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In vitro effects of the tyrosine kinase inhibitor Imatinib mesylate on cardiac stem cells

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Summary

Abstract

Cardiotoxic effects of some old generation antiblastic drugs represent an actual problem in oncologic chemotherapy. In this context it seems fundamental to introduce new chemotherapics drawn to interact with high specificity with essential proteins in tumorigenesis, decreasing toxicity in health cells (targeted therapies). The life expectancy in chronic myeloid leukemia, acute linphoblastic leukemia and gastrointestinal stromal tumor patients has been improved after the introduction of a tyrosine kinase inhibitor, Imatinib mesylate, object of this study.

Despite of the effectiveness of this drug, it is advancing the hypothesis that this molecule could have side effects on cardiovascular system. In particular, this drug could compromise the integrity of cardiac stem cells and their capability to replace damaged tissues, increasing the incidence of cardiovascular diseases. In this study we used cardiac stem cells: rat cardiac progenitor cells (CPCs) and human cardiac mesenchimal stem cells (C-MSCs). Cytotoxicity, genotoxicity, autophagy activation and oxidative stress induction have been evaluated.

Doxorubicin, an old generation chemotherapy, has been used to compare *in vitro* effects induced by IM treatment with those induced by a drug with known cardiotoxic effects. Furthermore the effects induced by IM and Doxorubicin have been evaluated on their target cell lines, K562 (chronic myelogenous leukemia cell line) for IM, and MCF7 (breast adenocarcinoma cell line) for Doxorubicin.

Data reported in this thesis underline IM toxicity induction in target and non- target cell lines. Furthermore comparison with cellular response induced by Doxorubicin confirms the higher specificity of IM. On the other hand it shows that an high IM concentration, that could result from a chronic treatment, could induce in cardiac stem cells a toxicity comparable with that observed in the same cell lines treated with Doxorubicin.

Given the increasing interest in autophagy, as cellular response pathway induced by chemotherapic treatment, assays have been performed to understand the role of this pathway in IM treated cell lines. Furthermore we have verified if autophagy modulation could decrease toxicity induced in non- target cell lines without affecting the drug efficacy.

Riassunto

Gli effetti cardiotossici di alcuni farmaci antiblastici di vecchia generazione rappresentano tuttora una problema aperto nella chemioterapia oncologica. In questo contesto assume sempre più importanza l'introduzione sul mercato di nuovi chemioterapici disegnati per interagire con elevata specificità con proteine fondamentali per la tumorigenesi e quindi in grado di ridurre al minimo gli effetti di tossicità sulle cellule sane (*targeted therapies).* La prospettive di vita di pazienti affetti da leucemia mieloide cronica, leucemia linfoblastica acuta e da tumore stromale gastrointestinale è stata migliorata dall'introduzione di un inibitore delle tirosina chinasi cellulari, Imatinib mesylate, oggetto di questo studio. Nonostante l'indiscussa efficienza di questo farmaco, sta lentamente avanzando l'ipotesi che questa molecola possa avere effetti collaterali negativi sul sistema cardiocircolatorio. In particolare la possibilità che questo farmaco possa compromettere l'integrità delle cellule staminali e la loro capacità di rimpiazzare il tessuto danneggiato aumenta la probabilità di sviluppo di patologie cardiovascolari. Per questo motivo in questo studio sono state utilizzate cellule staminali di origine cardiaca: progenitori cardiaci di ratto (CPCs) e cellule staminali mesenchimali cardiache di origine umana (C-MSCs). Su queste linee cellulari sono state valutate la citotossicità, la genotossicità, l'attivazione del pathway autofagico e l'induzione di stress ossidativo.

È stata inoltre condotta una comparazione degli effetti indotti su cellule staminali cardiache da IM con quelli dovuti al trattamento con un chemioterapico di vecchia generazione, la doxorubicina, nota per indurre tossicità cardiaca in soggetti esposti. Gli effetti indotti da questi due farmaci sono stati, inoltre, valutati sulle rispettive linee cellulari target, le K562 (linea cellulare di leucemia mieloide cronica) per IM e le MCF7 (linea cellulare di adenocarcinoma mammario) per la doxorubicina.

I dati riportati in questa tesi mettono in luce che il trattamento *in vitro* con IM può indurre tossicità non solo in cellule target, ma anche in linee cellulari non- target. Inoltre, la comparazione con la risposta cellulare indotta dal trattamento con Doxorubicina ha confermato la maggiore specificità d'azione di IM. Allo stesso tempo si è evidenziato che un'alta concentrazione di IM, come quella che potrebbe risultare da un trattamento cronico, può indurre nelle cellule staminali cardiache una tossicità comparabile con quella osservata nelle stesse cellule trattate con Doxorubicina.

Dato il crescente interesse nei confronti del pathway autofagico, come meccanismo di risposta cellulare indotto in seguito a trattamento con chemioterapici, sono stati condotti saggi al fine di comprendere il coinvolgimento di questo pathway nella risposta al trattamento con IM. Si è cercato inoltre di verificare se la modulazione della risposta

autofagica potesse diminuire la tossicità indotta in cellule non- target senza compromettere l'efficacia del farmaco stesso.

Introduction

Chemotherapy and cardiotoxicity

Cardiovascular diseases and cancer represent respectively the first and second cause of death in industrialized countries. These two conditions may become synergistic when cardiovascular complications of anti-cancer therapy are considered [Prezioso et al., 2010].

The National Cancer Institute defines cardiotoxicity in general terms as ‘‘toxicity that affects the heart’’ (http://www.cancer.gov/dictionary). This definition embraces a variety of side effects affecting both the heart and circulation: valvular injury, dysrhythmias, changes in blood pressure, arterial/venous thrombosis or impairment in myocardial contraction or relaxation (systolic and diastolic dysfunction) [Raschi et al., 2012]. We can distinguish two classes of cardiotoxicity: the acute or subacute one that occurs during the chemotherapy treatment, and the chronic one that can be early or late if the cardiac disorders appear within a year or after the end of an year of treatment respectively.

Cytostatic antibiotics of the anthracycline class (daunorubicin, doxorubicin and epirubicin) are perhaps the most notorious chemotherapeutics with cardiac side effects; other agents such as cyclophosphamide, ifosfamide, cisplatin, carmustine, busulfan, chlormethine, mitomycin, paclitaxel, etoposide, teniposide, the vinca alkaloids, fluorouracil, cytarabine, amsacrine, cladribine, asparaginase, tretinoin, and pentostatin have also been associated with cardiotoxicity [Jain, 2000; Ryberg et al., 1998; Schimmel et al., 2004]. Besides the typical chemotherapeutics, targeted therapies are also associated with a clinically relevant cardiotoxic profile [Geiger et al., 2010].

“Old fashioned” chemotherapies

Numerous are the old generation chemotherapies that is known to induce cardiotoxicity. Between these 5-Fluorouracil has been described to induce ischaemia, a phenomenon that is attributed to coronary vasospasm, and myocarditis [Dalzell et al., 2009]. In this categories we have to mention the family of Anthracyclines that are

chemotherapies belonging to antibiotic antineoplastic class. They represent the more effectiveness till now developed. One of their features is the broad spectrum of activity; they are active antineoplastic drugs able to improve survival in adult and pediatric patients with hematologic (like Hodgkin disease and acute leukemia) [Tilly et al., 2003] and solid (like breast, ovarian and hepatic cancer and sarcomas) tumors [Cadeddu 2006; Jones et al., 2006]. The use of anthracyclines as antineoplastic agents in the clinic is compromised by the risk of cardiotoxicity. It has been calculated that approximately 10% of patients treated with doxorubicin or its derivatives will develop cardiac complications up to 10 years after the cessation of chemotherapy [Yanti 2012]. Anthracyclines possess different mechanisms of action:

- intercalation into DNA, leading to inhibition of synthesis of macromolecules;
- generation of free radicals, leading to DNA damage or lipid peroxidation;
- DNA binding and alkylation;
- DNA cross-linking;
- interference with DNA unwinding or DNA strand separation and helicase activity;
- direct membrane effects;
- initiation of DNA damage via inhibition of topoisomerase II: Anthracyclines act by stabilizing a reaction intermediate in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II, eventually impeding DNA resealing.;
- induction of apoptosis in response to topoisomerase II inhibition [Minotti et al., 2004].

Targeted therapies

Many of the characteristics of cancer are manifestations of abnormal changes in the physiology of cancer cells, like self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion/metastasis. These physiological changes result from genetic alterations, and for any change, a different gene or group of genes might be involved. The biochemical events that lead to critical changes in the physiology of cancer cells are becoming the newest targets for anticancer therapeutics [Cattley et

al., 2004]. These new drugs are able to hit only specific cellular receptors responsible of the tumor development and growth, without damaging healthy cells. These molecules are named *targeted therapies* and are divided in: monoclonal antibodies generally direct against tyrosine- kinases or their ligands, (like trastuzumab and bevacizumab); little molecules targeted against specific kinases (KI) (like Imatinib and Erlotinib).
These drugs can offer new hope to cancer patients but treatment sometimes has been associated with cardiovascular complications including hypertension, left ventricular dysfunction, and/or heart failure [Cheng et al., 2010]. We can distinguish an ‘‘on-target’’ and an ‘‘off-target’’ drug side effects that lead to cardiotoxicity [Raschi et al., 2012].

‘‘On-target’’ toxicity occurs when the inhibited kinases in addition to cancer progression are important in other organ systems including the heart and vasculature [Cheng et al., 2010]. An example is the cardiotoxicity induced by trastuzumab, a monoclonal antibody used against ERBB2 receptor (HER2). HER2 is overexpressed in about 20% breast cancers and it is important for tumor progression. HER2 inhibition by trastuzumab increases the survival of patients but sometimes induces left ventricular dysfunction because HER2 is involved in cardiomyocytes proliferation during the development and in their survival.

“Off-target” toxicity is determined by the inhibition of non target kinases or of other kind of enzymes that play a key role in the heart. This kind of toxicity is linked to the low selectivity of certain KI because of the high dimension of human kinome [Cheng et al. 2010].

Tyrosine Kinases Inhibitors and Imatinib mesylate

Tumor sequencing projects allowed to individuate a lot of mutation in protein kinases. One study found that mutations in as many as 120 kinases (or ~20% of the kinome) could be present in individual cancers [Schwab et al., 1986; Haber et al., 2007]. Approximately 90 of the 518 kinases in the human kinome are tyrosine kinases (TKs). This has led to develop new drugs that have as target TKs (TK inhibitors [TKIs]). TKIs

typically compete with ATP for binding to the ATP pocket. If ATP cannot bind, phosphotransferase activity is blocked and downstream substrates cannot be phosphorylated. In the cell, ATP is present in mM concentrations but TKIs will be present in nanomolar to very low micromolar concentrations. Thus they have to possess a very high affinity for their binding site. Because the structure of the ATP pocket is highly conserved across the more than 500 kinases of the human genome, it is relatively easy to make an inhibitor that blocks a kinase of interest. On the other hand the lack of selectivity is the major issue of most kinase inhibitors that target the ATP pocket; the aspecific inhibition of non- target kinases can induce the so- called "off-target" effects that lead to toxicity. The relatively poor selectivity of these kind of TKI, called Type I inhibitors, can be addressed by targeting additional regions of the kinase [Okram et al., 2006]. The so-called type II inhibitors bind the ATP pocket and at the same time interact with a site adjacent to the pocket. This allows not only an enhanced selectivity but also the binding to the kinase when it is in the inactive conformation. Finally, type III inhibitors (for example the families of MEK inhibitors including PD98059 and UO126) bind to regions near the ATP pocket [Ohren et al., 2004]. These regions are typically not highly conserved, allowing an highest selectivity. Although type III agents are more selective, they are a small minority of agents in development because they are more difficult to design and not so predictably effective.

The first TKI to reach market was imatinib mesylate (Gleevec, Novartis) (Figure 1), and it was approved in 2001 [Sherbenou et al., 2007] . Imatinib revolutionized the treatment of chronic myeloid leukemia (CML). Before the introduction of imatinib, CML was fatal within 5 years, whereas now, ~90% of patients are alive 5 years after diagnosis. Imatinib is a type II inhibitor and was designed to target Bcr-Abl, which is causal in ~90% of cases of CML and ~20% of acute lymphocytic leukemia (ALL).

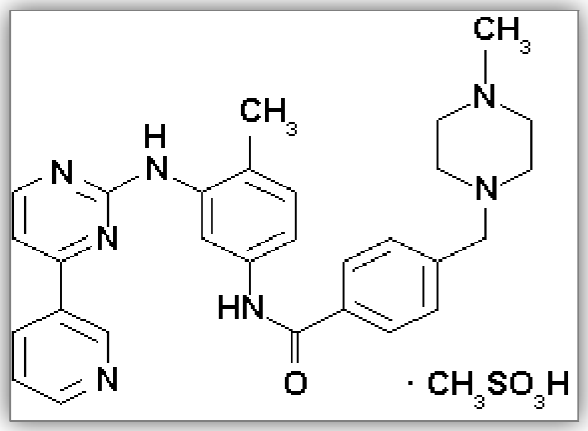


Figure 1: Structure of Imatinib mesylate.

The Bcr-Abl fusion protein is created by a balanced translocation in bone marrow progenitor cells that creates the Philadelphia chromosome. The protein consists of a nonkinase domain of Bcr (a kinase of unclear function) and the kinase domain of the nonreceptor TK c-Abl. The Bcr-Abl fusion dimerizes leading to cross-phosphorylation and constitutive activation of the Abl kinase domain. This protein acts suppressing apoptosis by activating the Ras-Raf-ERK pathway (which increases antiapoptotic Bcl2 expression), the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (which inhibits the proapoptotic factors Bad and FOXO3A), and STAT5 (signal transducer and activator of transcription 5) (which induces expression of antiapoptotic Bcl-x). Imatinib blocks all Bcr-Abl-dependent signaling, leading CML cells into apoptosis (figure 2) [Cheng et al., 2010].

Imatinib was found to inhibit two additional protein kinases that are involved in a variety of malignancies: c-Kit, the receptor for stem cell factor, which is overexpressed in gastrointestinal stromal tumors (GISTs) (a rare tumor of the upper gastrointestinal tract derived from cells of neuroendocrine origin) and is mutated in systemic mastocytosis; and platelet-derived growth factor receptors (PDGFRs). Fusion proteins such as FIP1L1-PDGFRα and ETV6- PDGFRβ are involved in rare diseases including hypereosinophilic syndromes, dermatofibrosarcoma protuberans, and chronic myelomonocytic leukemia. Mutations and overexpression of PDGFRs play key roles in other cancers including GIST and glioblastoma [Bantscheff et al., 2007].

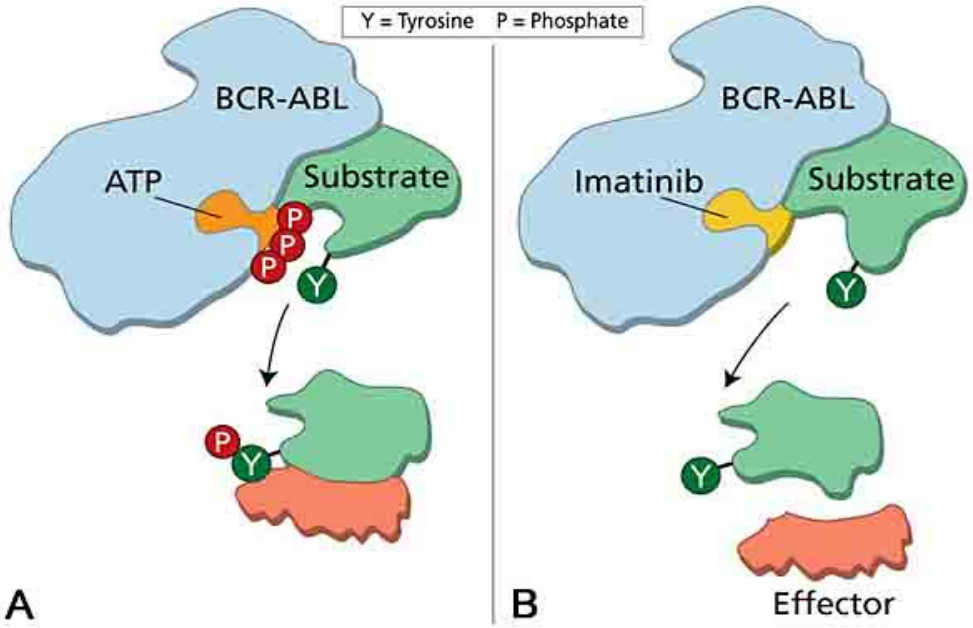


Figure 2: Schematic representation of Imatinib mesylate inhibition of tyrosine- kinase BCR-ABL .

