mediates NF-κB activation in response to T cell receptor (TCR) stimulation (Paul *et al.*, 2012). In regard to inflammation, macroautophagy can negatively regulate the production of anti-viral type I IFN (IFN-I) in response to both viral RNA and DNA through the modulation of receptor amount and intracellular trafficking (Figure 3, panel 5). It contributes to the degradation of mitochondria, which are a potential source of oxidative stress that amplifies IFN-I production (Tal *et al.*, 2009). The downstream adaptor protein IFN-β promoter stimulator 1 (IPS-1/MAVS/VISA/Cardif) integral to retinoid-induced gene-like receptor signalling is anchored on the cytosolic side of mitochondrial membranes, and is also degraded by macroautophagy. Then, certain proteins of macroautophagic machinery such as ATG9a or LC3-II

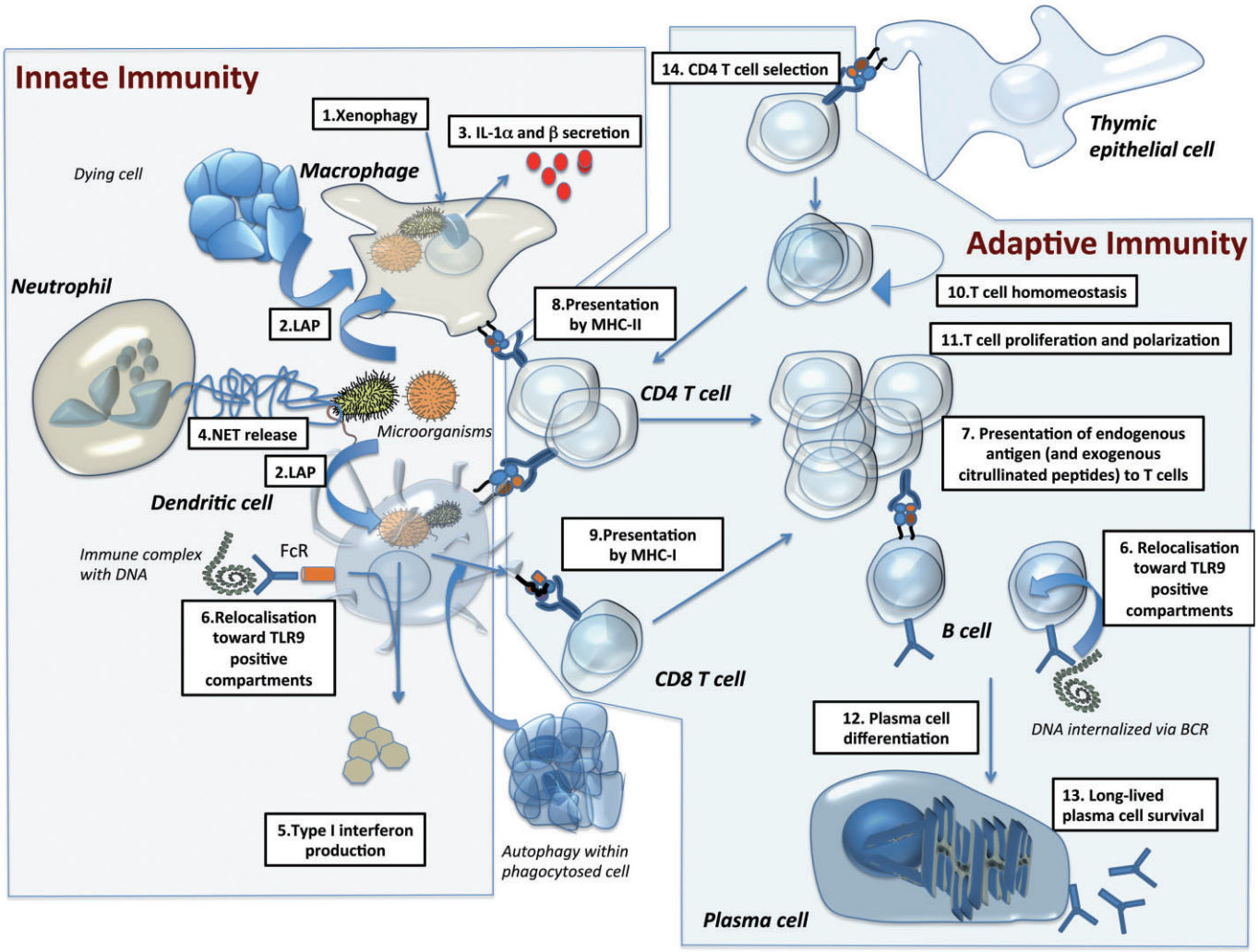


Figure 3

Macroautophagy in the immune system. Macroautophagy is involved in the regulation of innate immunity. (1) Macroautophagy facilitates the elimination of intracellular microorganisms by a mechanism called xenophagy. (2) Macroautophagy machinery contributes to phagocytosis of extracellular pathogens and dead cells by a mechanism called LAP. (3) Macroautophagy contributes to the limitation of IL1-α and IL1-β release by modulating the calpain pathway and the inflammasome respectively. (4) Macroautophagy is an important factor participating in NET release and NETosis of neutrophils. (5) Macroautophagy or its machinery regulates the intensity of IFN-I responses by modulating the trafficking and the amount of PRR. (6) Macroautophagy machinery can also positively influence IFN-I secretion by helping to the relocalization of DNA-containing immune complexes towards TLR9-positive compartments in plasmacytoid dendritic cells. The same mechanism is suspected for DNA containing antigens internalized after interaction with BCR at the surface of B cells. Macroautophagy activity is important at the interface between innate and adaptive immunity. (7) Macroautophagy promotes the presentation of intracellular viral epitopes and self peptides by MHC-II molecules. Macroautophagy is necessary for presentation by B cells of citrullinated epitopes from extracellular origin to CD4 T cells. (8) Macroautophagy contributes to the presentation of both intracellular and extracellular antigens to CD4 T cells by DCs and macrophages. It is also involved the presentation of citrullinated peptides. (9) Cross-presentation by DCs of exogenous antigens is more efficient under active macroautophagy within the antigen-presenting cells. Moreover, macroautophagy activity in phagocytosed cells improves the subsequent presentation of internalized antigens onto MHC-I molecules, to CD8 T cells. Macroautophagy is integral to adaptive immunity. (10) Macroautophagy is needed for peripheral T cell homeostasis and (11) for their survival, activation and polarization. (12) Macroautophagy contributes to B-cell differentiation into plasma cell and/or (13) plasma cell survival. (14) Constitutive macroautophagic activity in the thymus participates in the positive and negative selection of T cells and the prevention of autoreactivity.

modulate the production of IFN-I in response to cytosolic double-stranded DNA (dsDNA) by preventing the assembly of the cytosolic indirect DNA sensor STING (stimulator of IFN genes) and TANK-binding kinase 1 proteins (Saitoh *et al.*, 2009). A recent work from Jung and collaborators showed that recognition of microbial DNA by cyclic GMP-ATP (cGAMP) synthase, the direct sensor of DNA upstream of STING, activates Beclin-1 (Liang *et al.*, 2014). This activation not only directly inhibits cGAMP synthesis but also stimulates macroautophagy that mediates degradation of cytosolic

DNA. This mechanism limits excessive IFN production directly through GAMP level modulation and indirectly through the removal of pathogen DNA. Macroautophagy can also exert a positive role on IFN-I secretion. It has been shown to help to the relocalization of immune complexes internalized through Fc receptors towards TLR9-positive compartments in plasmacytoid dendritic cells (DCs), thus leading to the amplification of IFN-I responses to DNA-containing immune complexes (Figure 3, panel 6; Henault *et al.*, 2012). These observations can be related to data published by Susan Pierce and her colleagues who showed that material internalized via the BCR localized to autophagosome-like compartments (Chaturvedi *et al.*, 2008). Using pharmacological inhibitors of autophagy, the team found that macroautophagy could favour co-signalling of BCR and TLR9 pathways. This effect of macroautophagy might bridge innate-like signals to adaptive immunity.