Imatinib mesylate and cardiotoxicity

Despite the promise of their high selectivity that accompanied the ideation of this new generation drug a certain degree of toxicity was detected during the treatment. In particular Kerkela et al. in 2006 published a report of cardiotoxicity. They analyzed a case series of 10 patients who developed congestive heart failure while receiving Imatinib [Kerkelä et al., 2006]. Both a “on- target” and “off- target” toxicity have been detected for Imatinib. IM treatment determines the inhibition of different tyrosine kinases, like c-Abl and ARG (Abl-related gene or Abl2), PDGFRs, c-Kit, the Src family member Lck, CSF1R, Cdc2, and discoidin domain receptor (DDR) that induces alterations in cellular functions (Figure 3).

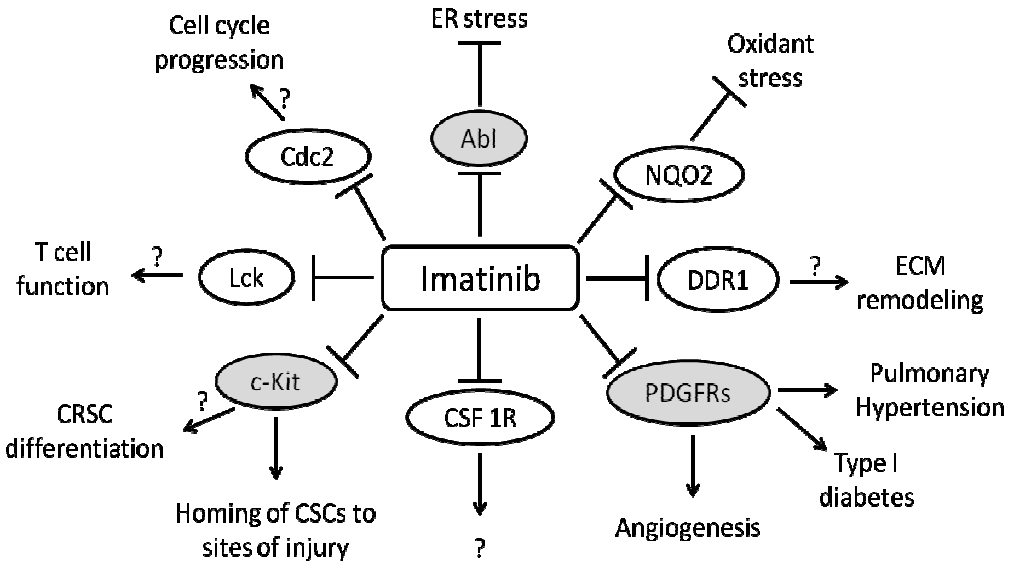


Figure 3: Possible targets of Imatinib mesylate and their effects.

In particular c-Abl inhibition causes induction of the endoplasmic reticulum (ER) stress response, with activation of two different kinases PERK and IRE1; PERK induces the phosphorilation and the activation of elF2 (eukaryotic initiation factor 2), that switches off mRNA translation and promotes the translation of specific mRNA coding chaperones to decrease the accumulation of unfolded proteins. IRE1 induces the splicing of the mRNA coding for XBP1 (X-box binding protein), that activates the unfolded protein response (UPR). Alternatively IRE1 activates the JNKs kinases that induce Bax release that can promote mithocondrial membrane permeabilization and caspases activation. These two pathway culminate in apoptotic cell death in cardiomyocytes (Figure 4) [Kerkelä et al., 2006].

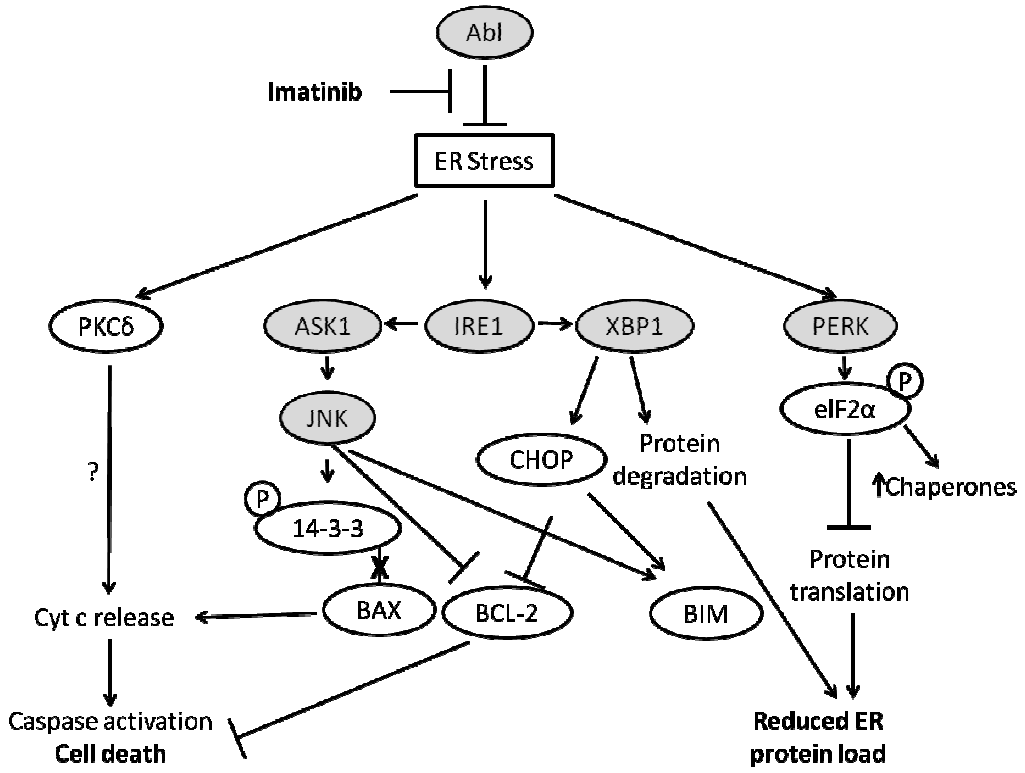


Figure 4: Effects of Abl inhibition by Imatinib mesylate.

c-kit is involved in cardiac resident stem cells differentiation (CRSC) [Li et al., 2008] and in cardiac stem cells bone marrow derivative migration to the lesion regions (CSC) [Ayach et al., 2006]. The PDGFR inhibition compromises angiogenesis in cardiac muscle [Chintalgattu et al., 2010]. It, in fact, seems to be important to induce angiogenesis during cardiac stress [Louvet et al., 2008]. IM toxicity is also induced by inhibition of off-target kinases such as Cdc2 and DDR1 and of the oxidoreductase NQO2 which plays a protective role in the cellular response to oxidative stress (Figure 3).

In a study Kerkela and collaborators identified mitochondria as a target of Imatinib. They observed an enhanced susceptibility to Ca^{2+} induced by opening of the mitochondrial permeability transition pore and a collapse of the mitochondrial membrane potential, a marked reduction in ATP concentration, and release of c cytochrome in the absence of any other inciting stimulus. Thus mitochondrial dysfunction and the consequent rundown of energy can represent a crucial factor in the cardiotoxicity [Kerkelä et al.,

2006]. IM is also known to generate reactive oxygen species (ROS) with an increase of oxidative stress [Blasiak et al., 2002]. Another important observation was that the decrease of left ventricular mass wasn't associated to the presence of fibrotic area [Kerkelä et al., 2006; Leipner et al., 2008]. Usually during a damage in cardiac tissue, like during an ischemia, cells activate necrotic death. If stem cells can't replace the damaged tissue, the organism induce an emergency mechanism that includes the activation of fibroblasts. They bring to the formation of a scary consisting of connective tissue that hasn't the mechanic, electric and biochemical properties of the original tissue. The absence of fibrotic tissue induces us to exclude the possibility that IM could induce necrotic death in cardiomyocytes.

These evidences make important to investigate alternative cell deaths, first of all autophagy. This pathway seems to assume always higher importance because of its fundamental role in finally differentiated cells, especially in cardiac cells [Rabinowitz et al., 2010]. Cardiomyocytes replacement is more complicated than in other tissue, thus more susceptible to stress [Srivastava et al., 2006]. These cells with a long life span, like neurons, undergo accumulation of cross-linked material of proteic or lipid nature that can compromise mitochondria and lysosomes [Grune et al., 2005; Terman et al., 2006]. Actively proliferating cells can reduce these accumulations during cell division, but this is impossible in terminally differentiated cells. In this contest autophagy appears really important to maintain cellular homeostasis [Terman et al., 2006].

Autophagy

The term autophagy comes from the Greek words 'phagy' meaning eat, and 'auto' meaning self and was founded in 1963 when de Duve described the presence of single- or double-membrane vesicles that contain damaged parts of cytoplasm and organelles. Researches on this topic began in the late 1950s, but progresses in molecular studies of this pathway have been made only on the past 15 years. Molecular studies, started in the late 1990s, have revolutionized the ability to detect and genetically manipulate this process. Autophagy was initially identified in mammals but a significant breakthrough has been made when it was discovered that it is a conserved cellular pathway from yeast to mammals that controls degradation of proteins and organelles, and it is essential in cellular survival, development and homeostasis. Cytoplasmic cargo are sequestered inside double-membrane vesicles and delivered to the lysosomes for degradation. This 'self-eating' process rids the cell of intracellular misfolded or long-lived proteins, superfluous or damaged organelles, and invading microorganisms, but it is also an adaptive response to provide nutrients and energy during various stresses. Moreover autophagy seems to be important for human health and is involved in physiology, development, lifespan and a wide range of diseases, including cancer, neurodegeneration, immune response and microbial infection [Yang et al., 2010].

The recent advances in the study of autophagy allowed us to understand the differences among three different autophagic pathways that co-exist in most mammalian cells: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy (Figure 5).

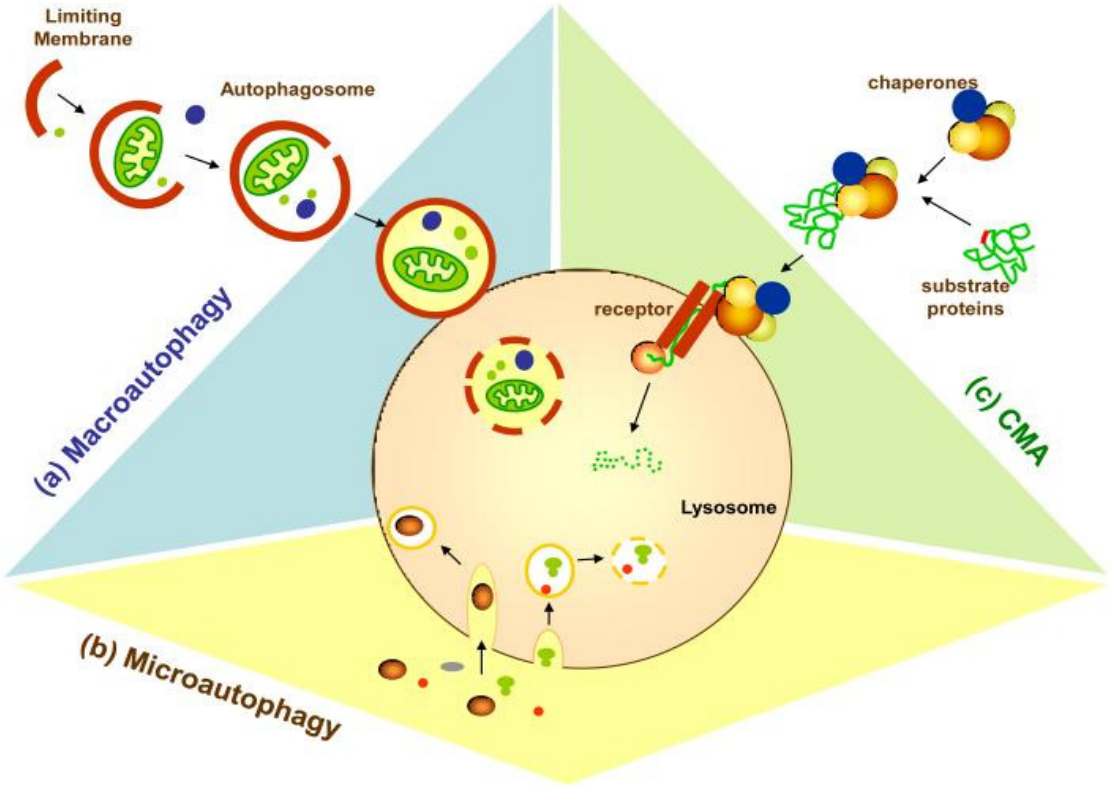


Figure 5: Three different autophagic pathways in mammalian cells: (a) Macroautophagy, (b) Microautophagy, (c) Chaperone- mediated autophagy (CMA).

Microautophagy

Between the three kind of autophagy, microautophagy is the less studied and the less understood. Studies on microautophagy have been focused on its characterization in yeast. In this pathway is the lysosomal membrane itself, which invaginates to form tubules or vesicles that sequester cytoplasmic components and organelles [Cuevo et al., 2010].

Chaperone-mediated autophagy (CMA)

CMA is a highly selective autophagy; only proteins with a specific signal sequences are recognized from chaperone complexes in the cytoplasm and transported to the lysosomes for degradation. Delivery of cargo via CMA does not require formation of intermediate vesicle compartments, membrane fusion or membrane deformity; specific

receptors (named Lamp2A) localized on lysosomal membrane link chaperone-protein complexes and allow their internalization [Cuevo et al., 2010]. CMA allows degradation of soluble proteins and requires the presence of three different proteins:

1. Cytoplasmic chaperones like Hsc70 recognizing signal sequences (KFERQ or QREFK), that link and drive proteins to lysosome.
2. Lamp2A receptors on the lysosomal membrane for the internalization of the complex in the lysosomal lumen.
3. Lysosomal chaperones like Hsc70-Lys indispensable for the transfer in lysosomial lumen [Li et al., 2011]

This pathway is constitutively active in almost all cells, but it is overexpressed during stress, like starvation, or in presence of damaged proteins [Orenstein et al., 2010]. It is involved in cellular homeostasis maintenance in normal conditions or during stress, recently it has been demonstrated its involvement in specialized functions like antigen presentation [Zhu et al., 2009].

Macroautophagy

Macroautophagy is the most important autophagic pathway. It allows the degradation of proteins, organelles and entire cytoplasmic regions. Cargo sequestration occurs in the autophagosome, a double membrane vesicle that origins from a series of interactions between more than 10 different proteins. The enzymes needed for degradation of the sequestered cargo are provided through fusion with lysosomes [Cuevo et al., 2009]. This pathway can be divided into different phases (figure 6):

1. Initiation: the pathway initiation is driven by a complex containing ULK1-2 (homologous to yeast Atg1), ATG13 and FIP200 kinases, during cellular stress like starvation or low ATP levels. The activated ULK1-2 kinases phosporilate ATG13 and FIP200 determining the activation of the entire complex and the localization near the membrane of the rising phagophore [Jung et al., 2009];
2. Autophagosome formation: this phase can be divided in three stage, nucleation, elongation and autophagosome completation. For the nucleation is essential a

complex composed of phosphatidilinositol-3-kinase, Vsp34, Beclin1 and p150 that produce PI3P, key component of the autophasomal membrane and of protein recruitment [Simonsen et al., 2009]. It's still unclear the mechanism of membrane formation; recently structures, named omegasomes, have been discovered; they origin near the endoplasmic reticulum (ER) during aminoacids deficiency [Axe et al., 2008]. These structures are rich of PI3P and other proteins typically expressed during autophagy, like LC3 and Atg5. The ER could act as a platform for PI3P synthesis and protein recruitment [Simonsen et al., 2009]. Beclin1 is another important protein in this pathway; it is formed by three domains: a N-terminal domain BH3, a central domain with coiled coil structure CCD and a C-terminal domain ECD. Everyone of this domain can interact with different proteins [Kang et al., 2011]. In condition of abundance of nutrients, Bcl2 (anti-apoptotic protein) link the BH3 domain of Beclin1 determining the detachment from the Vsp34 complex and inhibiting autophagy. The CCD domain interacts with Atg proteins like Atg14L, fundamental for complex assembly, PI3P production [Sun et al., 2008] and recruitment of protein like LC3 [Matsunaga et al., 2009]. The elongation stage requires two proteins ubiquitin-like, Atg12 and LC3. Atg12 is linked to Atg5 trough two enzymes, Atg7 and Atg10. A third protein, Atg16L, joins the Atg5-12 complex to form a tetrameric complex. The union of more tetramers produce a multimeric complex named ATG16L that acts like a platform for the autophagosome elongation [Nakatogawa et al., 2007]. ATG16L complex is needed for recruitment on the autophagosomal membrane of LC3 that has a key role in the elongation stage [Fujita et al., 2008]. The LC3 protein exists in two different form, a cytosolic LC3-I and a membrane form LC3-II. The cytosolic LC3 is cleaved by a Cys-protease (Atg4) to form LC3-I that is conjugated with phosfatidiletanolammine (one of the most important component of cellular membranes), generating LC3-II, that remain linked to the autophagosome membrane during all the autophagic process [Ohsumi et al., 2001].

3. Maturation and fusion with lysosome: autophagosome maturation requires various fusion events with endosomes, multivescicolar bodies and lysosomes;

different proteins are involved in this process, like SNAREs, Rab, ESCRT e Lamp, that take part in many pathways of vesicles fusion. Lysosomes usually are located in perinuclear position near the Microtubules organization center (MTOC), autophagosomes instead can be formed in every region of cytoplasm [Jahreiss et al., 2008]. A transport dinein-dependent is fundamental for the transfer of autophagosomes to perinuclear region [Ravikumar et al., 2005]. The interaction between autophagosomes and dinein is probably mediated by LC3, an injection of antibodies anti-LC3 inhibits their movements [Kimura et al., 2008]. The FYCO1 protein was recently discovered to interact with LC3 and Rab7 to form a complex that promote transport along microtubules [Pankiv et al., 2010]. Lysosomial proteins Lamp1-2 finally interact with Rab7 to promote autophagosome maturation and acidification [Saftig et al., 2008]

4. Cargo degradation and nutrient release: after autophagosome - lysosome fusion, lumen acidification and lipases (like Atg5) activation induce the autophagosome membrane degradation [Nakamura et al., 1997]; the proteic components of autophagosome are degraded by hydrolases like Pep4. Cargo degradation through proteinase A is linked to vesicle acidification determined by H^+ATPase activation that induce a pH reduction. Degradation products, like aminoacids and monosaccarides are transported in cytoplasm by lysosomal efflux transporters (like Aut4, SLC36A1 and LYAAT 1-2) [Lloyd et al., 1996; Sagné et al., 2001; Yang et al., 2006].

In addition to the degradation and the nutrients recycle, macroautophagy is important for cell viability maintenance and it has a key role in various human diseases like cancer and neurodegenerative disorders. This pathway is activated as response to different stimuli like starvation, ipoxia, oxidative stress, reticulum endoplasmic stress and after chemotherapic treatments. Alterations in this pathway can be linked to the development of various human pathologies, like tumors, inflammatory diseases and neurodegenerative disease.

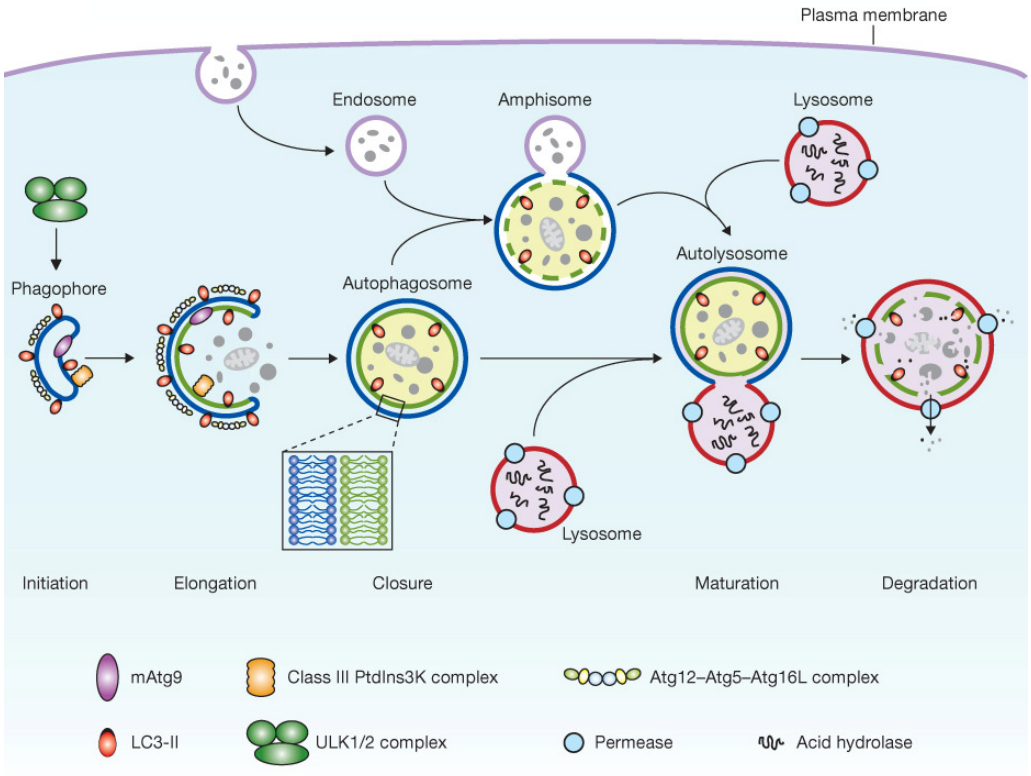


Figure 6: Stages of autophagosome formation and cargo degradation.

Autophagy and cellular homeostasis maintenance

Autophagy is a very controversial topic. It seems to be important in cellular defence but at the same time under stress condition it can induce programmed cell death [de Bruin et al., 2008; Chen et al., 2008].

This pathway is activated as response to different cellular stress (Figure 7); senescent mitochondria, for example, have a more permeable membrane that increases protein release (like c citocrome) that induces a pro-apoptotic signal. In this contest mitophagy (mitochondria degradation through autophagy) seems to be a cytoprotective pathway [Nishida et al., 2009]. Reticulum endoplasmic stress (ER) also can induce autophagy activation. ER provides a calcium store and is the site where post-translational modification take place. ER homeostasis alteration cause an accumulation of unfolded or misfolded proteins that induces the “unfolded protein response” (UPR) [Kaufman et al., 2002; Ron et al., 2002]. UPR induces the damaged protein sequestration and the

inhibition of mRNA translation to decrease the ER charge. Autophagy activation can help ubiquitin-proteasome system in this purpose [Ding et al., 2007; Rouschop et al., 2010].

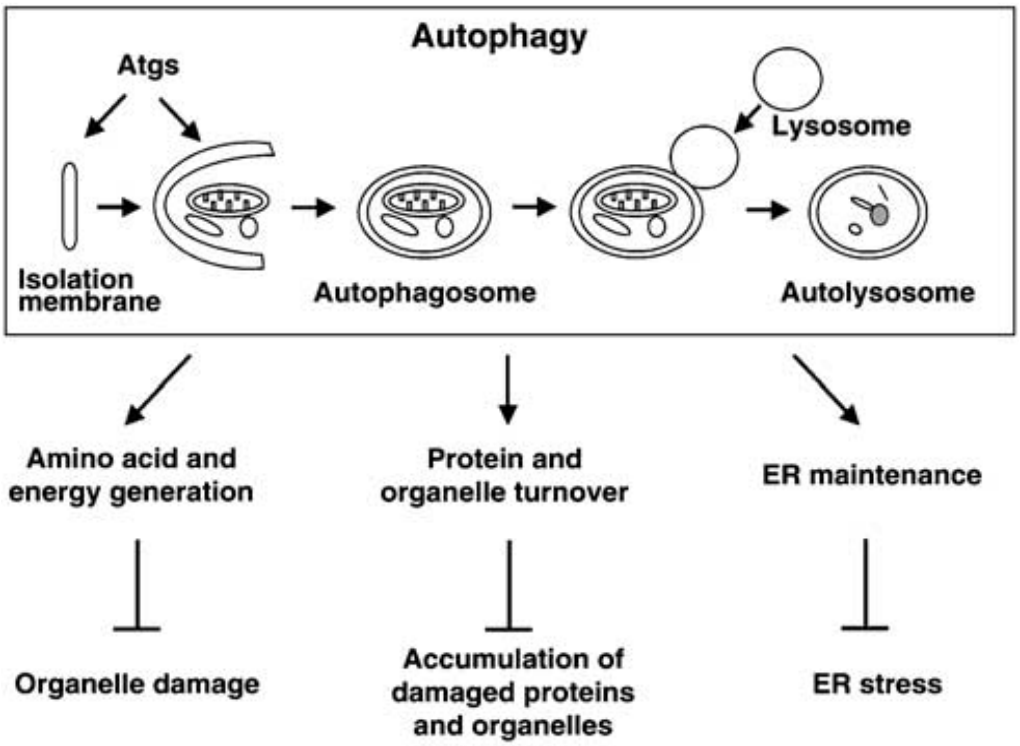


Figure 7: Autophagy protective role in cellular homeostasis maintenance [Nishida et al., 2009] .

Hypoxia induce autophagy via HIF1α; this protein acts as a sensor detecting oxygen concentration lower than 3% [Majmundar et al., 2010] and activates BH3-Only, BNIP3 and BNIP3L transcription. These proteins link Bcl2 determining its detachment from Beclin1 that can induce autophagy [Kroemer et al., 2010].

Oxidative stress also could acts as autophagy inducer [Chen et al., 2008]. High levels of cytoplasmic ROS induce activation of ATM (Ataxia-Telangectasia Mutated) that determine TSC2 phosphorylation by AMPK; active TSC2 inhibits mTORC1 with consequent autophagy activation [Alexander et al., 2010]. Alternatively ROS could induce MAPKs, like JNK1, that active autophagy [Wong et al., 2010] (Figure 8). Although it is still unclear what could be autophagy role, it is known that this pathway is induced also by DNA damage [Rodriguez-Rocha et al., 2011]. Cells constantly undergo conditions

that damage DNA determining block of replication, mutations and sometimes cell death. Harmful agents can come from external environment (like ionizing radiations, UV rays, alkalizing agents, intercalating agents) or from normal physiological pathways that generate ROS or reactive species. Some important DNA damage sensors, like FOXO3a and ATM, are also important autophagy regulators. In presence of DNA damage FOXO3a activate transcription of pro-autophagic genes like LC3 and BNIP3 and induce ATM self-phosphorilation and activation [Chiacchiera et al., 2010]. As already seen ATM itself can induce autophagy activation through TSC2 phosphorilation during oxidative stress (Figura 8).

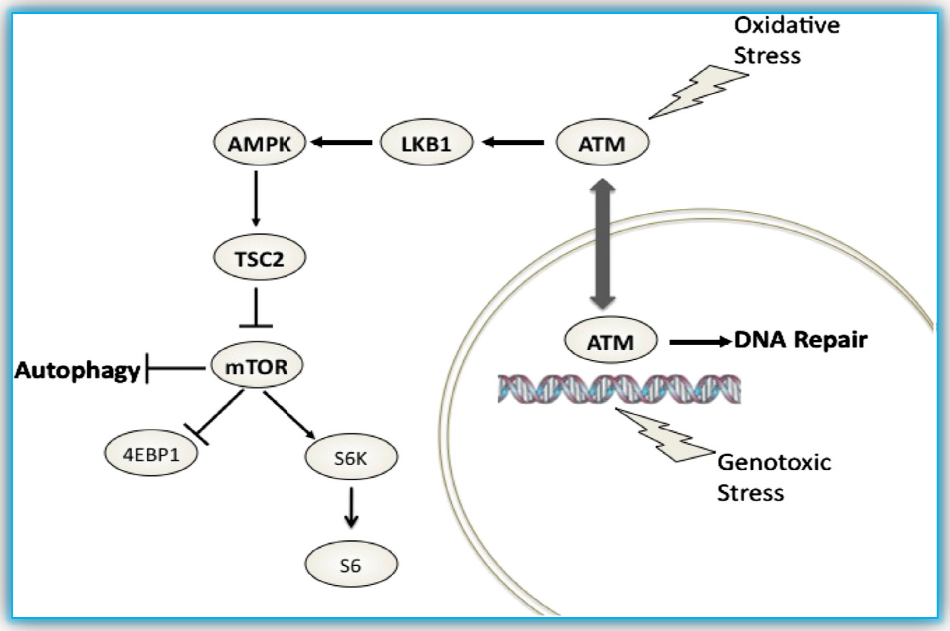


Figure 8: ATM as autophagy regulator during oxidative or genotoxic stress [Alexander, et al. 2010].

Alternatively DNA damage induce PARP1 (poly ADP-ribose polymerase1), an enzyme that uses NAD^+ and ATP to form poly-ADP chains. A genotoxic stress can activate this polymerase with a consequent imbalance of ADP/ATP ratio; this causes AMPK activation that induces autophagy [Muñoz-Gàmez et al., 2009].

p53 also can act as autophagy regulator. Under normal conditions cytoplasmatic form of p53 inhibits autophagy, but during a genotoxic stress it induces this pathway activating the transcription of pro-autophagic genes like PTEN, TSC2 e AMPK and inhibiting mTOR [Tasdemir et al., 2008].

Many studies have shown that alterations in autophagy induce an increase of DNA damage and promote tumor and neurodegenerative diseases occurrence, highlighting the autophagy role in maintenance of genomic stability [Rodriguez-Rocha et al., 2011]. Under DNA damage conditions autophagy could serve as a source of energy during cell cycle arrest and for repair mechanisms. On the other hand autophagy seems to act also degrading some components of repair machinery [Robert et al., 2011].

Although autophagy seems to act as surviving mechanism, it is emerging that sometimes the final result of autophagic pathway could be cell death, named type II cell death [Levine et al., 2005]. Apoptosis cell death is characterized by membrane vesiculation, chromatin condensation and nucleus and cytoplasm fragmentation in apoptotic bodies; autophagic cell death instead is characterized by cell shrinkage and formation of multiple autophagosomes [Tsujimoto et al., 2005]. While many studies have made a distinction between apoptosis and autophagy [Guillon-Munos et al., 2005; Reef et al., 2006], others pointed out that the two mechanisms can be interconnected [González-Polo et al., 2005; Sadasivan et al., 2006]. Many signaling pathways that regulate apoptosis also regulate autophagy (Figure 9) [Zhou et al., 2011]. Beclin1 for example induces autophagy and at the same time inhibits apoptosis blocking tBid migration to the mitochondria membrane and consequently limiting membrane permeabilization. On the other hand active caspases cut Beclin1 inhibiting autophagy [Kang et al., 2011]. Atg5 also regulates both the pathways; It is important for autophagy initiation, but its proteolitic cleavage inhibits autophagy and induces formation of a 24KDa fragment that migrates to mitochondria promoting membrane permeabilization and c cytochrome release [Yousefi et al., 2006].