Another important role occurring at this interface relates to the contribution of macroautophagy to presentation of antigens by major histocompatibility complex (MHC) molecules (Figure 3, panels 7–9; Blum *et al.*, 2013). One of the first described roles of macroautophagy was its necessary involvement for presentation of the Epstein–Barr (EB) nuclear antigen-1, encoded from EB virus (EBV) genome, by B lymphocytes to specific CD4 T cell clones (Figure 3, panel 7; Paludan *et al.*, 2005). The precise role of macroautophagy in the *in vivo* immune response to EBV, however, remains to be deciphered further. Yet, starvation-induced macroautophagy in a human B-lymphoma cell line was found to favour the loading of peptides from the cytoplasmic origin onto MHC class II (MHC-II) molecules (Dengjel *et al.*, 2005). Macroautophagy also participates in antigen presentation by phagocytes as macrophages of both intracellular and extracellular pathogens (Figure 3, panel 8; Brazil *et al.*, 1997; Romao *et al.*, 2013). Successive studies also implicated macroautophagy in the presentation of antigens by professional antigen-presenting cells such as DCs. *In vitro* experiments showed that transfected DCs expressing an antigenic peptide-encoding sequence fused with LC3 more efficiently presented antigens to CD4 T cells. This enhancement was dependent on macroautophagy (Schmid *et al.*, 2007). In a more physiological setting, using mice harbouring a specific deficiency for autophagy in DCs, Lee *et al.* (2010) showed the crucial importance of macroautophagy in the presentation of herpes simplex virus antigens and subsequent immune response. Surprisingly, these authors also showed that ATG5 could contribute to the presentation of antigens from an extracellular source, when combined with TLR ligands. Although nowadays it seems clear that the macroautophagic machinery contributes to the presentation of extracellular antigens at least, in macrophages, DCs, and probably also in B cells (Crotzer and Blum, 2010), canonical macroautophagy was not involved in this particular case, as no typical double-membrane-shaped vesicles could be observed (Lee *et al.*, 2010).

The contribution of macroautophagy to antigen presentation by MHC-I molecules is also well documented (Figure 3, panel 9). In some settings, elements of macroautophagic machinery seem to be involved in the presentation of certain intracellular viral epitopes (Tey and Khanna, 2012). The hypothetic mechanism relies on an exchange of peptides at the crossroads between endosomes carrying MHC-I molecules, which are recycled from the surface, and autophagosomes in a so-called amphisome. Macroautophagy is also suspected to be involved in the 'classical' cross-presentation of extracellular antigens on MHC-I molecules by DCs, although the mechanism still remains elusive (Fiegl *et al.*, 2013). The contribution of macroautophagy to cross-presentation does not only reside within the DC. Indeed, cellular sources of antigens with active macroautophagy have been shown to promote the presentation of these antigens after phagocytosis. Tumour cells bearing antigens, or virus-infected cells competent for macroautophagy, are known to favour cross-presentation onto MHC-I molecules after uptake by DCs (Li *et al.*, 2008; Uhl *et al.*, 2009). This raises two interesting basic issues that need to be further investigated. First, autophagosomes *per se* could be a source of concentrated antigens when cells undergo macroautophagy. Second, in certain settings, autophagosomal compartments can be secreted. Whether macroautophagy participates in a particular form of exosome generation adapted to capture by DCs and more prone to enter cross-presentation trafficking is a seductive hypothesis that is currently being examined.

Macroautophagy importantly contributes to lymphocyte biology and notably in lymphocyte homeostasis. Chimeric mice reconstituted with *Atg5*–/– bone marrow (Pua *et al.*, 2007) and conditional deletion of essential autophagy genes *Atg5* (Stephenson *et al.*, 2009), *Atg7* (Pua *et al.*, 2009; Jia and He, 2011), *Atg3* (Jia and He, 2011) showed that macroautophagy is critical to the survival of peripheral T cells (Figure 3, panel 10). Most of these studies concluded that macroautophagy is integral to CD4 and CD8 T cell survival in the periphery. T cells deficient for autophagy also showed defects in proliferation upon activation (Figure 3, panel 11). It has to be noted, however, that models published to date use early deletion promoters, active since the thymic stages, preventing therefore any definitive conclusion to be drawn on the role of macroautophagy in the periphery, as survival defects could well be linked to developmental failures originated in the thymus. Other publications used conditional deletion models invalidating *Beclin-1* (Kovacs *et al.*, 2012) or *Vps34* (Willinger and Flavell, 2012; Parekh *et al.*, 2013) genes specifically in T cells. The authors observed the same phenotype, namely, T cell loss of function and compromised survival. These global defects in survival and activation might be linked to defective energy mobilization in response to TCR stimulation and/or homeostatic proliferation (Hubbard *et al.*, 2010). It might also involve defective clearance of damaged mitochondria (Pua *et al.*, 2009; Stephenson *et al.*, 2009) and ER in excess (Jia and He, 2011). Also, macroautophagy-deficient T cells accumulated pro-apoptotic molecules sensitizing them to cell death (Kovacs *et al.*, 2012). The same group reported that macroautophagy deficiency in T cells affected more Th1, Th2 and Th0 cell survival than Th17 cells. Others also noted this apparent selective effect of macroautophagy on Th-cell polarization. A few years earlier, it was effectively shown that macroautophagy was involved in a pro-death mechanism, upon growth factor withdrawal, especially in Th2 cells (Li *et al.*, 2006). Finally, macroautophagy can selectively participate in T cell death in the context of human immunodeficiency virus (HIV) infection (Espert *et al.*,

2006). HIV-infected T cells induced death of non-infected T cells through macroautophagy induction, and this triggering effect involved the viral-encoded protein Env and its interaction with the chemokine receptor CXCR4.

Macroautophagy is involved in B-cell development in BM precursors (Pua *et al.*, 2007) but seems poorly involved in B-cell survival in the periphery. Mice with conditional deletion of autophagy in B cells exhibit, however, a reduction of B1 innate-like B cells, emphasizing its possible role in the homeostasis of this self-renewed population with low replenishment from BM-derived cells (Miller *et al.*, 2008). Two recent studies showed a role for macroautophagy in plasma cell differentiation and/or survival (Figure 3, panels 12 and 13), and thus in antibody production. The first investigation did not exclude a role for macroautophagy in early B-cell differentiation into plasma cells (Conway *et al.*, 2013), as defective macroautophagy in B cell compromises the expression of plasma cell-specific transcription factors. The second one, in contrast, stated that the impact of macroautophagy deficiency in antibody production is restricted to long-lived plasma cell survival defects and not to initial B-cell activation. They found, however, no difference in plasma cell number in the BM between mice harbouring macroautophagy-competent and macroautophagy-deficient B cells (Pengo *et al.*, 2013). To tentatively reconcile their findings with the previous ones, Pengo and collaborators argued on the incomplete deletion of *Atg5* in B cells. They further show that there is an enrichment of autophagy-competent plasma cells in the BM that could explain their observation, and identify a specific defect in the survival of plasma cells expressing antibodies against the model antigen used for the immunization. The discrepant conclusions between these two studies regarding the precise stage in B-cell differentiation where macroautophagy intervention would be needed remain puzzling. More trivially, these discrepancies could also be explained by differences regarding the mode of immunization and of *in vitro* stimulation. The first paper includes infections by pathogens while in the second one, the data were generated mainly with soluble model antigens for which presentation by MHC-II molecules implies different requirements. It would be interesting to try conciliating these two series of data by testing other mouse models with complete deletion and/or analysing under scrutiny B-cell differentiation with other antigens linked to infection or immune defects. Finally, macroautophagy is implicated in tolerance induction, and polarization of T cell populations as discussed in the next section.

In contrast to macroautophagy, relatively little is known about the role of CMA in the immune system. This lack of information results from the fact that the influence of CMA in this setting is difficult to analyse, both *in vitro* and *in vivo*, due to the lethality of mice lacking HSPA8 or LAMP-2. An important contribution to this aspect came from Janice Blum and her colleagues who succeeded in deciphering the key steps of CMA in the presentation of the cytosolic glutamic acid decarboxylase antigen to CD4 T cells (Zhou *et al.*, 2005). Because CMA is theoretically able to translocate 30% of cytosolic proteins to competent lysosomes, CMA is suspected to favour MHC-II antigen presentation of a rather large diversity of cytosolic antigens to CD4 T cells, as observed in macroautophagy.

Autophagy and autoimmunity

With regard to the central influence that different forms of autophagy display on innate and adaptive immune systems, any alteration of autophagy processes were effectively anticipated to have downstream consequences.

Apart from the suspected contribution of macroautophagy deregulation in autoinflammatory syndromes such as Crohn's disease, alteration of macroautophagy is also suspected to be involved in autoimmune diseases *stricto senso*. The potential links between autoimmunity and autophagy has been previously reviewed (Pierdominici *et al.*, 2012; Zhou and Zhang, 2012; Gianchecchi *et al.*, 2014) and could stand at the level of inflammation regulation, central and peripheral tolerance regulation, and immune cell homeostasis.