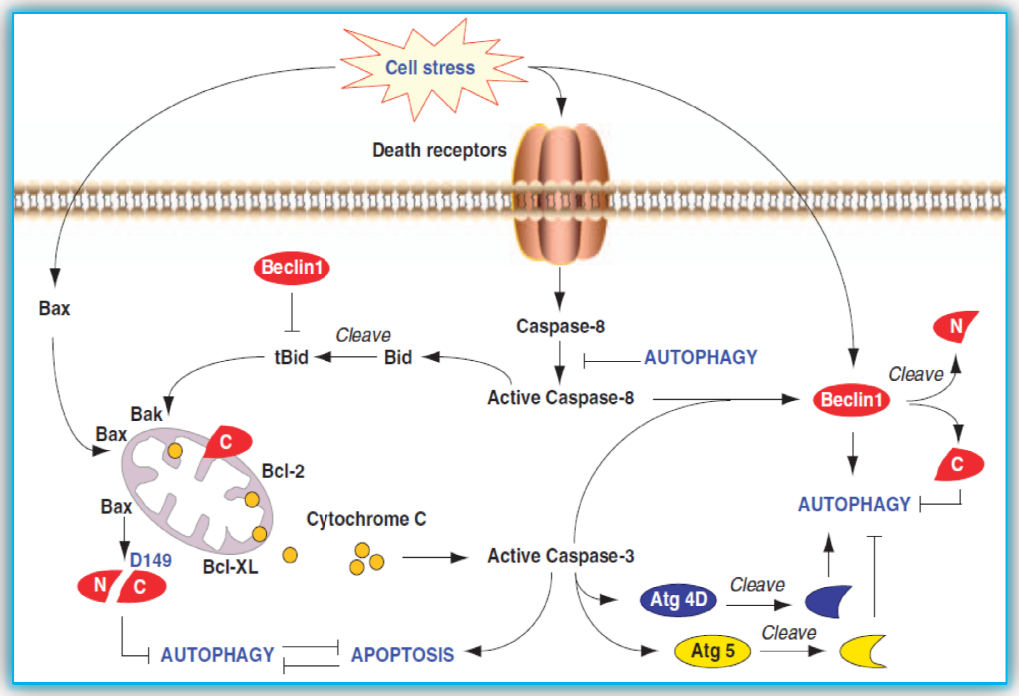


Figure 9: interaction pathways between apoptosis and autophagy [Kang, et al. 2011].

Autophagy could represent a first cellular attempt during the early damage stages for cellular homeostasis maintenance; in presence of an extensive damage apoptosis prevails inhibiting autophagy. Autophagy itself can sometimes induce cell death as a result of an excessive degradation of cellular components. This pathway can prevent accumulation of damaged or misfolded proteins and senescent organelles that can induce cellular or tissue damages. Alterations in this pathway are associated with a variety of degenerative processes characterized by movement disorders [Uttenweiler et al., 2005; Massey et al., 2006], like in Parkinson disease or in Huntington Corea [Massey et al., 2006; Xilouri et al., 2011]. Autophagy inhibition in nervous cells cause the generation of neuronal aggregates and cell death [Shibata et al., 2006].

Alterations in autophagic pathway and human diseases development

Many studies have underlined the relationship between autophagy alterations and human diseases development. Tissue specific gene knock-out experiments in transgenic mice have permitted to analyze autophagy role in organs and tissues.

The inhibition of autophagy in liver determine the accumulation of damaged mitochondria in hepatocytes and the emergence of typical symptoms of hepatic diseases [Komatsu et al., 2005]. Pathway inhibition in β cells determines an increase of cell death during high - calories diet [Jung et al., 2008].

Basal levels of autophagy seem to be important also during hematopoiesis and for muscular and nervous tissue homeostasis. It was demonstrated in 2007 the correlation between autophagy deficiency and Crohn's disease [Rioux et al., 2007]. Among diseases associated with autophagy alterations there are neurodegenerative diseases like Parkinson's disease, Huntington Corea and Alzheimer's disease, cancer and cardiac diseases.

Neurodegeneration

Proteic aggregates formation in the cytoplasm of neuronal cells is a common feature of many neurodegenerative diseases. Some examples are the Lewy's bodies, constituted of α- Sinuclein accumulation in Parkinson's disease, or the huntingtin's aggregates in Huntington's disease. The toxicity of these aggregates is due to their capacity to aggregates each other and with other proteins. Usually these aggregates can be sequestered by autophagosomes and eliminated [Mariño et al., 2011].

Alzheimer's disease (AD) also is characterized by the formation of cytoplasmic Tau proteins aggregates and the accumulation of extracellular aggregates, named amyloid plaques, constituted of β- amyloid protein. In AD patients' brain is visible an accumulation of autophagosomes and autolysosomes. An overexpression of autophagy in neuronal cells seems to reduce intracellular levels of β-amyloid and Tau proteins, and at the same time a reduction of amyloid plaques deposition, determining a neurodegeneration reduction [Mariño et al., 2011]. As in all finally differentiated cells, autophagy has a primary role in neurons. In this cells cellular waste can't be redistribute in daughter cells and a reduction of autophagy levels induce a organelles/proteins accumulation that contribute to accelerate neurodegenerative process. The

pharmacological regulation of autophagy with inhibitors or inducers could represent a new therapeutic approach of various neurodegenerative diseases.

Autophagy and cardiac tissue homeostasis

Cardiac tissue is continuously subjected to stress; a rapid turn-over of cellular components is indispensable in physiological conditions also. As for neurons autophagy is a key pathway to maintain cellular homeostasis in cardiomyocytes reducing cellular waste. Moreover cardiomyocytes are long life cells and for this motif susceptible to aging that can hit cellular structures like mitochondria and lysosomes. Numerous studies have demonstrated that alterations in autophagic pathway induce cardiac dysfunctions; Atg5 deficient mice died after birth and showed a low free aminoacid concentration and a lack of energy in cardiac cells [Kuma et al., 2004]. Atg5 knockout in mouse's hearth induced a cardiac hypertrophy, ventricular dilatation and contractile dysfunctions, associated with proteic aggregates and damaged mitochondria accumulation in cytoplasm [Nakai et al., 2007]. Removal of mitochondria is particularly important during ischemia/reperfusion injury because oxygen starvation and subsequent re- oxygenation leads to the uncoupling of oxidative phosphorylation, opening the mitochondrial permeability transition pore, and subsequent pro-apoptotic factors release and damages to the mitochondria induction [Halestrap et al., 2007]. It has been demonstrated that ischemia induces autophagy up-regulation through various factors. Hypoxia in cardiomyocytes for example activates BNIP3 that induce autophagy inhibiting Bcl2; during energy lack autophagy is induced by AMPK an indicator of ADP/ATP ratio. If during ischemia autophagy represents an adaptive response to stress condition, its upregulation during reperfusion can induce autophagic cell death (Figure 10) [Ravikumar et al., 2010].

It remains still unclear the real role of autophagy in the heart, its protective or adverse effects on cells seem depend on the different pathways that participate in (figure 10). Better understanding the autophagic pathway could allow the development of new

drugs that can improve the cellular energetic metabolism in cardiac cells under stress conditions [Loos et al., 2011].

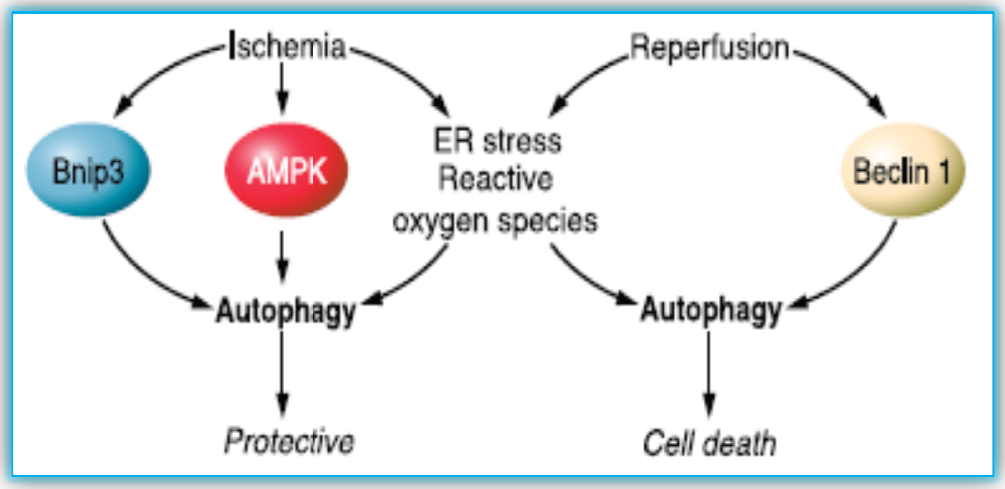


Figure 10: Different autophagy roles during ischemia and reperfusion [Ravikumar, et al. 2010].

Cancer and autophagy: suppressor or promoter of tumor growth?

The autophagy role in cancer is complex and highly discussed. It is known that autophagy is a protective system able to prevent accumulation of cellular waste and damaged organelles that could promote the conversion of healthy cells in cancer cells, acting as cancer suppressor. On the other hand the capability to allow surviving in cancer cell under stress conditions, like hypoxia and starvation, gives to autophagy an important role in tumor growth and progression. In this contest the autophagy role can be double and can change according to the kind of tumor, cancer genetic features and microenvironment. Between the autophagic genes with suppressor features there are Beclin1, Atg5, Atg4, PTEN, TSC1-2. Atg4 knockout mice treated with carcinogens had an higher probability to develop cancer than wild type mice [Mariño et al., 2007]. Moreover mutations in pro-autophagic genes, like UVRAG and Beclin1, have been detected in various cancers. A monoallelic mutation of Beclin1 has been found in 40-70% of breast, ovarian and prostatic sporadic tumors [Liang et al., 1999]. The elimination of mitochondria, named mitophagy, seems to have a key role in tumor

suppression; this pathway allows to control ROS production, preventing DNA damage and metabolic alterations under stress conditions [Karantza-Wadsworth et al., 2007; Mathew et al., 2007]. In presence of autophagic defects cancer seems characterized by p62 accumulation that induces ROS production and an increase of DNA damage [Mathew et al., 2009]. On the other hand high levels of autophagy have been registered in tumor regions more subjected to metabolic stress, acting as tumor promoter [Roy et al., 2010]. Furthermore the PI3K/Akt/mTOR pathway, one of the higher autophagy regulators, is often altered in many tumors; conversely no homozygosis mutations in Beclin1 have been found in cancer cells. Beclin1 deletion in cancer cells decrease tumor growth and increase apoptotic cell death [Karantza-Wadsworth et al., 2007]. Pharmacological autophagy inhibition in colorectal cancer induces cell death from nutrient lack, hypothesizing that this pathway provide energy for cellular growth, proliferation and survival [Sato et al., 2007]. Degenhardt demonstrated that autophagy is highly induced in regions exposed to hypoxic stress, promoting surviving. This finding is really important because hypoxic condition due to low vascularization is associated with severe phenotype, with high predisposition to metastasis. Under hypoxic condition HIF1α induces transcription of genes involved in angiogenesis and autophagy. In this contest autophagy appears to promote tumor proliferation [Degenhardt et al., 2006].

In order to better understand the dual role of autophagy we can take as an example the stromal tumor. Cancer cells induce oxidative stress in adjacent stromal fibroblasts that activate autophagy determining high degradation of mitochondria. Mitochondrial pool reduction drives fibroblasts to use aerobic glycolysis with consequent lactate and glycolytic intermediate release in extracellular microenvironment. These components can serve as fuel for the adjacent tumor cells. Moreover the increase of ROS in extracellular environmental due to oxidative stress in fibroblasts induces casual mutations in adjacent epithelial cells promoting genomic instability and tumor evolution. Tumor growth seems to be promoted by autophagy and stromal fibrobalsts that provide energy. On the other hand direct induction of autophagy in epithelial tumor cells trough HIF1α induces a drastic reduction of tumor growth. Autophagy

activation can promote or suppress tumor progression depending on the cell type in which it occurs [Martinez-Outschoorn et al., 2010].

Anticancer drugs and autophagy

Autophagy induced through chemotherapy treatments can have a positive or negative effect on cell viability, according to the kind of tumor treated and the drug utilized. Sometimes autophagy can induce treatment resistance as, for example, in esophageal cancer cells treated with 5-fluoracil [O'Donovan et al., 2011], or in gastrointestinal stromal tumor cells (GIST) treated with Imatinib mesylate [Gupta et al., 2010] and in pulmonary cancer cells treated with Cetuximab [Li et al., 2010]. In other cases autophagy can help drug action inducing cancer cells death as for example Ewing's sarcoma cells treated with 2-Metoxyestradyol [Lorin et al 2009], or in glioma cells treated with Temozolomide (Figure 11) [Voss et al., 2010].

Autophagy activation has been observed after treatment both with traditional chemotherapy like Doxorubicin or Daunorubicin than with new generation drugs like targeted therapies [Wu et al., 2011]

Agents	*Class of agent*	*Cancer cell types*	*Role of autophagy*
5-Fluorouracil	Antimetabolite	Esophageal cancer cells	Pro-survival
ABT737	BH3 mimetic	Cervical cancer cells	Pro-survival
Arginine deiminase	Enzyme	Prostate cancer cells	Pro-survival
Celecoxib	COX-2 inhibitor	Colon cancer cells	Pro-survival
Cetuximab	EGFR-blocking antibody	Lung cancer cells	Pro-survival
Imatinib mesylate	Multiple kinase inhibitor	GIST cells	Pro-survival
INNO-406	Bcr-Abl TK inhibitor	CML cells	Pro-survival
MG-132	Proteasome inhibitor	Gastric cancer cells	Pro-survival
Perifosine	Alkylphospholipid	Lung cancer cells	Pro-survival
PI-103	Dual PI3K/mTOR inhibitor	Glioma cells	Pro-survival
Tamoxifen	Estrogen receptor antagonist	Breast cancer cells	Pro-survival
TRAIL	Death receptor ligand	Colon cancer cells	Pro-survival
(−)-gossypol	BH3 mimetic	Prostate cancer cells	Pro-death
$[pIC]^{PEI}$	dsRNA mimetic	Melanoma cells	Pro-death
2-Methoxyestradiol	Angiogenesis inhibitor	Ewing sarcoma cells	Pro-death
Ad.mda-7	Recombinant adenovirus	Glioma cells	Pro-death
Ionizing radiation	Radiation	Lung cancer cells	Pro-death
Sodium selenite	Inorganic compound	Glioma cells	Pro-death
STF-62247	Novel compound	Renal cancer cells	Pro-death
Temozolomide	Alkylating agent	Glioma cells	Pro-death
Voacamine	Phytochemical	Osteosarcoma cells	Pro-death
Shikonin	Phytochemical	Breast cancer cells	Bystander

Figure 11: Autophagy activation after chemotherapy treatment [Wu, et al. 2011].

Aim of Study

This thesis is the result of a research program made in collaboration between the Life Sciences Department and the Clinical and Experimental Medicine Department of the University of Parma in a project supervised by Professor F. Quaini in collaboration with Professor A. Buschini. Principal aim of this study was to evaluate Imatinib mesylate (IM) controversial effects on cellular and on organ- systemic level. To reach this goal I peformed *in vitro* assays like: cytotoxicity assay MTS, single cell gel electrophoresis (SCGE or Comet assay) to determine genotoxicity, the assessment of autophagy through a fluorescent assay and the evaluation of gene expression modification through real time PCR (RT-PCR). The adverse effects of IM could be due to inhibition of aspecific tyrosine kinases. It is known that IM can link, even if with minor affinity, other proteins like c-kit that is fundamental for cardiac stem cells (CSC) and cardiac resident stem cells (CRSC) differentiation in cardiomyocytes and for CSC migration from bone marrow to hearth. Alterations in stem cell migration and maturation could prejudice their capability to replace damaged tissue and repair lesions, increasing the possibility of cardiac diseases development. To investigate the possibility that this drug could compromise integrity of stem cells, the activity of IM has been evaluated on two cardiac stem cells lines (rat cardiac progenitor cells- CPCs and human cardiac mesenchimal stem cells- C-MSCs). Furthermore I have compared IM effects on these stem cell lines with that induced on IM target cell line K562 (chronic myelogenous leukemia cell line).