Thus, macroautophagy has been shown to be up-regulated in synovial fibroblasts from patients with rheumatoid arthritis (RA) after TNF-α stimulation (Connor *et al.*, 2012). Macroautophagy in this particular context might explain the prolonged survival of fibroblasts mediated by TNF-α. A recent study further showed that macroautophagy is increased in osteoclasts from RA patients, with an up-regulation of Beclin-1 and ATG7 expression. In that case, macroautophagy was activated in a TNF-α-dependent manner. In addition, mouse models deficient for *Atg7* in osteoclasts were less sensitive to bone erosion mediated by TNF-α, pointing out a likely contribution of macroautophagy to bone destruction in RA. Thus, it is possible that beneficial effects of anti-TNF-α treatments observed in RA are at least in part mediated by inhibition of autophagy.

Although not yet proven, macroautophagy deregulation could also contribute to several diseases by initiating the loss of central tolerance (Figure 3, panel 14). Macroautophagy is involved in the generation of autoantigenic peptides presented by thymic epithelial cells (TECs) dedicated to thymocyte education. Knowing that MHC-II molecules are expressed by TECs and that these cells are poorly phagocytic, the question of the source of peptides presented could not be explained by the sole canonical endocytic route. Several studies reported an intense constitutive macroautophagic activity in TECs (Nedjic *et al.*, 2008; Kasai *et al.*, 2009). Moreover, Nedjic and colleagues showed, with *Atg5*-deficient thymus graft models in *Nude* mice, that macroautophagy plays an important role in positive selection. Such grafted mice develop severe autoimmune colitis due to TCR repertoire skewing towards autoreactivity. This probably reflects the importance of macroautophagy in providing antigens from an intracellular source for MHC-II presentation. Another recently published work enlarges the role for macroautophagy in thymic negative selection (Aichinger *et al.*, 2013). Aichinger and colleagues used an elegant mouse model where the expression of a test antigen was restricted to the medullary TECs (mTECs). This expression could induce deletion of T cells specific for this particular antigen but only if mTECs were macroautophagy competent. Thus, macroautophagy contributes to central tolerance induction regarding CD4 T cells. Although not yet proven, such a deregulation of macroautophagy could also contribute to human autoimmune diseases.

As described in the previous section, the fine-tuning of macroautophagy in the immune peripheral compartment

might constitute an important factor in the control of autoimmunity. Thus, in studies focused on T cells, it has been shown, for example, that *ATG5* expression was increased in lymphocytes infiltrating lesions in multiple sclerosis (Alirezaei *et al*., 2009). Kovacs *et al*. (2012) further showed that mice deficient for macroautophagy in total T cells lost sensitivity to experimental autoimmune encephalomyelitis induction. A role of macroautophagy in T cells of individuals with systemic autoimmune diseases such as RA or systemic lupus erythematosus (SLE) is also suspected. The first study that pointed out a potential deregulation of autophagy in lupus was investigating the pro-autophagic role of antibodies obtained from patients suffering from type 2 diabetes with neuropathy (Towns *et al*., 2005). The authors showed that autoantibodies, possibly autoreactive, purified from SLE patients' serum, could induce autophagy in a neuroblastoma cell line. A few years later, genome-wide association studies were performed and allowed identification of new candidate genes for systemic autoimmunity development. In SLE, several SNPs located on autophagy-related genes have thus been associated with the disease occurrence (Harley *et al*., 2008; Orozco *et al*., 2011). One SNP located in the intergenic region between *ATG5* and *PRDM1* was found to correlate with a greater expression of *ATG5* mRNA (Zhou *et al*., 2011b). The genetic association between *ATG5* and susceptibility to SLE has been confirmed in individual studies, but not found in others (Jarvinen *et al*., 2012). Interestingly, a recent study showed strong association of SNPs on *DRAM1* with SLE susceptibility. This gene encodes an activator of macroautophagy in response to p53-mediated stress signals. On this basis, we then were the first to report macroautophagy deregulation in SLE (Gros *et al*., 2012). We found that autophagosomal load was increased in T cells isolated from two genetically unrelated lupus-prone mouse strains and also from SLE patients. This deregulation was even more obvious when T cells were stimulated by chemical activators of TCR-related signalling pathways. The increase of autophagic compartments was not due to a complete block of autophagic flux as under lysosomal protease inhibition, autophagosome-associated marker expression was further increased. It is important to insist on the fact that this deregulation is not the sole result of a systemic inflammation as *in vivo* administration of lipopolysaccharide in normal mice did not increase macroautophagic activity in their T cells. Interestingly, we showed that a phosphopeptide, called P140, developed in our team inhibited lysosomal processes, including macroautophagy, even if macroautophagy may not be the main target of P140 as explained in the next section (Page *et al*., 2011). A few months after the publication of our results, Alessandri *et al*. (2012) also showed an increase of the autophagosome-associated LC3-II isoform in T cells, which interestingly mainly occurred in naïve CD4 T cells isolated from SLE patients. This result suggests that there is an intrinsic deregulation of autophagic activity in SLE T cells. However, the authors concluded from their observation that SLE T cells are resistant to macroautophagy induction and could thus become more prone to apoptosis. They came to this conclusion by re-stimulating T cells with rapamycin or with autologous serum. It is possible, however, that SLE T cells are already at the maximum of autophagosome load and that re-exposure to their own serum is unable to further increase autophagic activity. Interestingly, they confirmed the pro-autophagic role of SLE serum on normal T cells. The authors did not evaluate the effects of TCR-related stimulation, which would have been particularly relevant on naïve T cells isolated from SLE patients. Two other studies also recently showed an increase of autophagic activity first, in peripheral blood lymphocytes from SLE patients and second, in CD4 T cells (Caza *et al*., 2014; Clarke *et al*., 2014). Thus, the question still remains open – is macroautophagy activity globally increased or decreased in lupus T cells? It seems well established, however, that in the setting of lupus, more autophagosomes are generated than degraded. The signalling pathways responsible for such a deregulation remain to be defined. Any information on this finely regulated mechanism could also give us precious insights regarding the regulation of autophagy in normal lymphocytes, which nowadays is still poorly known.

Most interestingly, another set of studies showed a distinct picture in RA T cells. Weyand and colleagues showed that an enzyme involved in glycolysis, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), was defective in RA T cells (Yang *et al*., 2013). This defect was associated with the inability of these cells to trigger macroautophagy in conditions of energy demand. This phenomenon could contribute to the enhanced cell death upon stimulation, which is a characteristic of RA T cells, and to the mildly lymphopenic environment suggested to promote autoimmunity. Forced expression of PFKFB3 in T cells restored glycolysis and autophagy. Altogether these different investigations show that macroautophagy is strongly related to the abnormal survival/death balance observed in T cells during systemic autoimmune pathologies.

In B cells, macroautophagy deregulation may also be involved in the development of autoimmunity. If account is taken of the fact that macroautophagy influences plasma cell differentiation and survival, macroautophagy in excess could certainly allow maintenance of autoantibody production. This effect has effectively been proposed to occur in a recent study showing that macroautophagy is increased in certain B-cell subsets collected from SLE patients and lupus-prone mice (Clarke *et al*., 2014). At this stage, however, additional studies are needed to determine at which level along the differentiation process macroautophagy could play a role. Thus, a complex picture about the role of macroautophagy in lymphocytes emerges that seems to differ in different autoimmune settings. Mouse models that are autoimmune prone with an absence of macroautophagy specifically in lymphocyte subpopulation may help deciphering the *in vivo* role of lymphocyte macroautophagy in systemic autoimmunity.

A very interesting point that remains to be studied in detail is the contribution of macroautophagy to the presentation of autoantigens in the periphery. Considering both canonical and non-canonical autophagy processes, it is possible that macroautophagy participates in the processing of both extracellular and cytoplasmic antigens. An interesting study published by Unanue and colleagues identified macroautophagy as a mechanism favouring presentation of citrullinated antigens by DCs, macrophages and B cells (Figure 3, panels 7 and 8; Ireland and Unanue, 2011). Given the high frequency of lymphocytes reactive to citrullinated antigens observed in RA, a deregulation of macroautophagy in regard

to MHC-II antigen processing in this pathology is very probable. In this context, it should be borne in mind that, as reported above, NETosis and NET formation (NETs that are a source of citrullinated antigens) requires active autophagy (Remijsen *et al.*, 2011).

Macroautophagy, or at least its machinery, can also control autoimmunity development by its role played on the innate immune system. Phagocytes deficient for MAP1LC3A, an isoform of LC3, clear dead cells less efficiently, because they are unable to trigger LAP (Martinez *et al.*, 2011; Figure 3, panel 2). At the level of phagocytes, LAP could therefore also participate in the scavenging role of macrophages *in vivo*, preventing these individuals from autoimmunity. Furthermore, as mentioned above and particularly relevant in the case of SLE, macroautophagy defects could participate in the deregulation of inflammatory cytokine release, such as type I-IFN in response to nucleic acids as PRR ligands (Saitoh *et al.*, 2009; Tal *et al.*, 2009; Liang *et al.*, 2014). Moreover, the regulatory role of macroautophagy on IL-1α and β secretion (Deretic *et al.*, 2013) could affect several autoimmune diseases, for example, SLE, RA, vitiligo, multiple sclerosis, autoimmune Addison's disease, celiac disease and type 1 diabetes. It has to be noted, however, that a strict causal relationship between these autoimmune syndromes and IL-1β secretion is not firmly proven or remains confusing in the existing literature (Shaw *et al.*, 2011; Doria *et al.*, 2012).

The effect of CMA activity on several human autoimmune diseases is unknown until now. Recently developed molecules targeting HSPA8 or other specific molecules of the CMA process could give new information about CMA regulation and its possible impact in certain autoimmune diseases.

Pharmacological regulators of autophagy

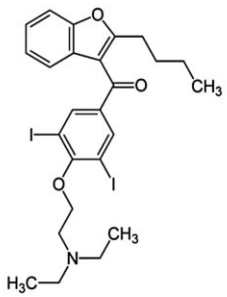
As our knowledge of autophagy processes progressed and highlighted its central role for maintaining cellular homeostasis and energy balance, and therefore as the list extended regarding pathological situations possibly resulting from deregulation of this central machinery, an intense effort to design, synthesize and evaluate autophagy-regulating small molecules for therapeutic applications has strengthened throughout the years. During the last years, much has also been done to better define more relevant and specific *in vitro*/*ex vivo* assays for the evaluation of such molecules (Mizushima *et al.*, 2010; Klionsky *et al.*, 2012). A number of comprehensive review articles have been published listing exhaustively various families of compounds, activators and inhibitors, which have been generated to modulate autophagy directly or indirectly (Fleming *et al.*, 2011; Baek *et al.*, 2012; Cheong *et al.*, 2012; Rubinsztein *et al.*, 2012). It is noticeable that among these active compounds, some small-molecule regulators of autophagy were identified using sophisticated assays, such as a high-throughput image-based screens, for example (Zhang *et al.*, 2007), while others have been discovered by serendipity and some others, used for years, have been found much later to target one or another type of autophagy processes. Currently in fact, there are few specific compounds and even less with detailed structural insight enabling us to study a precise step of the autophagy pathways. Quite surprisingly, the targets of some autophagy regulators that are widely prescribed to patients are not really known. Here, we briefly describe the characteristics of some chemical classes of chemical compounds that have been shown to induce or inhibit autophagy with a particular emphasis on low MW drugs or drug candidates developed to modulate inflammatory and autoimmune diseases as well as some cancers. Further details, both structural and pharmacological, can be found in more specialized reviews (Renna *et al.*, 2010; Fleming *et al.*, 2011; Baek *et al.*, 2012; Rubinsztein *et al.*, 2012), which contain rather exhaustive tables describing the structure and physical characteristics of these regulatory chemical compounds.

Low MW inducers targeting mTOR-dependent autophagy

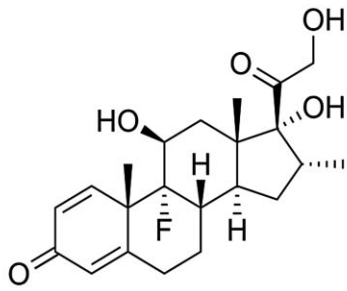
As underlined above, autophagy is negatively regulated by mTOR, which is downstream in the PI3K pathway (Figure 2). A large number of molecules target the mTOR pathway, the most widely used being rapamycin/sirolimus (Figure 4), an immunosuppressant and anti-fungal agent, which forms a complex with the immunophilin FKBP-12 and inhibits the kinase activity of mTORC1, thus leading to autophagy induction (mTORC2 is largely resistant to rapamycin; Thomson *et al.*, 2009). Other chemical inhibitors of mTORC1 include torin-1, rottlerin, niclosamide, perhexiline and amiodarone, for example (Figure 4), as well as glucose and glucose-6 phosphate. The anti-diabetes drug metformin also inhibits mTORC1 axis, acting indirectly via its property to activate AMPK that inhibits mTOR activity by a different mechanism, notably by directly targeting raptor, a component of mTORC1, absent in mTORC2 (Gwinn *et al.*, 2008). Interestingly, both rapamycin and metformin, while using two different mechanisms, seem to enhance T cell memory formation by inhibiting mTORC1 and facilitating the switch of effector T cells to memory T cells (Araki *et al.*, 2009). It should be emphasized at this stage that rapamycin, a notable immunosuppressive drug used to treat transplant rejection and some autoimmune diseases, can display opposite effects and notably exacerbates autoimmunity depending upon the dose which is administered (Zhou *et al.*, 2005). It is possible that these dual properties are mediated by the distinct cellular mechanisms they use to exert their effect (Araki *et al.*, 2011).

Torin-1 is a potent inhibitor of both mTORC1 and C2 with IC_{50} values of 2 and 10 nM. Unlike rapamycin, this anti-fungal drug fully inhibits mTORC1 (Thoreen and Sabatini, 2009; Thoreen *et al.*, 2009). Torin-1 is efficacious at a dose of 20 $mg^{-1} \cdot kg^{-1}$ in a U87MG xenograft model and demonstrates good pharmacodynamic inhibition of downstream effectors of mTOR in tumour and peripheral tissues. *In vivo*, torin-1 also affects expression of cell proliferation, angio/lympho-genesis and stemness markers such as Ki67, DLL1, DLL4, Notch, Lgr5 and CD44 (Francipane and Lagasse, 2013).

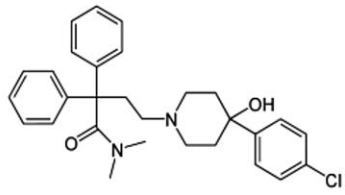
Initially thought to act as a selective PKC-δ inhibitor, rottlerin was shown later that it was not the case and that it blocks other kinase and non-kinase proteins *in vitro*. This compound is a potent large conductance potassium channel (BK_{Ca}) opener, a property that is beneficial for post-ischaemic changes in vasodilation. It seems that by decreasing ATP



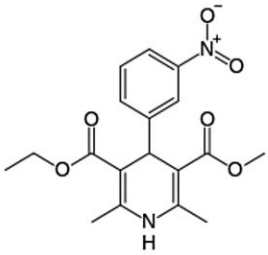
Amiodarone



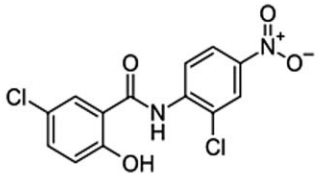
Dexamethasone



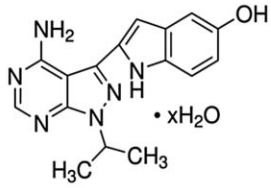
Loperamide



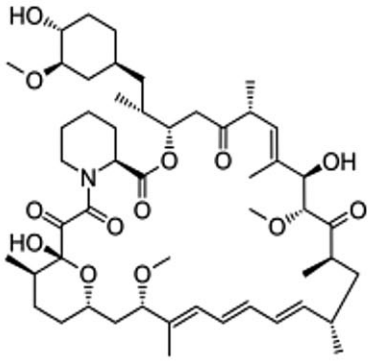
Nitrendipine



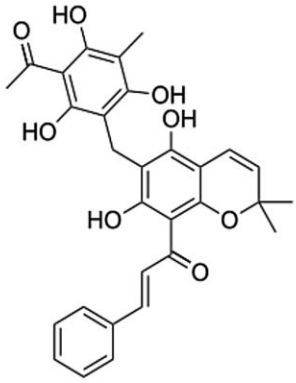
Niclosamide



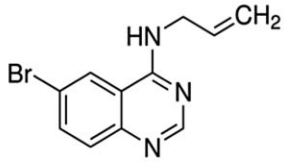
PP242



Rapamycin



Rottlerin



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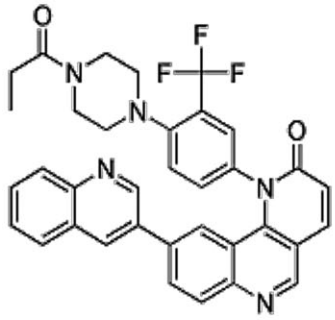
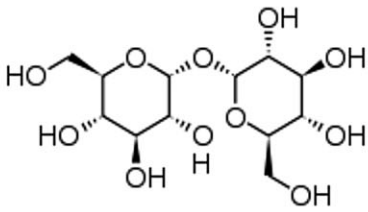
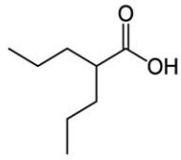