Since oxidative stress seems to be involved in IM toxicity I've assessed through a fluorescent assay the formation of reactive oxygen species, and their effects on cell physiology evaluating autophagic pathway activation (through a transfection assay) and the variations in gene expression after IM treatment by RT-PCR.

To evaluate the role of autophagy induced by IM treatment I have analyzed the effects of co- treatments with autophagic regulators. Autophagy is a complex pathway that could be regulated by different stimuli. Numerous are the molecules that can interact with proteins involved in this process, inducing its activation or inhibition. Among autophagy inducers rapamycin and lithium salts (LiCl e $LiHCO_3$) are the most used for *in vitro* studies. Rapamycin is a natural antibiotic isolated from *Streptomyces hygroscopicus* that inhibits mTOR kinase activity, allowing the formation of

ULK1/FIP200/Atg13 autophagy start complex [Ravikumar B, et al. 2010]. Lithium salts are used for treatment of neurological disorders. Lithium action is mTOR- dependent and interferes in phosphoinositol pathway; it inhibits two enzymes, IPPase and IMPase, involved in IP_2 conversion into free inositol that induces a decrease of intracellular levels of IP3 and the inhibition of IP_3 receptor (IP3R), driving to autophagy activation. Moreover lithium induces decrease of inositol blocking inositol transporter (SMIT) [Sarkar S, et al. 2006] (Fig. 12).

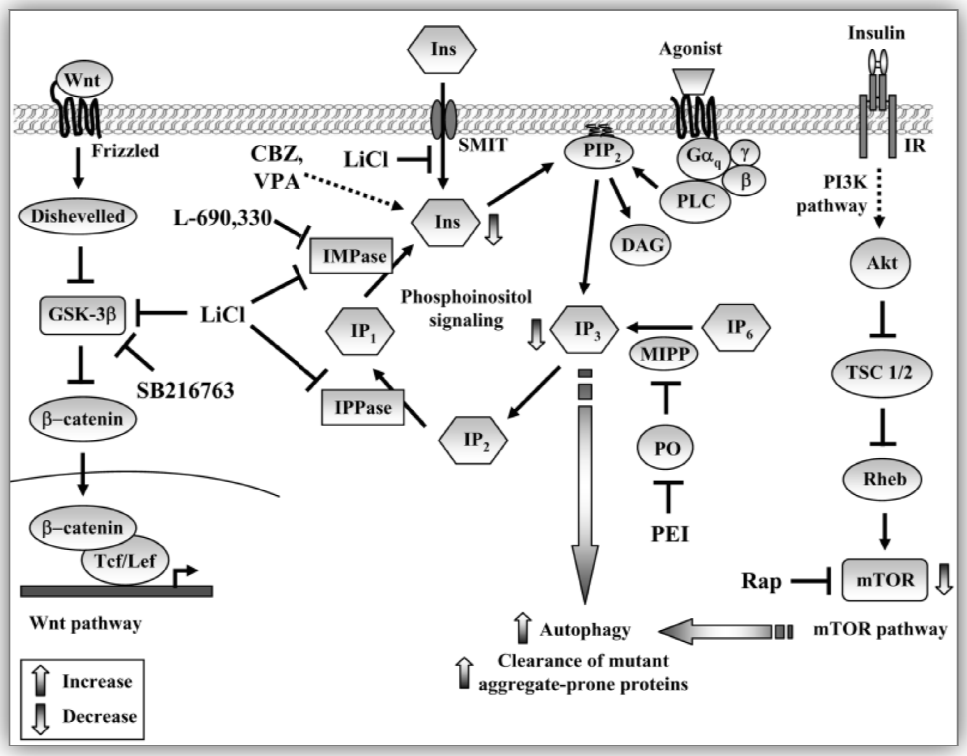


Figure 12: Mechanism of action of autophagy inducers that are involved in phospoinositol pathway [Sarkar S, et al. 2006].

Autophagy inhibitors can also act in different phases of this pathway; they can block autophagy in initial phase or during the degradation of autophagosomal content (Fig. 13). Among the molecules that act upstream in the autophagic pathway there are 3-Metiladenine (3-MA) and Wortmannin. They link Vps34 subunit of class III PI3K and interfere with the autophagosomal nucleation [Kroemer G, et al. 2005]. Between the downstream inhibitors we can find cloroquine (CQ) and hydroxychloroquine (HCQ), two

antimalarial drugs. They belong to the 4- aminoquinoline family and differ each other only for an hydroxyl group on the lateral chain of HCQ. These molecules are able to inhibit the acidification of lysosomal lumen; they determine an increase of lysosomal pH from 4.0 to 6.0, blocking the activity of lysosomal hydrolases and inducing a cytoplasmic accumulation of no-degraded autophagosomes.

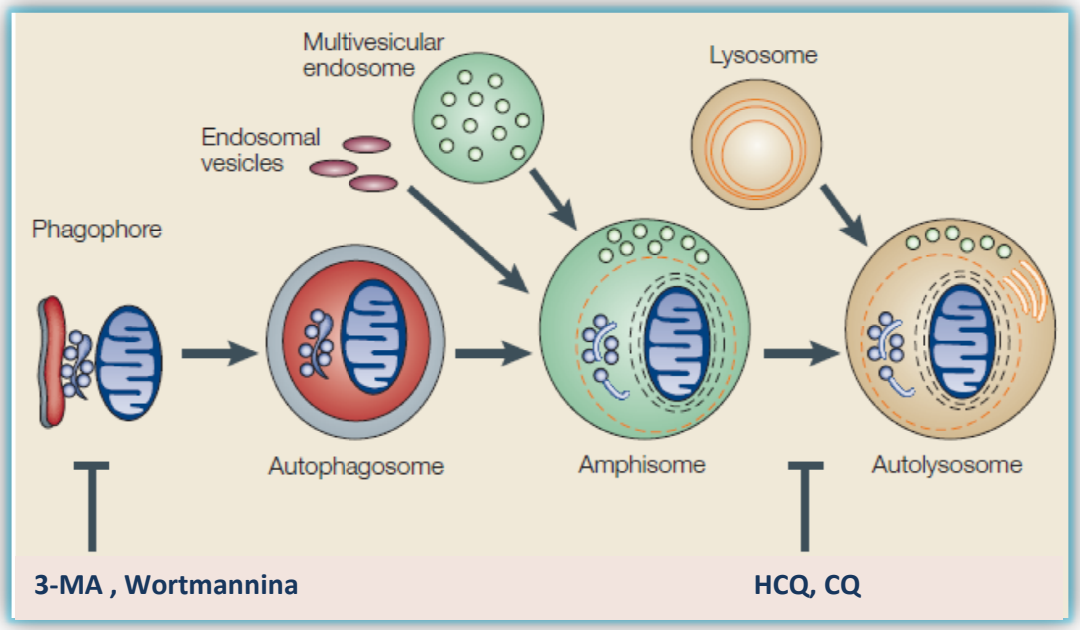


Figure 13: Inhibitors of different steps of the autophagic pathway.

Since autophagy can represent a defence mechanism during chemotherapic treatment, their role of autophagic inhibitors made CQ and HCQ ideal candidates for the development of new antitumor therapies. Numerous phase I and II studies are evaluating the combined administration of CQ or HCQ and chemotherapic drugs [Yang Z,et al. 2011] (tab. 1).

CQ/HCQ and lithium chloride are drugs used for many years in treatment of human diseases, so their absorption kinetics and collateral effects are known. This aspect led me to use during my study lithium chloride as autophagic inducer and chloroquine as inhibitor.

Tumor type	Development status	Therapeutic combination
Colorectal cancer	*In vitro, in vivo*	CQ + bortezomib
		CQ + vorinostat
	Phase II	HCQ + XELOX + bevacizumab
Gastrointestinal stromal tumor	*In vitro, in vivo*	CQ + Imatinib
Prostate cancer	*In vitro, in vivo*	CQ + Src Kinase inhibitors
Vulvar cancer	*In vitro*	CQ + cetuximab
Chronic myelogenous leukemia	*In vitro*	CQ + vorinostat
	PhaseII	HCQ + Imatinib
Lymphoma	*in vivo*	CQ + cyclophosphamide
Pancreatic cancer	Phase II	HCQ only
	Phase I/II	HCQ + gemcitabine
Prostate cancer	Phase II	HCQ + docetaxel
Lung cancer	Phase II	HCQ + erlotinib
Glioblastoma multiforme	Phase I/II	HCQ + temozolomide + radiation
Multiple myelome	Phase I/II	HCQ + bortezomib
Renal cell carcinoma	Phase I	HCQ only
Breast cancer	Phase II	HCQ only
Chronic lymphocytic leukemia	Phase II	HCQ only
Advanced solid tumor	Phase I	HCQ + sirolimus or vorinostat
	Phase I	HCQ + temsirolimus
	Phase I	HCQ + sunitinib
	Phase I	HCQ + temozolomide

Abbreviations: CQ, chloroquine; HCQ hydroxychloroquine

Table 1: Clinical and pre-clinical phase studies that use CQ and HCQ as autophagy inhibitors during tumor treatment [Yang Z,et al. 2011].

In the last period of my work I have compared the in vitro effects induced on target and non- target cell lines by IM treatment with those induced by a drug with known cardiotoxic effects. For this reason I have used doxorubicin, an old generation chemotherapy. Doxorubicin effects have been evaluated on a human breast adenocarcinoma cell line (MCF7), target cell line of doxorubicin, and on human cardiac mesenchimal stem cells (C-MSCs).

Materials and Methods

Cell culture

In vitro effects of IM were investigated on a human chronic myeloid leukemia cell line, K562, on a rat cardiac progenitor cell line (CPCs), isolated from untreated rat hearth as described by Beltrami and collaborators trough Langhendorff perfusion apparatus [Leri et al, 1999], and on a human cardiac mesenchimal stem cell line (C-MSCs), obtained from human bone marrow. Doxorubicin activity was evaluated using a human breast adenocarcinoma cell line (MCF7) as target cell line and the human cardiac mesenchimal stem cell line, C-MSCs. Prior to the experiments, cells were thawed and grown in tissue culture flasks as monolayer in IMDM (Iscove's Modified Dulbecco's Medium), supplemented with 1% L- glutamine (2mM), 1% penicillin (5000U/ml)/streptomycin (5000 μg/ml), 1% ITS (insulin, transferrine sodium selenite) and 10% fetal bovine serum (FBS), or RPMI (Roswell Park Memorial Institute) or DMEM (Dulbecco's Modified Eagle Medium), supplemented with 1% L- glutamine (2mM), 1% penicillin (5000U/ml)/streptomycin (5000 μg/ml) and 10% fetal bovine serum (FBS) at 37°C in a humidified CO_2 (5%) incubator. The cardiac cell lines were trypsinized with trypsin/EDTA for a maximum of 5 min and seeded with a subcoltivation ratio of 1:3-1:8. Determination of cell numbers and viabilities was performed with the trypan blue exclusion test.

Antiproliferative activity

The antiproliferative activity was evaluated by a colorimetric assay that allows us to determine the number of viable cells in proliferation. This assay (CellTiter96R AQueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. In order to determine cell viability, in the exponential phase of growth, cells were seeded at 5 x 10^4/ml in 96-well plates, in IMDM or RPMI or DMEM. After seeding (24h), cells were treated, in quadruplicate, with 5-50 μM IM or 5 μM IM/3 μM chloroquine or 5 μM IM/10mM Lithium chloride and incubated for 6-12-24-48h. The cytotoxicity assay was performed by adding a small amount of the CellTiter96R AQueous One Solution Cell Proliferation Assay directly to culture wells, incubating for 4h and then recording the absorbance at 450 nm with a 96-well plate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland).

The same experimental protocol was applied on MCF7 cell line and C-MSCs cell line after treatment with 0.1 ÷50 µM Doxorubicin.

Comet assay

The Comet Assay or Single-Cell Gel Electrophoresis (SCGE) assay is an useful approach for assessing DNA damage evaluating the presence, after electrophoresis, of fragmented DNA outside the core of the nucleus. Relaxed and/or broken DNA fragments, negatively charged, migrate towards the anode and the resulting image has the appearance of a comet (fig. 14). The amount of DNA migrated from the "head" of the comet indicates the extent of the DNA damage. This quantity is dependent on the size of DNA fragments and the number of broken ends in the strands.

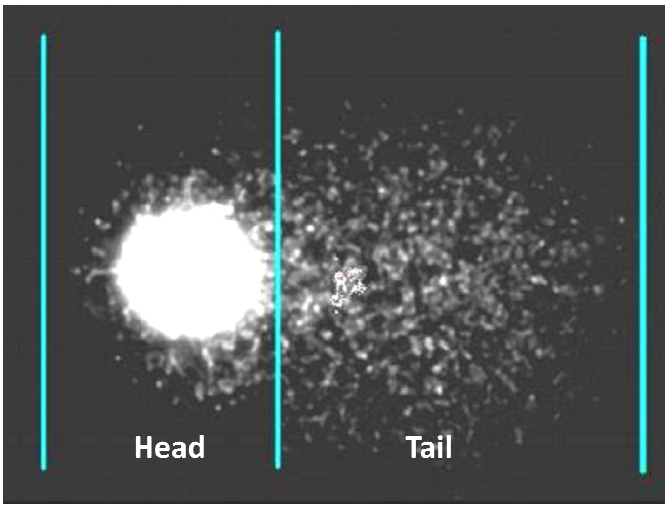


Figure 14: An example of DNA molecule visualized through Comet Assay.

The Comet Assay is usually performed at pH> 13 to detect, in addition to single and double strand breaks, "alkali-labile" sites (adducts, apurinic and apirimidinic sites, oxidation of the nitrogenous bases, etc.). DNA damage was evaluated on different cell lines after 3-6-12-24h treatment with 5-50 µM IM and after co-treatment 5 µM IM/3 µM chloroquine or 5 µM IM/10mM Lithium chloride. Cells were seeded at 5 x 10^3/ml in 6-well plates in IMDM or DMEM or RPMI supplemented as described above. After seeding (24h), cells were treated with drugs and incubated at 37°C for the different times of treatment. After treatment a single cell suspension was obtained and resuspended in 1ml IMDM or DMEM or RPMI; the suspensions were centrifuged (1 min, 800g) to recover the cells and the cell pellets were resuspended in 90 µl Low Melting

Agarose 0.7% (LMA), transferred onto degreased microscope slides previously dipped in 1% Normal Melting Agarose (NMA) for the first layer. The agarose was allowed to set for 15 min at 4°C before the addition of a final layer of LMA. Cell lysis was carried out at 4°C overnight by exposing the cells to a buffer containing 2.5 M NaCl, 100 mM Na2EDTA, 8 mM Tris-HCl, 1% Triton X-100 and 10% DMSO, pH 10. The electrophoretic migration was performed in an alkaline buffer (1 mM Na2EDTA, 300 mM NaOH, 0°C) at pH>13 (DNA unwinding: 20 min; electrophoresis: 20 min, 0.78Vcm-1, 300mA). DNA was stained with 75 µl ethidium bromide (10 µg/ml) before the examination at 400x magnification under a Leica DMLS fluorescence microscope (excitation filter BP 515-560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay III – Perceptive Instruments Ltd, UK). Total % fluorescence in tail (TI, tail intensity) provided representative data on genotoxic effects. For each sample, coded and evaluated blind, 100 cells were analyzed and the mean value of TI was calculated.
The same experimental protocol was applied on MCF7 cell line and C-MSCs cell line after treatment with 1-10-100 µM Doxorubicin.