Torin-1



Trehalose



Valproic acid

Figure 4
Examples of low MW compounds that are autophagy activators.

levels it can block PKC-δ tyrosine phosphorylation and activation indirectly. In human pancreatic cancer stem cells, rottlerin was found to induce autophagy followed by induction of apoptosis via inhibition of PI3K/AKT/mTOR pathway and activation of the caspase cascade, an effect that is not affected by PKC-δ silencing (Singh *et al.*, 2012).

Niclosamide, a well-known protonophoric anti-helminthic drug (Figure 4), was only recently identified as an inhibitor of mTORC1 signalling after a cell-based screen of >3500 chemicals (Balgi *et al.*, 2009). In this screen, rottlerin as well as two other approved drugs, namely, perhexiline (a carnitine palmitoyl-acyltransferase inhibitor developed to treat symptoms in patients with hypertrophic cardiomyopathy) and amiodarone (an anti-arrhythmic drug used to treat ventricular tachycardia or ventricular fibrillation; Figure 4), were also picked out. Biochemical assays showed that niclosamide, as well as the two other molecules, did not inhibit mTORC2, suggesting that they do not inhibit mTOR catalytic activity properly but rather inhibit signaling to mTORC1. The effect of these molecules was reversible but was essentially irreversible in the case of amiodarone (note that the latter is also mentioned as a Ca^{2+} channel blocker that reduces intracytosolic Ca^{2+} levels). Further studies notably showed that niclosamide provokes apoptosis of myelogenous leukemic cells, via the inactivation of NF-κB and ROS generation (Jin *et al.*, 2010), and that of stem-like cells in breast cancer (Wang *et al.*, 2013). Niclosamide in this latter system was found to inhibit the expression of cyclin D1 (by 33%), Hes1 (by 57%) and PTCH (by 79%), which are target genes for Wnt, Notch and Hh signalling pathways respectively. These effects might be related to the ability of niclosamide to inhibit the STAT3 signalling pathway (Ren *et al.*, 2010), a property that could be used advantageously to reduce resistance to radiotherapy in lung cancer (You *et al.*, 2014).

PP242 [2-(4-amino-1-isopropyl-1H-pyrazolo[3,4-d] pyrimidin-3-yl)-1H-indol-5-ol] (Figure 4) is a cell-permeable adenine-mimetic pyrazolopyrimidine compound that acts as a potent, reversible, ATP-competitive inhibitor against both mTORC1 and mTORC2 (IC_{50} = 30 and 58 nM, respectively, with 100 μM ATP). It blocks the phosphorylation of AKT at Ser^{473} and prevents its activation. Reported as a complication of PP242, this compound also mediates ERK activation by a mechanism that remains to be elucidated (Hoang *et al.*, 2012). It appears however that its mode of action differs from the one of rapalogs where ERK activation is mediated by a p70S6K- and PI3K-dependent activation of upstream kinases of the MAPK ERK cascade.

Dexamethasone (DEX; Figure 4) is a very potent, if not the most effective with prednisone, anti-inflammatory and immunosuppressive drug used today in clinical medicine. It is a member of the glucocorticoid class of steroid drugs. Its ability to induce lymphocyte apoptosis is exploited in many haematology (lymphoid) malignancies where it is employed as a chemotherapeutic agent. Its ability to induce macroautophagy in lymphocyte cells lines and in primary lymphoblastic leukaemia cells was also described (Swerdlow *et al.*, 2008) and studied in detail (Molitoris *et al.*, 2011). In particular, DEX induces the expression of a gene encoding the stress response protein Dig2/RTP801/REDD1, and the elevation of Dig2/RTP801/REDD1 contributes to the induction of macroautophagy. A few years before this discovery, Dig2/RTP801/REDD1 had been described as a negative regulator of mTOR signalling that exerted its effect by displacing TSC2/tuberin from the 14-3-3 binding protein, allowing the formation of the TSC1/hamartin-TSC2 complex, which inhibits mTOR (DeYoung *et al.*, 2008). Thus, DEX, by augmenting expression of Dig2/RTP801/REDD1 protein, would inhibit mTOR and favour upstream steps of the macroautophagy axis. It should be mentioned that depending on the dose and the type of cells, the effect of DEX on Dig2/RTP801/REDD1 was not equivalent (less dependence at high DEX dose). Other mechanisms of action of glucocorticoids were also described, notably when the studies were investigating the effects at later stages (~20 h culture instead of ~4 h), acting then through the AKT axis or the inositol pathways (Harr *et al.*, 2010).

Low MW inducers targeting mTOR-independant autophagy

One of the mTOR-independent pathways is regulated by intracellular Ca^{2+} levels, and involves L-type Ca^{2+} channels, or implies a GPCR to which ligand of this receptor binds (Figure 2). Under stress conditions or through binding of GPCR antagonists or an L-type Ca^{2+} channel, mTOR-independent autophagy will be promoted by lowering IP_3 levels or blocking activities of Ca^{2+}-dependent cysteine proteases, the calpains (Fleming *et al.*, 2011; Baek *et al.*, 2012; Rubinsztein *et al.*, 2012; Decuypere *et al.*, 2013).

In the group of small chemical molecules that activate mTOR-independent macroautophagy, we can emphasize some compounds that act as L-type Ca^{2+} channel antagonists (Figure 2). These positive regulators of autophagy, a number of which are used in the clinic (e.g. the anti-arrhythmic verapamil, anti-diarrhoeal loperamide, anti-hypertensive nimodipine and nitrendipine; Figure 4), induce autophagy by preventing the influx of Ca^{2+} and therefore decreasing intracytosolic Ca^{2+} levels, which, as a consequence, leads to the inhibition of Ca^{2+}-dependent calpain proteases. Other compounds, such as calpastatin and calpeptin, act directly on calpain as inhibitors. In calpain-deficient cells, autophagy is impaired with a resulting dramatic increase in apoptotic cell death (Demarchi *et al.*, 2006).

Among the molecules that activate mTOR-independent macroautophagy, we can also mention the anticonvulsants sodium valproate and carbamazepine (myo-inositol-1-phosphate synthase inhibitors) or lithium and L-690,330 (inositol monophosphatase inhibitors), which inhibit inositol synthesis and decrease IP_3 levels (Figure 2). Such substances might have beneficial effects in neurodegenerative diseases. We will see, however, that some molecules of this family seem to be deleterious in susceptible individuals as they can trigger lupus-like disease.

Reduction of cAMP levels by antagonists of GPCR Gα5 (NF 449) or AC (2′5′-dideoxyadenosine), or by imidazole-1 receptor agonists (clonidine used to prevent migraines and recurrent vascular headaches, rilmenidine used as an anti-hypertensive drug) also trigger autophagy (Figure 2). Although these drugs apparently work differently, they all affect different parts of the same cyclic pathway in which cAMP regulates IP_3 levels, which increase calpain activity, which cleaves and then activates $G\alpha_s$, which in turn regulates cAMP levels. Intervention at any point was shown to be effective in inducing autophagy.

The BH3 mimetics, ABT737 and HA14-1, induce autophagy by competitively disrupting the inhibitory interaction between the BH3 domain of Beclin-1 and the anti-apoptotic proteins Bcl-2 and Bcl-X(L). *In vitro*, ABT737 exerts its BH3 inhibitory activity against Bcl-xL, Bcl-2 and Bcl-w with EC_{50} values of 78.7, 30.3 and 197.8 nM respectively. Contrary to *in vitro* affinity studies, however, ABT-737 targets Bcl-2 in preference to Bcl-xL and Bcl-w in cellular settings (Rooswinkel *et al.*, 2012). Although ABT737 and HA14-1 also modulate other pro-autophagic pathways, including the mTOR axis (Malik *et al.*, 2011; Marquez and Xu, 2012), which can represent a difficulty in their pharmacological development, they show promise for cancer therapy. Thus, in an aggressive leukaemia model, ABT-737 suppressed the leukaemia burden by 53%, with significantly extended survival of mice (Konopleva *et al.*, 2006). ABT-737 showed impressive single-agent activity, in particular against leukaemias, lymphomas and small-cell lung cancer, but resistance was unfortunately often encountered. Combination of ABT-737 with other anti-cancer drugs is being evaluated (see Zinn *et al.*, 2013).

Another very promising compound was recently described, which corresponds to a peptide construction encompassing amino acid residues 267–284 of Beclin-1 at its C-terminal end associated via a diglycine linker to a 11-amino acid residue cell-permeable peptide at its N-terminal end, corresponding to the HIV-1 Tat protein transduction domain (Shoji-Kawata *et al.*, 2013). Three substitutions were introduced in the Beclin-1 moiety present in the final construct to increase its solubility and binding, namely, H275E, S279D and Q281E. The peptide of Beclin-1 that was selected binds the HIV-1 Nef protein. C57BL/6J mice pretreated with Tat–Beclin-1 construct showed reduced mortality when infected with the West Nile or Chikungunya viruses. None of the neonatal mice pretreated with the control Tat-scrambled peptide construct and infected with Chikungunya virus were found to survive as compared with 62.5% of mice treated with Tat–Beclin-1 peptide that were protected. Additionally, human cells treated with Tat–Beclin-1 *in vitro* were resistant to HIV and other pathogens. The effectiveness of the peptide against HIV *in vivo* remains to be tested. The demonstrated therapeutic value of the Tat–Beclin-1 synthetic construct, which may extend to neurodegenerative diseases and cancer, lies in its ability to induce autophagy as shown using several independent methods, namely, immunoblot assays (p62 degradation, LC3 conversion), detection of GFP–LC3 puncta with fluorescence microscopy, radiolabelled long-lived protein degradation and electron microscopy. This peptide construct interacts with a newly identified negative regulator of autophagy, called Golgi-associated plant pathogenesis-related protein 1 or GAPR-1 (also named GLIPR2). GAPR-1 associates with lipid rafts at the cytosolic leaflet of the Golgi membrane.

Another molecule that targets a mTOR-independent pathway is Xestospongin B. The latter is a macrocyclic bis-1-oxaquinolizidine alkaloid initially extracted from the marine sponge *Xestospongia exigua*. Inhibition of the IP_3R by Xestospongin B induces autophagy by disrupting the IP_3R–Beclin-1 complex (Figure 2) and, as a result, the Bcl-2/Beclin-1 autophagy inhibitory complex (Vicencio *et al.*, 2009).

A number of so-called small-molecule enhancers (SMERs) of autophagy have been identified from an initial library of 50 729 compounds using a functional cell-based screen, either by measuring the clearance of autophagy substrates or autophagy flux (Sarkar *et al.*, 2007b). Among them, SMER10, SMER18 and SMER28 (Figure 4) were found to be active independently or downstream of mTOR; their precise target is unknown. These SMERs increase autophagosome synthesis and enhance clearance of model autophagy substrates such as mutant huntingtin, which is prone to aggregate. They proved to be protective in a *Drosophila* model of Huntington disease.

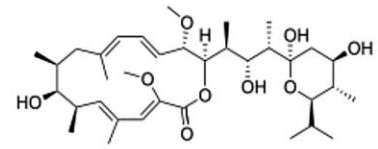
Trehalose disaccharide (Figure 4) is another molecule that activates mTOR-independent macroautophagy (Sarkar *et al.*, 2007a) but the target of this non-toxic molecule that is widely used for its cryoprotective properties and acts as a chemical chaperone is unknown. Interestingly, it enhanced clearance of mutant huntingtin and protect cell models from Huntington disease (Tanaka *et al.*, 2004; Sarkar *et al.*, 2007b), as other SMERs (see above). Trehalose has been claimed to represent a possible candidate for the treatment of certain neurodegenerative disorders (Sarkar and Rubinsztein, 2008).

A substantial number of autophagy mTOR-dependent and mTOR-independent, low MW, activators have been identified (extensively reviewed in Sarkar and Rubinsztein, 2008; Renna *et al.*, 2010; Fleming *et al.*, 2011; Baek *et al.*, 2012; Rubinsztein *et al.*, 2012; He *et al.*, 2013). Although their target and mechanism of action remain often to be experimentally determined, some of them have shown promising properties, both in terms of efficacy in model settings and pharmacological properties, and could have a potential therapeutic future. It has to be noted here that combining rapamycin with certain mTOR-independent small-molecule autophagy enhancers, such as trehalose, SMERs, L-690,330 or calpastatin, has been shown to give additive effects, yielding much higher autophagy up-regulation than that produced by individual compounds (see Ravikumar *et al.*, 2010). Also, thalidomide derivatives combined with temozolomide were more potent as autophagy enhancers on glioma cells *in vitro* (Gao *et al.*, 2009) and resveratrol and spermidine, used in suboptimal conditions where alone they have no effect, synergised to induce autophagy (Morselli *et al.*, 2011).

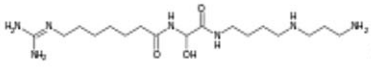
Low MW inhibitors of autophagy

Using the same methodology as the one designed to generate activators, numerous studies have been undertaken to identify low MW compounds that selectively inhibit autophagic processes. To date, very few drug candidates have emerged from extensive screening studies of libraries of high MW compounds. The available inhibitor compounds tend to target those key steps of the autophagy machinery that are also targets of autophagy activators. Most generally, as also observed in the case of autophagy activators, they are not specific for a particular link in the chain of autophagy events but also affect other cellular pathways, thus limiting the effect of some data and the robustness of the results.

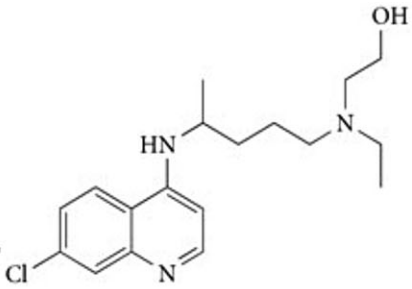
They are notably inhibitors of the PI3K pathway, wortmannin, LY294002, 3-methyladenine, KU55933 and Gö6976 (Figure 5). While some of these compounds have been claimed to be rather selective (Farkas *et al.*, 2011), some others display dual effects. For example, 3-methyladenine can promote or suppress autophagy according to the experimental conditions that are applied (Wu *et al.*, 2010). Thus, as pointed out more than 20 years ago (Caro *et al.*, 1988),