Measurement of reactive oxygen species (ROS) production

ROS production was measured by fluorescence assay, using 2',7'-dichlorfluorescein-diacetate (DCFH-DA - Sigma). DCFH-DA is a nonfluorescent compound that can pass through cell membranes. Once it is in the cytoplasm, cellular esterases remove the acetates to produce 2', 7'-dichlorodihydrofluorescein (DCFH), which because of its polarity is not cell permeable. DCFH is easily oxidized to 2', 7'-dichlorofluorescein (DCF), an highly fluorescent compound [E. J. Bland et al., 2001]. In a typical experiment cells seeded at 2,5 x 10^5/ml in 6-well plates in IMDM or DMEM or RPMI supplemented as described above. After seeding (24h), cells were washed with PBS and pre-incubated for 30 min (37 °C) with DCFH-DA (10 µM) which was added from a stock solution in DMSO and diluted in PBS. The quantity of DMSO never exceeded 0.1%, and was also added to the blank. Cells were washed with PBS to remove extracellular DCFH-DA and treated with 1-5 µM IM and incubated at 37°C for 3h. Then medium was removed, and a solution of Tris–HCl-TritonX and a cell dissociation solution (Sigma) was added for 10 min. Cell lysates were scraped from the dishes and the extracts were centrifuged. The supernatant was collected, and the fluorescence was immediately read with a fluorescence spectrophotometer (Cary Eclipse, Varian, Palo Alto, CA, USA) looking at the fluorescence peak between 510 and 550 nm (excitation = 480 nm).

Autophagy assessment

Autophagic pathway activation has been evaluated through a transfection protocol using a plasmid encoding the autophagosome marker LC3 fused with the fluorescent protein EGFP (pEGFP-LC3 human, Addgene). Transfection is performed using Lipofectamine™ reagent (Invitrogen®), consisting of lipidic subunits that can form liposomes in an aqueous environment that entraps plasmid and drives it inside cells. Transfection allows cells a constitutive LC3-EGFP fusion protein synthesis. Its nuclear and cytoplasmic distribution confers an uniform fluorescence to the cell; autophagy activation induces the formation of LC3-EGFP aggregates that determine the fluorescent signal amplification, conferring a punctuated morphology with green spot in cytoplasm exclusively (fig. 15).

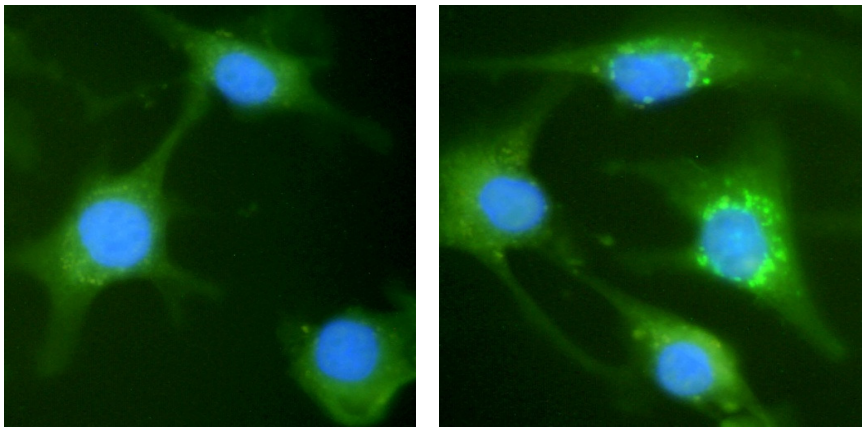


Figure 15: left panel: example of transfected cells; right panel: example of transfected and autophagic cells.

For the assay execution $2.5*10^3$ cells were seeded in 1ml of growth medium in cell chamber slides and incubated at 37°C. After 24h cells were trasfected with the plasmid described above according to the following protocol: 4 µg of plasmid were diluted in 200 µl of Opti-MEM (Invitrogen®), at the same time as 5 µl of Lipofectamine™ are gently mixed with 200 µl Opti-MEM. After a first incubation of 5–10 min, the diluted plasmid solution and diluted Lipofectamine™ solution were gently mixed and incubated for another 20 min to promote the formation of Lipofectamine™:plasmid complexes; 30 µl of solution containing the Lipofectamine™:plasmid complexes were added to each well and cells incubated at 37°C in 5% CO2 atmosphere for 24 h. At the end of transfection cells were treated with 5-50 µM IM or with 0.5-1 µM Doxorubicin for 6-12-24 h. After treatment growth medium was removed, cells were washed twice with PBS and fixed in 400 µl of fixative solution for 30 min at RT; fixative solution was removed, cells were

washed three times with PBS, chamber slides were disassembled and cover slips were mounted onto slides using Vectashield mounting medium with DAPI. For the visualization of LC3-EGFP aggregates, cells were examined through a fluorescent microscopy using an oil immersion objective (100× magnification). For each sample 200 transfected cells were analyzed; in autophagy negative cells LC3-EGFP exhibits a diffuse cytoplasmic signal; when autophagy is induced, LC3-EGFP chimeric proteins aggregate in autophagic vacuoles, leading to a punctuate cytoplasmic staining [E. Tasdemir et al., 2008].

Quantitative real time- PCR (RT- PCR)

A quantitative real-time PCR was performed to evaluate the expression of genes involved in DNA damage checkpoints (ATM and ATR) and in autophagy (LC3, Beclin1, mTOR, Bcl2). The use of an housekeeping gene as Actina B (ActB) allows relative quantification of gene expression. This method consists of three phases: RNA extraction, c-DNA synthesis and RT-PCR.

RNA extraction:
Total RNA extraction was performed through RNeasy Mini Kit (QIAGEN); 1-2*10^6 cells were seeded in Petri plates in 12 ml of growth medium and incubated 24h at 37°C ; then cells were treated with 1-5 µM IM for 3-12h or with Doxorubicin 0.5-1 µM. After treatment a single cell suspension was obtained; cells were disrupted in Buffer RLT and homogenized by a 20 gauge syringe needle. Then Ethanol was added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants were washed away by washes and centrifugation in a microcentrifuge. Then RNA was eluted in nuclease-free water. Eluted RNA concentration was evaluated through spectrophotometer Eppendorf Biophotometer.

c-DNA synthesis:
2.5 µg RNA were used for c-DNA synthesis. A Genomic Elimination Mixture (GE) was prepared in a sterile PCR tube as follows:

Total RNA	2.5-5 µg
GE (5x gDNA Elimination Buffer)	2.0 µl
H2O	To 10.0 µl
Total volume	10.0 µl

GE was mixed, centrifuged and incubated at 42°C for 5' and then transferred in ice for 1'. A retrotranscriptase cocktail (RT-cocktail) was prepared as follows:

RT Cocktail	1 reaction
BC3 (5x RT Buffer 3)	4 µl
P2 (Primer & External Control Mix)	1 µl
RE3 (RT Enxzyme Mix 3)	2 µl
H_2O	3 µl
Total volume	10 µl

10 µl of RT-cocktail were added to every 10 µl of GE and incubated at 42°C for 15'; reaction was then inactivated at 95°C for 5'. 91 µl of H_2O were added to every 20 µl of reaction and sample were stored at -20°C till next step.

Real time PCR:
Every PCR reaction was performed in triplicate preparing a reaction mix as follows:

RT^2 SYBR Green qPCR Master Mix	12.5 µl
H2O	10.5 µl
gene-specific 10 µM PCR primer pair stock	1.0 µl
RT^2 First-Strand cDNA (template)	1.0 µl
Total volume	25.5 µl

PCR reaction was performed through a thermocycler using the following program:

Cycles	Time	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	30 seconds	55°C
	30 seconds	72°C

Statistical analysis

Data reported in this thesis have been evaluated through SPSS 21.0 (Inc., Chicago, Illinois, U.S.A.). A univariate variance analysis (ANOVA) has been performed, followed by Bonferroni multiple comparison post-hoc test.

Results

Evaluation of *in vitro* IM effects

As first step of this work I have evaluated the cytotoxicity induced by Imatinib mesylate (IM) treatment in K562 cell line and in cardiac cell lines, CPCS and C-MSCs. I have chosen to use two doses of IM: 5 µM, comparable with plasmatic drug dose in patients after *in vivo* treatment, and 50 µM to induce an acute response to mimic drug accumulation due to a prolonged treatment.

Cytotoxicity

IM treatment induces cytotoxicity, evaluated through MTS assay, in all the cell lines analyzed in a dose and time dependent manner. In particular an high cytotoxicity is detectable in K562 cell line with both tested doses: the higher dose (50µM) induces a significative cytotoxicity at all time points analyzed ($p<0.001$), meanwhile 5 µM IM induces a significant reduction of cell number in culture only after 24h treatment ($p<0.001$) (Fig. 16). High cytotoxicity is detectable with IM 50 µM ($p<0.001$) (fig. 17a-b) in the two cardiac cell lines, the lower dose induces a significant reduction of cell proliferation only in CPCs cell line after 24h treatment ($p<0.01$) (fig. 17a).

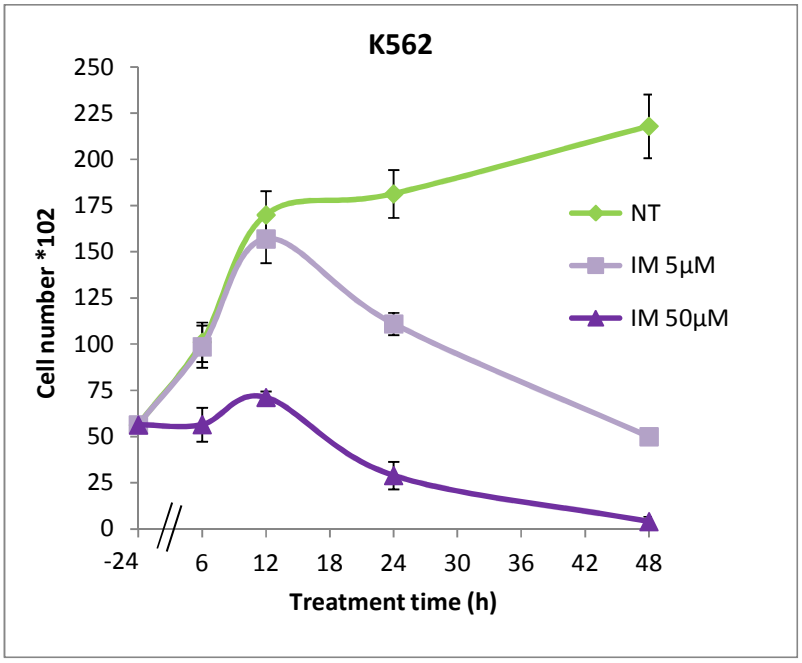


Figure 16 - cytotoxicity (MTS assay) induced on K562 cell line after 24 and 48 h treatment with Imatinib mesylate (5-50 µM) represented in terms of cell number in culture (cell number $*10^2$); NT= untreated

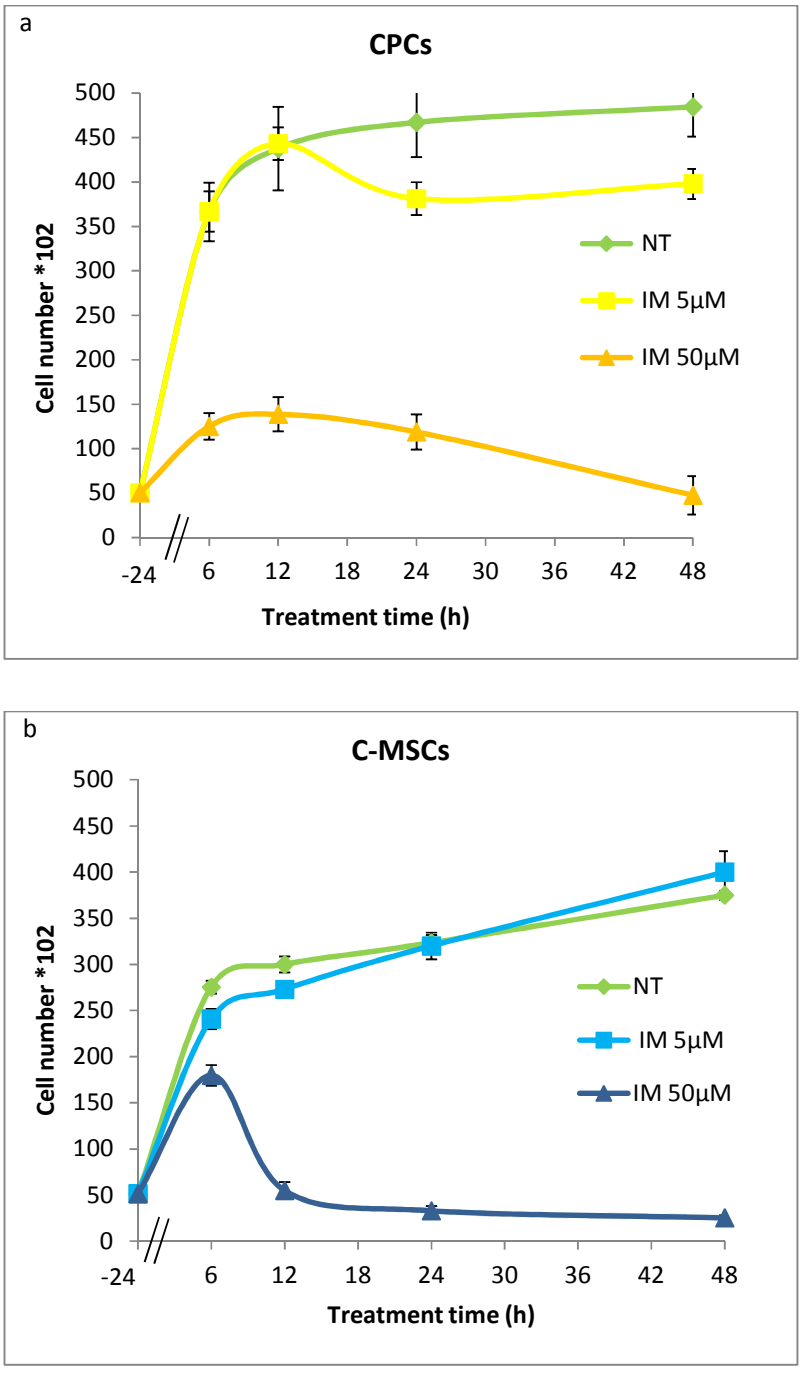


Figure 17 - cytotoxicity (MTS assay) induced on CPCs (a) and C-MSCs (b) cell lines after 24 and 48 h treatment with Imatinib mesylate (5-50 µM) represented in terms of cell number in culture (cell number $*10^2$); NT= untreated

The high toxicity found in non- target cell lines led me to investigate the mechanism involved in treatment response. I have considered different cellular endpoints related to cellular stress: genotoxicity, reactive oxygen species (ROS) production and induction of autophagy.

Genotoxicity

The induction of DNA damage has been evaluated through the Alkaline version of Comet Assay; this version of Comet Assay is usually performed at pH> 13 to detect single and double strand breaks, and "alkali-labile" sites (adducts, apurinic and apirimidinic sites, oxidation of the nitrogenous bases, etc.). Comet Assay was performed on the different cell lines after treatment with 5-50 µM IM for 3-6-12-24h. An increment of DNA migration, related to DNA damage induction, is detectable after IM treatment in the cell lines tested.