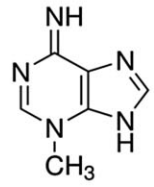
Bafilomycin A1



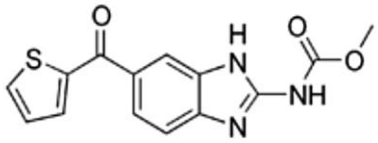
15-Deoxyspergualin



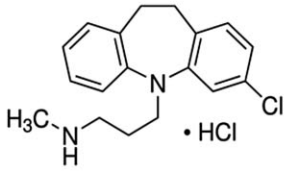
Hydroxychloroquine



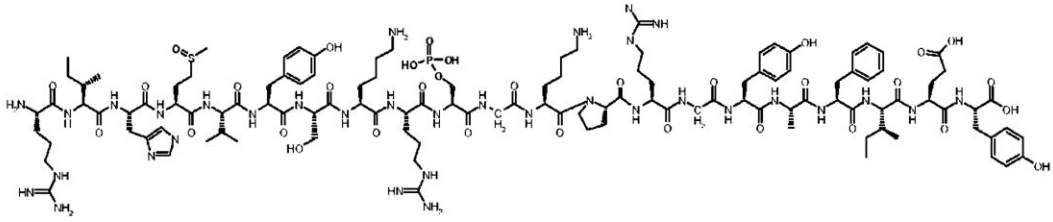
3-Methyleadenine



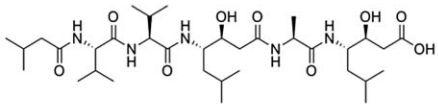
Nocodazole



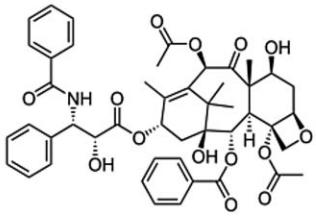
Norclomipramine



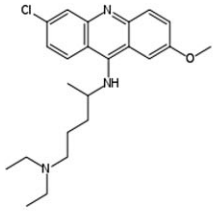
Peptide P140



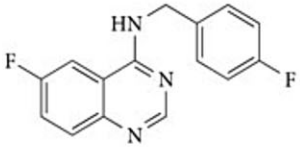
Pepstatin A



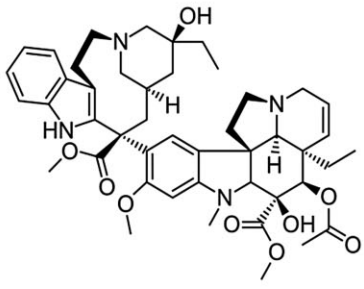
Paclitaxel/Taxol



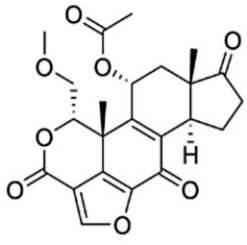
Quinacrin



Spautin



Vinblastine



Wortmannin

Figure 5
Examples of low MW compounds that are autophagy inhibitors.

3-methyladenine, which is used as a classical reagent in routine laboratory experiments, should be used with caution. In our hands, for example, 3-methyladenine showed no effect on autophagy flux in primary lymphocytes purified from lupus-prone MRL/lpr and (NZBxNZW)F1 mice (F Gros and S Muller, unpublished observation).

The chemotherapeutic (anti-mitotic) agent paclitaxel/taxol (Figure 5) also inhibits autophagy by blocking the activation of PI3K. A distinct mechanism dependent on cell cycle stage was recently described. In non-mitotic paclitaxel-treated cells, the formation of autophagosomes was effective but their movement and maturation was inhibited (Veldhoen *et al.*, 2013).

In the case of CMA, although chemical activators of this pathway are currently available (Anguiano *et al.*, 2013), considerably less progress has been made in the identification of specific inhibitors. Particularly, some low MW compounds described earlier as specific CMA modulators have since been shown to exhibit other activities, which somewhat diminishes their initial interest (Finn *et al.*, 2005; Cuervo and Wong, 2014). In this context, P140 peptide deserves particular attention. P140 is a 21-mer linear peptide (sequence 131–151) that is derived from the small nuclear ribonucleoprotein U1-70K and that is phosphorylated at the Ser^{140} position (Monneaux *et al.*, 2003; Figure 5). From the ongoing research, it seems that, in MRL/lpr mice that developed a spontaneous lupus-like disease, its primary target is the lysosome. P140 peptide enters MRL/lpr B lymphocytes via a clathrin-dependent endo-lysosomal pathway and accumulates at the lysosomal lumen. Although its mechanism of action within the lysosome remains to be exactly determined, parallel experiments showed *in vitro* using B cells purified from MRL/lpr lupus-prone mice that P140 peptide might directly affect the HSP90 and HSPA8 chaperoning functioning and hamper the lysosome-specific LAMP-2A protein to exert its functions in the lysosomal lumen (S Muller and AM Cuervo, unpublished data). It should be noted to this regard that expression of HSPA8 and LAMP-2A, the two main CMA components, which is increased in MRL/lpr B cells, is down-regulated after treating mice with P140 peptide given i.v. to mice (Page *et al.*, 2011; unpublished data). Using two independent *in vitro* assays for CMA activity, it was clearly established that P140 peptide specifically represses this autophagy pathway. P140 also reduces autophagic flux in MRL/lpr B cells treated *in vitro* (Page *et al.*, 2011). In a multicenter, randomized, placebo-controlled phase IIb study, P140/lupuzor was found to be safe and met its primary efficacy end points in lupus patients (Zimmer *et al.*, 2013).

Other low MW compounds target lysosomes and therefore affect the autophagic flux and as a consequence the autophagosome–lysosome fusion (see below). The two well-known members of this family of molecules are chloroquine (CQ) and HCQ, which are widely used for the prophylactic treatment of malaria. Both molecules differ by the presence of a hydroxyl group at the end of the side chain of HCQ (Figure 5). They have similar pharmacokinetics, with quick gastrointestinal absorption and elimination by the kidneys. Although their mechanism of action is not fully elucidated, these molecules display pleiotropic activity and also, unfortunately, important deleterious properties. As lysosomotropic agents, CQ and HCQ raise intralysosomal pH, impairing autophagic protein degradation. CQ/HCQ-mediated accumulation of ineffective autophagosomes may then result in cell death in tumour cell lines. CQ/HCQ may also affect peptide degradation within lysosomes due to the pH effect on lysosomal cathepsins and therefore the entire process of antigen presentation by MHC molecules in the class II compartment leading to activation of autoreactive T cells. Based on these properties, HCQ has been used for many years in the treatment of inflammatory autoimmune diseases, SLE, RA and Sjögren's syndrome. CQ has been shown to reduce the severity of EAE and the mechanism of action that was previously known to involve in part regulatory T cells has been recently established in much more details (Thome *et al.*, 2014). In this setting, CQ had a direct effect on DCs, and this result might be of importance in the aim of developing strategies avoiding CQ toxicity as CQ-treated DCs was given to model mice by adoptive transfer and notably, recipient mice showed a reduction of clinical signs of the disease. CQ/HCQ as well as quinacrine (also known as mepacrine or atebrine; Figure 5) also inhibit activation of endosomal TLRs (Kuznik *et al.*, 2011), which are involved in inflammatory responses through activation of the innate immune system (see above). This effect may explain in part their efficacy in certain indications. It seems that the anti-malarial drugs CQ, HCQ and quinacrine operate by interacting directly with TLR ligands and not through an effect on the lysosomal pH, for example (Kuznik *et al.*, 2011). Other properties of CQ, such as radiosensitizing and chemosensitizing properties, might also have promising future as anti-cancer drugs in humans. It should be reminded, however, that CQ/HCQ toxicity, in particular in the eye (cornea and macula) and in the occurrence of cardiomyopathies (Sumpter *et al.*, 2012), remains a major disadvantage. Ocular toxicity is related to the total cumulative dose rather than the daily dose; therefore, it becomes a serious potential problem in the cases of long-term use. A number of HCQ analogues and mimics have been tentatively designed to retain the beneficial activities without these side effects. Ongoing research should provide such safe molecules in the future.

Besides HCQ and CQ, other alkalinizing lysosomotropic drugs that alter lysosome functioning are amodiaquine and azithromycin. The latter is a potent macrolide antibiotic, and its primary mode of action is through its binding to the 50S subunit of the bacterial ribosome that affects mRNA translation. In clinic, it is notably used for treating chronic inflammatory lung diseases such as cystic fibrosis (CF). A deleterious effect of long-term use of azithromycin was shown in CF patients who paradoxically developed infection with non-tuberculous *Mycobacteria*. It was shown later in primary human macrophages that azithromycin affects lysosome acidification, thus leading to a blockade of autophagosome clearance and a much weaker intracellular killing of mycobacteria (Renna *et al.*, 2011).

Among the class of autophagy inhibitors that rather target downstream events, they are potent blockers of the autophagosome–lysosome fusion. Note that all those molecules described above that inhibit acidification of lysosomes affect this fusion step as well. Examples of such small molecules are bafilomycin A, vinblastine and nocodazole (Figure 5). Among other effects, these three molecules possess anti-neoplastic activities (Cheong *et al.*, 2012). Bafilomycin

A1 notably displays anti-bacterial, anti-fungal and immunosuppressive activities. Their individual mode of action is multiple and different. Thus, bafilomycin A, a macrolide antibiotic isolated from *Streptomyces* sp., specifically acts in a rat hepatoma cell line by inhibiting the vacuolar H^+ ATPase that is essential for acidifying the lumen lysosomes (Yamamoto *et al.*, 1998), while vinblastine and nocodazole both disrupt microtubules but do so on distinct types of microtubules (Xie *et al.*, 2010).

Other molecules able to hamper the formation of autolysosomes are pepstatin A and E64d, which are widely used as protease inhibitors in experimental assays designed to measure autophagy flux (Mizushima *et al.*, 2010; Klionsky *et al.*, 2012). It is worth mentioning that these classical lysosome inhibitors as well as CQ were unexpectedly found to also inhibit mTORC1 in a Rag-dependent manner (Li *et al.*, 2013). These findings have several implications. In particular they suggest that lysosomes can be mechanistically involved in autophagy activation in addition to their well-known implication in autophagic degradation. This work proposes another interactive link between CMA and macroautophagy.

15-Deoxyspergualin (DSG; 1-amino-19-guanidino-11-hydroxy-4, 9, 12-triazanona-decane-10, 1–3-dione) is a synthetic analogue of spergualin, a natural product of the bacterium *Bacillus laterosporus* (Maeda *et al.*, 1993; Figure 5). A long list of more stable analogues have been designed, synthesized and evaluated over years. 15-DSG is a potent immunosuppressant, which showed immunosuppressive activity both *in vitro* and *in vivo*, affecting B lymphocytes, T lymphocytes and macrophage/monocyte functions. DSG binds with high affinity to HSPA8 at a site that is apparently different from the one(s) recognized by P140 peptide (Stricher *et al.*, 2013); it also binds to HSP90 and modulate the functions of both HSPs. 15-DSG blocks the NF-κB pathway and antigen presentation, causing alteration in the activation of immune cells, notably monocytes and DCs. It also inhibits AKT kinase activation and phosphatidylcholine synthesis (Kawada *et al.*, 2002). DSG was also shown to suppress the progression of polyclonal B-cell activation and lupus nephropathy in lupus-prone MRL/lpr mice. However, in a short trial, two of three DSG/Gusperimus-treated SLE patients showed infectious episodes and the trial was interrupted (Lorenz *et al.*, 2005). 15-DSG was used clinically in the therapy of renal transplant rejection and Wegener's granulomatosis (Ohlman *et al.*, 1994; Flossmann and Jayne, 2010).

Comments

In many studies, autophagy activators and inhibitors have been used in investigations dealing with a better definition of specific pathways, regulation steps or physio(pathological) conditions in which autophagy processes had been suspected. We should keep in mind that most, if not all of the molecules described above, exhibit complex pleiotropic properties, and can notably influence different autophagy pathways (e.g. mTOR dependent and independent) as well as other quality control mechanisms affecting the cell life/death balance. Several widely used molecules exert dual (opposite) effects on upstream and downstream molecular events of the autophagy axes. The large majority of these compounds have been initially evaluated in cell culture conditions (some have emerged from cellular screens) and their mechanism of action largely depends on the selected cell type (immortalized cell lines, primary cells; cancer cells or non-cancer cells), concentration and time of exposure. These considerations are fundamental to analyse the conclusions that can be raised with most caution. It is worth repeating that studies based on such autophagy inducers/inhibitors should always be completed by other sets of experiments using independent strategies, distinct molecular tools and settings, notably cell types and animal models. Knock-down and knockout experiments, although somewhat tricky to set up, can reinforce the findings raised with chemical blockade or activation to conclude on cell-specific functions or effects. In this regard, the extensive work carried out to establish international guidelines for standardizing research in autophagy and in particular to propose relevant methodologies for monitoring autophagy that are accepted by the whole community is totally unique (Klionsky *et al.*, 2012). It should be exploited by investigators who evaluate new molecules designed to selectively target key steps of autophagy or who develop new high-throughput screening methods for autophagy-modulating compounds.

As underlined above, the arsenal of specific agonists and antagonists of autophagic activity remains minimal, particularly for CMA, and programmes dealing with drug screening of such low MW compounds should be established, which may yield additional therapeutic targets. Molecules interfering with autophagic processes are mainly clinically evaluated in neurodegenerative diseases and cancer. Theoretically, modulating the autophagy axes is extremely promising; the success of these therapeutic options in these and other indications will largely depend on minimal toxic and side effects, careful (possibly personalized) dosage and decisions regarding their mode, and schedule of administration.

Link between pharmacological regulators of autophagy and inducers or inhibitors of lupus disease

At this stage of our discussion, a striking observation that deserves to be mentioned is that in the list of compounds that are claimed to positively or negatively regulate autophagy processes, a number of small molecules are also regarded as activators or negative modulators of lupus disease. There are increasing numbers of reports and case series of patients who develop a lupus-like disease apparently resulting from exposure to certain drugs (Marzano *et al.*, 2009; Vedove *et al.*, 2009). Although in some cases evidence remain relatively weak and the observation might be coincidental or fortuitous, in general the definitive cases of drug-induced lupus (DIL) are interesting because they inform us of specific metabolic dysfunctions and cellular pathways that are important in the triggering of the lupus syndrome. Since the first paper published in 1945 reporting a case of lupus-like disease following exposure to a drug – a 19-year-old man who had received sulfadiazine – the list of drugs definitely associated with DIL has expanded and nowadays includes over 80 pharmacological agents. Among the historically recognized molecules are chlorpromazine, hydralazine, isoniazide, methyldopa, procainamide and quinidine. In the list of drugs that have been associated with DIL, we note in particular the

presence of autophagy activators amiodarone, carbamazepine, chlorpromazine, clonidine, lithium, minocycline, valproic acid and verapamil. The semi-synthetic tetracycline antibiotic minocycline has been used for decades as a prolonged treatment for acne (in particular pustular-type acne) and other skin infections, as well as for Lyme disease. Major side effects of minocycline include dizziness, nausea, vomiting, skin pigmentation changes, tooth discoloration and development of DIL (Schlienger *et al.*, 2000). Minocycline was shown to induce autophagy in glioma cell culture and in a xenograft tumour model of glioma cells through a process that appears to involve suppression of the AKT/mTOR/p70S6K signalling pathway and activation of ERK pathway (Liu *et al.*, 2011). Chlorpromazine, an anti-psychotic agent, induces autophagy by interfering with the PI3K/AKT/mTOR pathway in glioma cells and as minocycline, chlorpromazine also induces autophagy in a xenograft tumour model of glioma cells implanted into the brain of athymic nude mice (Okazaki *et al.*, 2002; Alinari *et al.*, 2011).

Special mention has to be made here regarding trehalose, which has been recognized to display pro-autophagic properties (see above). We discovered that trehalose alone tends to accelerate the lupus disease in lupus-prone mice (proteinuria and survival). It also showed some negative effects in patients who received trehalose alone in the placebo arm of a phase IIb clinical trial designed to evaluate the P140 peptide in lupus patients (Muller and Wallace, 2014). This harmful effect disappeared after the cessation of trehalose administration. This observation is important as trehalose is classically used as an excipient in pharmaceutical formulations.

DIL remains a relatively rare complication (~5% of the patients who have received hydralazine for long periods develop DIL). Typically, there is a resolution of clinical features on discontinuation of the drug although the circulating antibodies may persist. The causes for developing DIL are not fully understood; they appear to be individual and seem to be related to genetic factors. So-called slow acetylators showing a genetic deficiency in N-acetyltransferase would be at higher risk of developing DIL compared with fast acetylators (Davies *et al.*, 1975; Foad *et al.*, 1977; Schur, 1995).

To pursue this line of thought, we note with interest that, conversely, some low MW drugs or drug candidates that are given to lupus patients, or have shown some promise for the treatment of lupus patients (Monneaux and Muller, 2009), are known autophagy inhibitors. This includes CQ and HCQ, FTY720 (a synthetic analogue of sphingosine; Okazaki *et al.*, 2002; Alinari *et al.*, 2011), and P140 peptide. 15-DSG could also be inserted in this class of components. Whether other small molecules that display inhibitory autophagic activity might have some application in the treatment of lupus deserves much more investigation.

Concluding remarks

In the foregoing sections, we have described a large number of molecules that positively and negatively regulate autophagy. Some of them are widely used as classical reagents in *in vitro* experiments and sometimes in animal models to answer biological and cellular questions. We must realize, however, that only a few are effectively potent activators or inhibitors endowed with experimentally demonstrated selective properties, and relatively few also are used currently clinically or evaluated in clinical trials. Much effort is therefore expended to discover novel candidate molecules that show high selectivity and minimal toxicity while keeping suitable pharmacokinetics and stability characteristics to make acceptable drugs. The possible indications are vast and diversified, from neurodegenerative diseases, to metabolic and inflammatory diseases, infections and cancer. The road is long from the initial target identification and validation, assay development, eventual high-throughput screening of diversified libraries of compounds, hit identification, lead optimization and finally selection of candidates for preclinical and clinical development. Additional basic research remains important in order, hopefully, to develop entirely new approaches to discover relevant targets and pathways that are pertinent.

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Conflict of interests

The authors declare no conflicts of interests.

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