K562: IM treatment induces a significant increase of DNA migration in all tested time ($p<0.001$) (fig. 18); the high genotoxicity found after 3h of IM treatment seems to reduce over time. This behavior could be explained as the capability of cell to recover DNA damage or as a selection of a resistant cell population; this thesis could be confirmed by data of cytotoxicity reported above (fig. 16)

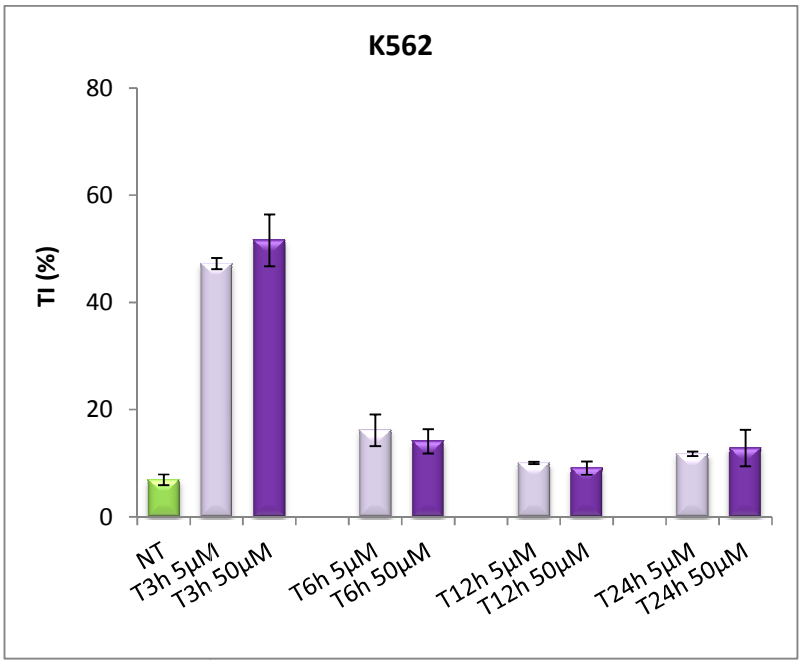


Figure 18: Genotoxicity (Comet assay) in K562 cell line after 3, 6, 12, 24 h treatment with Imatinib mesylate (5-50 µM) evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated; T= treated.

CPCs: 50 µM IM induces a relevant increase of genotoxicity with time ($p<0.001$)(fig. 19a); no significant increase in DNA migration is detectable after 5 µM IM treatment
C-MSCs: 50 µM IM determines an high rate of DNA damage ($p<0.001$) that culminates with an acute toxicity after 24 h treatment (fig. 19b), as confirmed by MTS assay (fig. 17b); 5 µM IM induces a significative migration only after 12 hours of treatment.

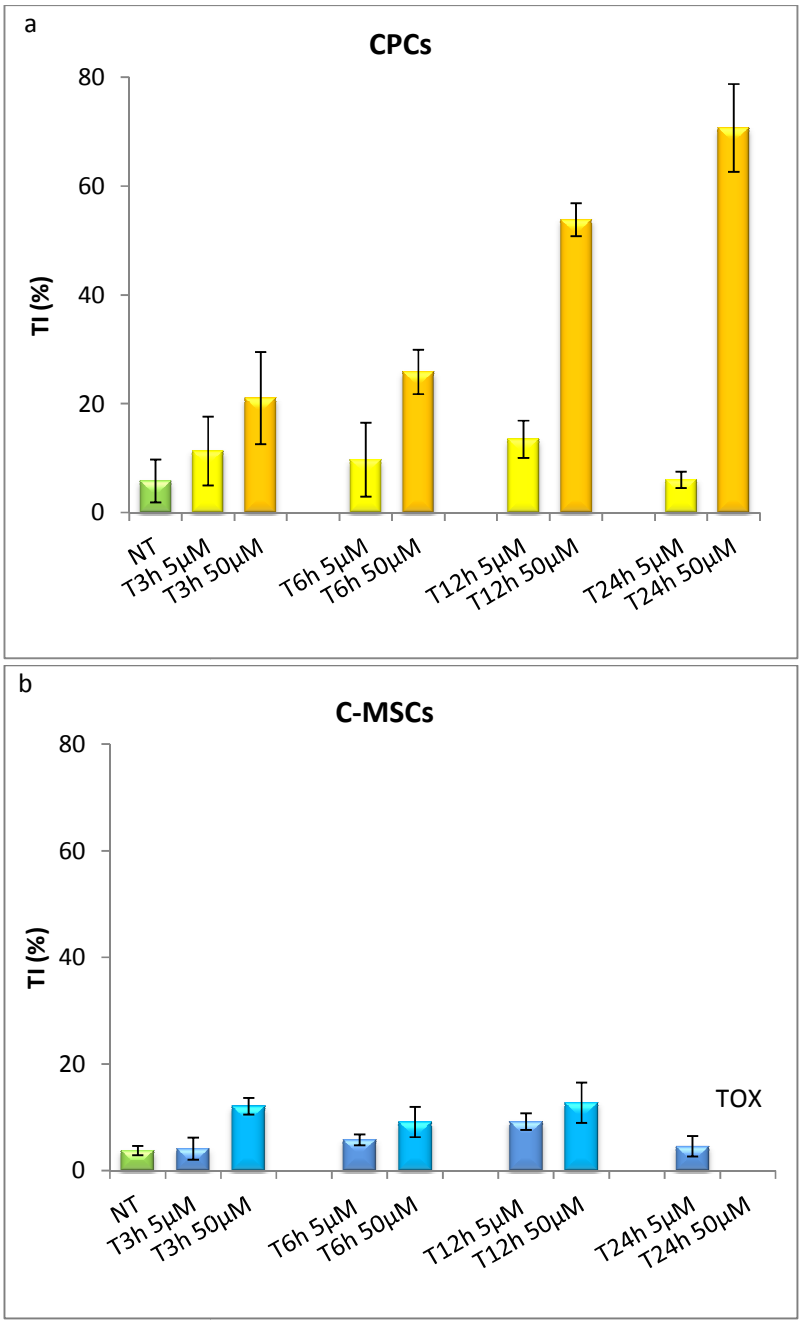


Figure 19: Genotoxicity (Comet assay) in CPCs (a) and C-MSCs (b) cell lines after 3, 6, 12, 24 h treatment with Imatinib mesylate (5-50 µM) evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated; T= treated.

Reactive Oxigen Species (ROS) detection

The early induction of DNA damage in cells treated with xenobiotics could be related to ROS production. What I detect through Comet Assay, a rapid increment of DNA migration especially in K562, drives me to analyze the ability of IM to induce oxidative stress. A fluorescent assay, after 1-5 µM IM treatment, was performed on K562 and CPCs cell lines; an increase of reactive oxygen species was registered in both cell lines (tab. 2). The use of Menadione (25 µM) as a positive control allowed me to compare the oxidative stress induced by IM with the one induced by a known oxidant. Interestingly CPCs seem to have an higher resistance against oxidative stress as shown by the different impact of Menadione on this cell line than on K562 cell line.

	ROS increment (%)	
Cell line	**IM 5µM**	**Menadione**
K562	300,15	1224,15
CPCs	20,70	47,04

Table 2: ROS induction in K562 and CPCs cell lines after 3h IM (5 µM) and Menadione (25µM) treatment, expressed in terms of ROS increment percentage.

Autophagy

Autophagy was evaluated as mechanism of cellular stress response. A transfection assay was conducted using a plasmid encoding the autophagosome marker LC3 fused with the fluorescent protein EGFP (Fig. 20).

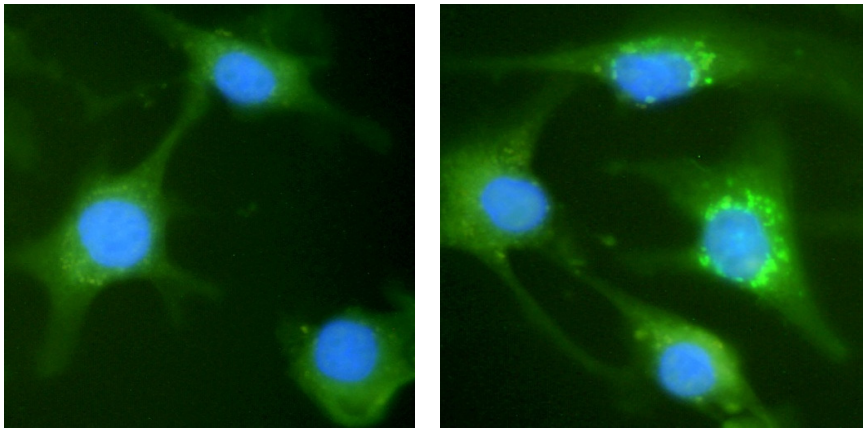


Figure 20: left panel: example of transfected cells; right panel: example of transfected and autophagic cells.

An evident activation of autophagic pathway was found in the cell lines tested (fig.21-22).

Because of the high cytotoxicity induced by IM (fig. 16), autophagy detection in K562 cell line after 50 µM IM treatment was performed only after 3-6 hours of treatment (fig. 21). Interestingly basal levels of autophagy in cardiac cells seem to be higher than in K562 cell line (fig. 22a- b).

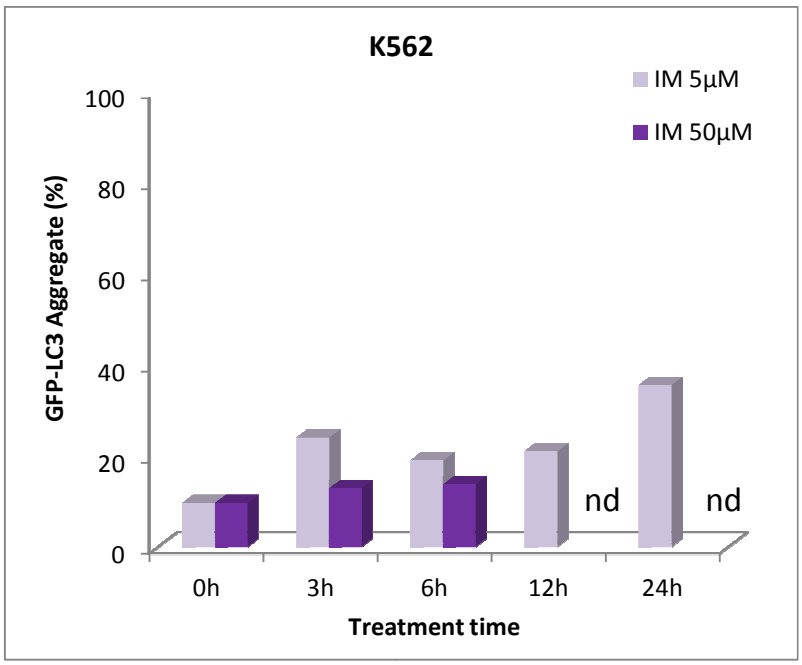


Figure 21.: Autophagy induction in K562 cell line presented in terms of cells with GFP- LC3 aggregates on 100 cells (GFP-LC3 aggregates %).
nd: not determined.

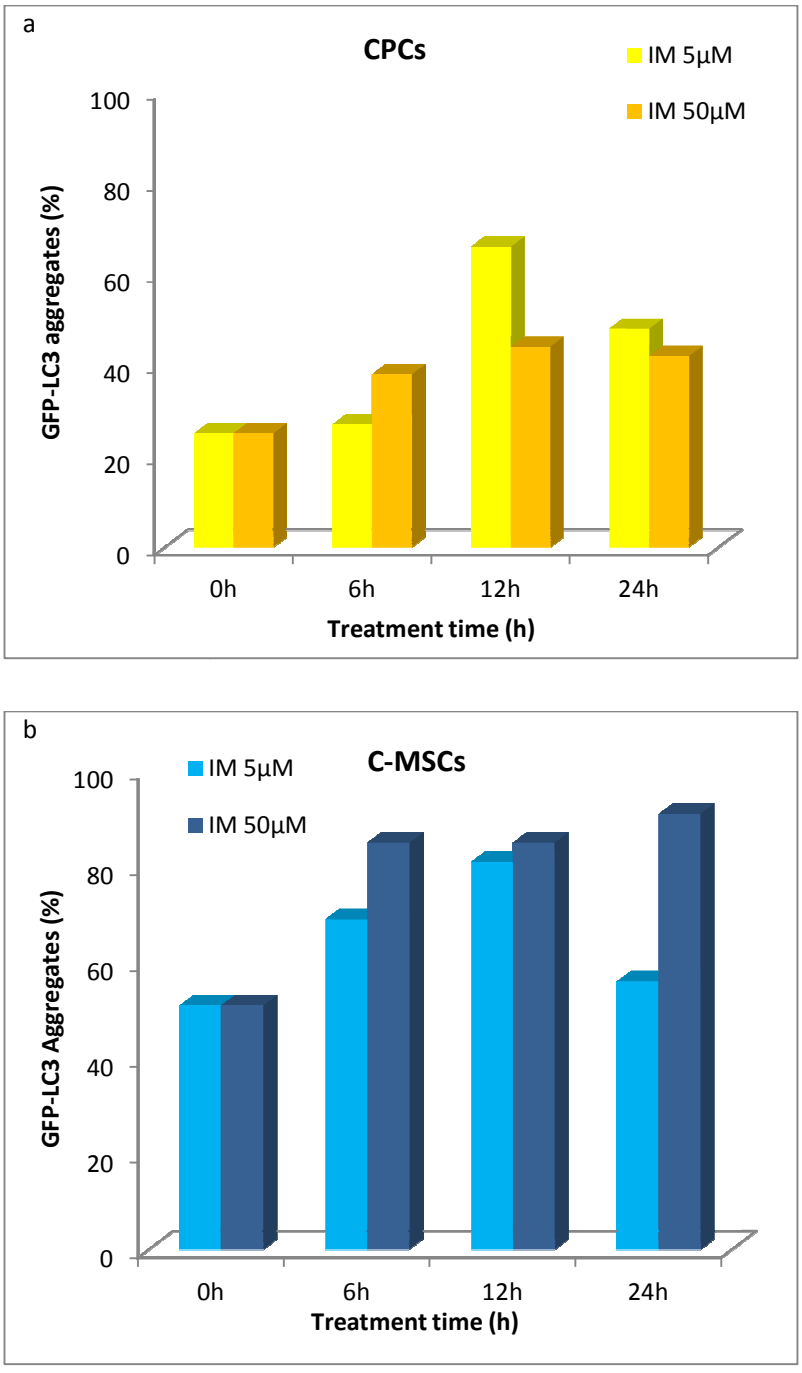


Figure 22: Autophagy induction in CPCs (a) and C-MSCs (b) cell lines presented in terms of cells with GFP-LC3 aggregates on 100 cells (GFP-LC3 aggregates %).

Evaluation of transcriptional profile

To confirm the involvement of DNA damage response and autophagic pathways, a quantitative real time PCR (RT-PCR) was performed on K562 and C-MSCs cell lines after 12h treatment with 1-5 μM IM. The higher dose (50 μM) hasn't been used because of the high cytotoxicity that it induces in all cell types. Furthermore, I introduce a subtoxic dose (1 μM IM) to better understand the biological activity of the drug.

I have analyzed the expression of genes encoding the proteins involved in DNA damage response, ATM and ATR, and genes encoding the proteins involved in autophagic pathway, LC3 and Beclin1, that have a key role during autophagic pathway, and mTOR and Bcl2, that act as regulators of autophagy.

The activation of ATM and ATR genes in K562 cell lines confirms the induction of DNA damage (fig. 23a) and, at the same time, the induction of LC3, Beclin1, Bcl2 and mTOR (fig. 24a) demonstrates the involvement of autophagy. Interestingly the activation of transcription seems to be higher after treatment with the lower dose than with the higher one. This reduction of gene expression observed with 5μM IM could be due to the reduction of cell proliferation observed with MTS assay (fig. 16).

In C-MSCs cell line a variation of transcriptional levels of gene involved with autophagy is observable only with the higher dose (5 μM) (fig. 24b). On the other hand, a significant induction of ATR, a gene encoding the a protein activated in response to DNA damage and cell cycle arrest during replication, is observable with both the concentrations used. Surprisingly ATM, encoding a protein activated in presence of DNA double strand breaks, doesn't show variation in expression after IM treatment (fig. 23b).

Gene	K562		C-MSCs	
	ΔΔCt (1 μM)	**ΔΔCt (5 μM)**	**ΔΔCt (1 μM)**	**ΔΔCt (5 μM)**
ATM	-4,84	-0,44	4,49	1,75
ATR	-3,46	-0,21	-7,10	-11,33
LC3	-2,88	-0,78	0,61	-3,94
Beclin1	-2,53	-1,03	0,16	-3,23
mTOR	-1,28	0,07	0,40	-4,33
Bcl2	-3,72	0,47	3,50	-1,37

Table 3: mRNA expression of genes involved in DNA damage response and in autophagy in K562 cell line and in C-MSCs cell line after 1-5 μM IM treatment.

ΔΔCt values = ΔCt of treated − ΔCt of control; ΔCt = (Ct of sample − Ct ActB)

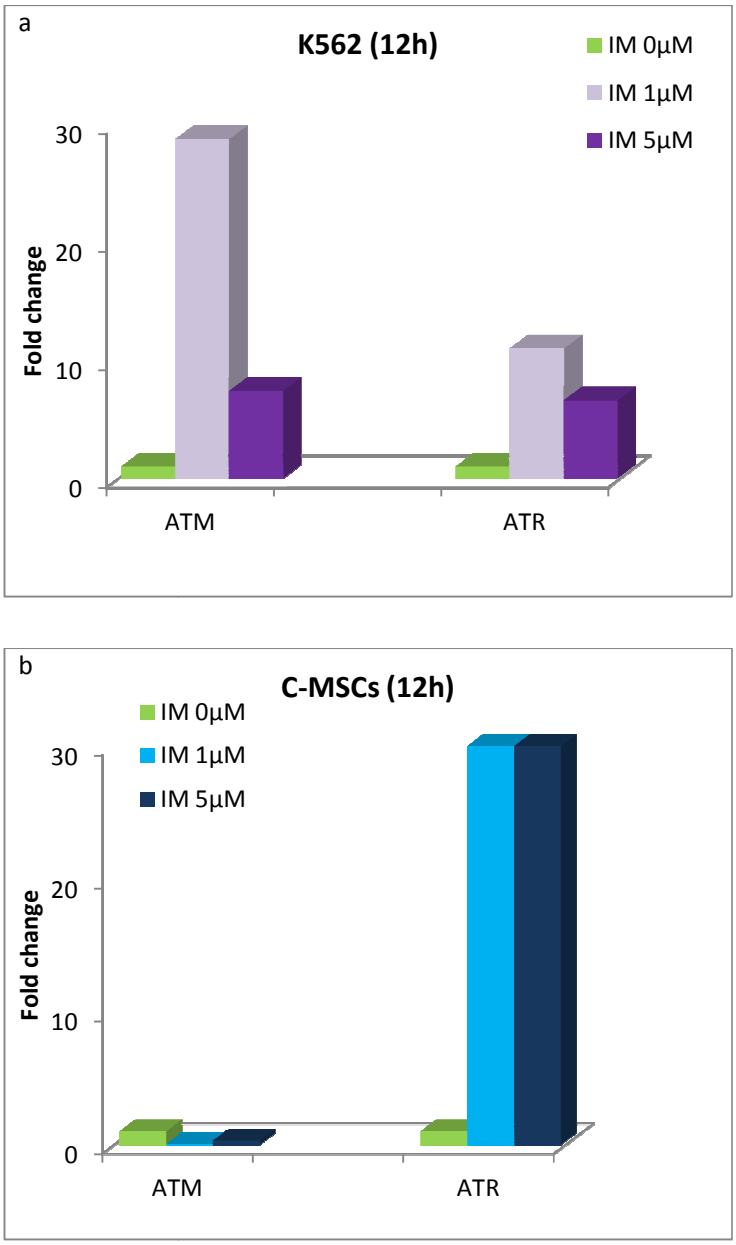


Figure 23: evaluation of expression of genes involved in DNA damage cell response after IM (1- 5 µM) treatment in K562 cell line (a) and in C-MSCs cell line (b).

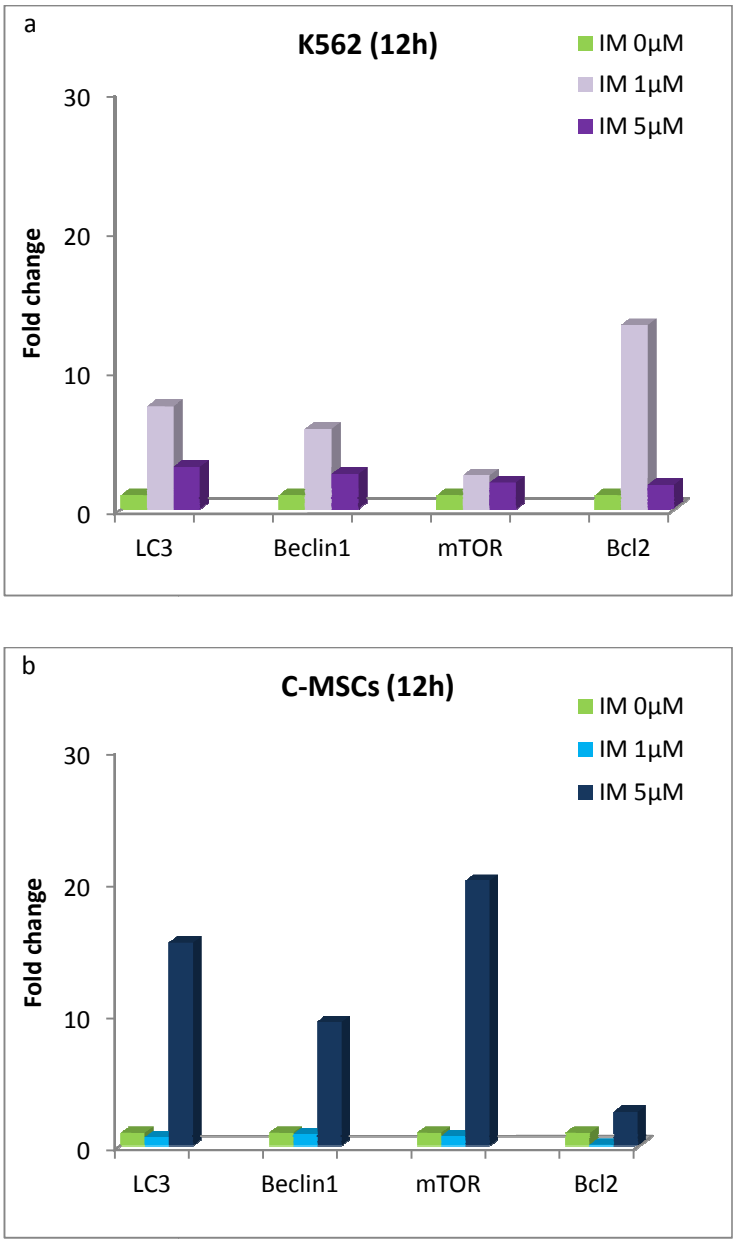


Figure 24: evaluation of expression of genes involved in apoptotic and autophagic pathways after IM (1- 5 µM) treatment in K562 cell line (a) and in C-MSCs cell line (b) .

Autophagy modulation

The evidences that autophagy could have a key role in chemotherapy, as pro-survival or pro-death mechanism, led me to investigate the role of autophagy in IM treatment. Co-treatments with IM 5 µM and autophagy regulators were performed; CQ/HCQ (autophagy inhibitor) and lithium chloride (autophagy inducer) are drugs used for many years in treatment of human diseases, so their absorption kinetics and collateral effects are known. This aspect led me to use these two molecules during my study.

Cytotoxicity

A variation in terms of cell number in culture was detected in all tested cell lines; in particular in K562 cell line autophagy inhibition through cloroquine induces a reduction of cell proliferation ($p<0.01$), conversely autophagy activation by lithium chloride improves survival in this cell line ($p<0.001$) (fig. 25). In the two cardiac cell lines both chloroquine and lithium chloride induce a reduction of cell proliferation after 24h treatment compared to the cell population treated with only IM (CPCs $p<0.01$; C-MSCs $p<0.001$)(fig. 26a- b).

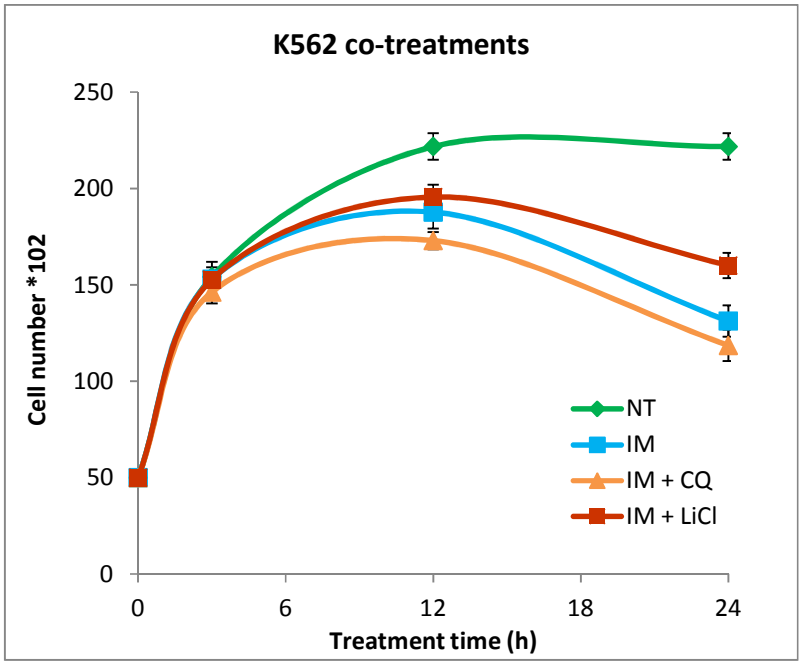


Figure 25: cytotoxicity (MTS assay) induced on K562 cell line after 24h treatment with Imatinib mesylate (5 µM), Imatinib mesylate plus chloroquine or Imatinib mesylate plus lithium chloride represented in terms of cell number in culture (cell number $*10^2$); NT= untreated, CQ= chloroquine, LiCl= lithium chloride.

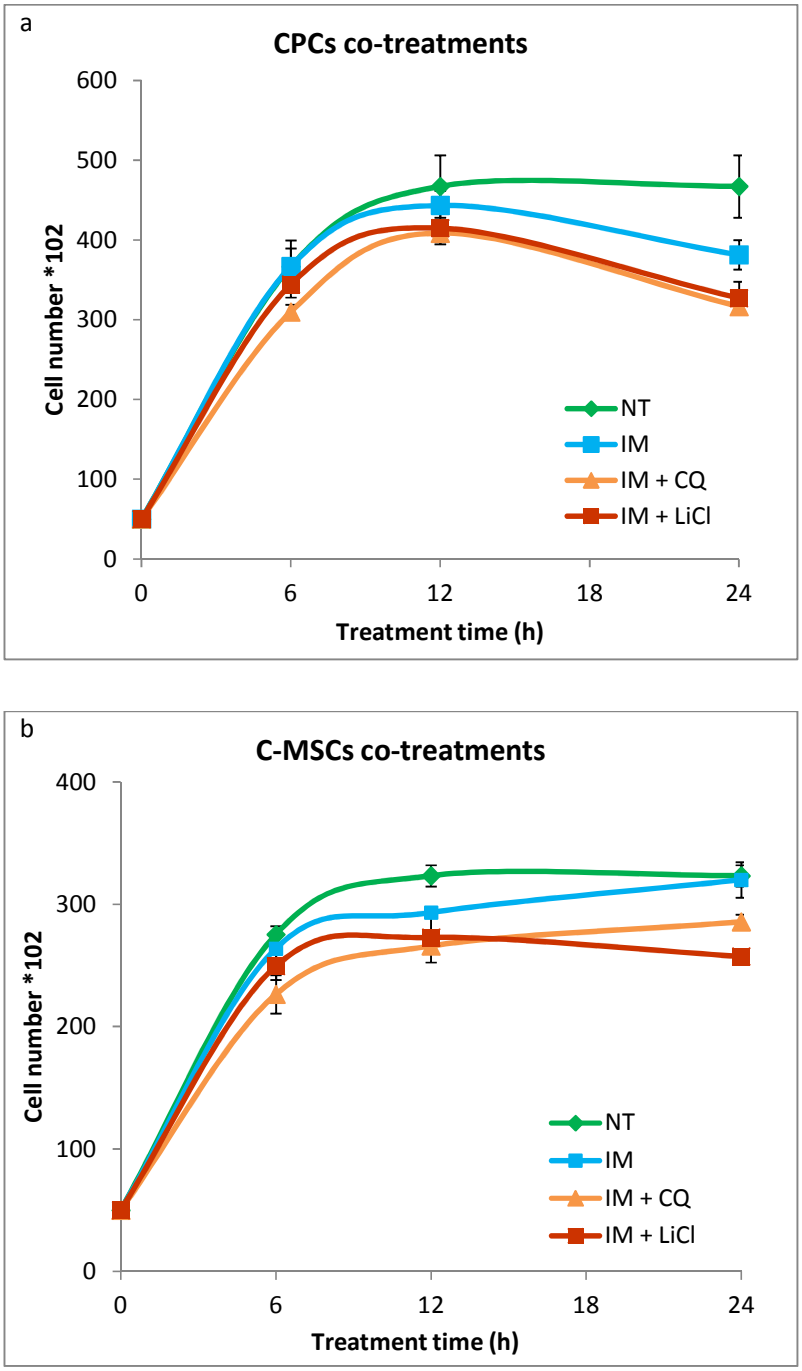


Figure 26: cytotoxicity (MTS assay) induced on CPCs (a) and C-MSCs (b) cell line after 24 h treatment with Imatinib mesylate (5 µM), Imatinib mesylate plus chloroquine or Imatinib mesylate plus lithium chloride represented in terms of cell number in culture (cell number $*10^2$); NT= untreated, CQ= chloroquine, LiCl= lithium chloride.

Genotoxicity

To evaluate variation in DNA damage the alkaline version of Comet Assay has been performed on human cell lines: K562 treated for 3-6 hours, C-MSCs treated for 6-12 hours. I used different treatment times in relation with the time course of DNA damage induction for each cell line analyzed. A variation of DNA damage represented in terms of percentage of tail intensity was detected in all tested cell lines after co- treatments with IM and autophagy inhibitor Chloroquine; in particular a similar behavior is observable in the two human cell lines: both in K562 than in C-MSCs cell line co- treatment induces a reduction of DNA damage (fig. 27- 28). DNA migration decrease observed in K562 cell line could be related to high rate of cytotoxicity observed through MTS assay (fig. 16)

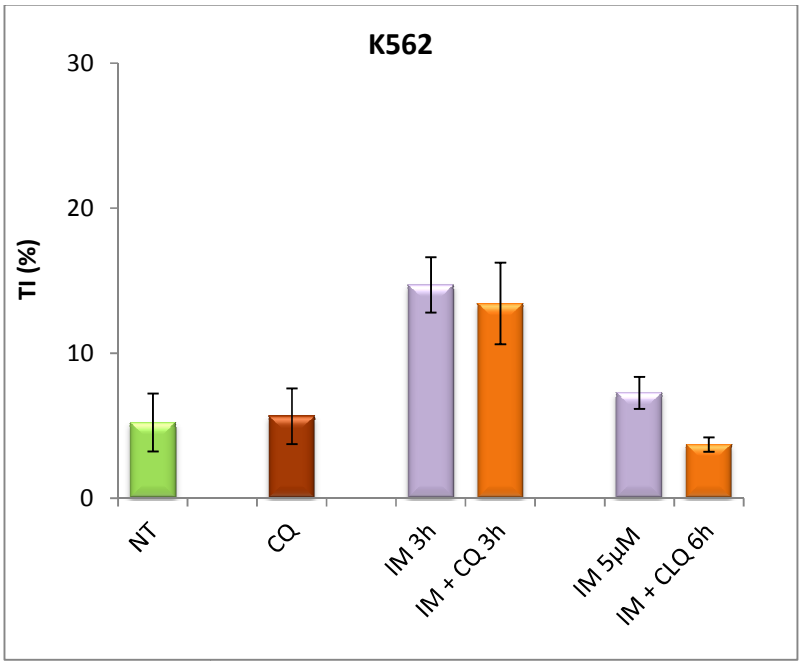


Figure 27: Genotoxicity (Comet assay) in K562 cell line after treatment with Imatinib mesylate (5 µM) and Imatinib mesylate plus chloroquine evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated; CQ= chloroquine.

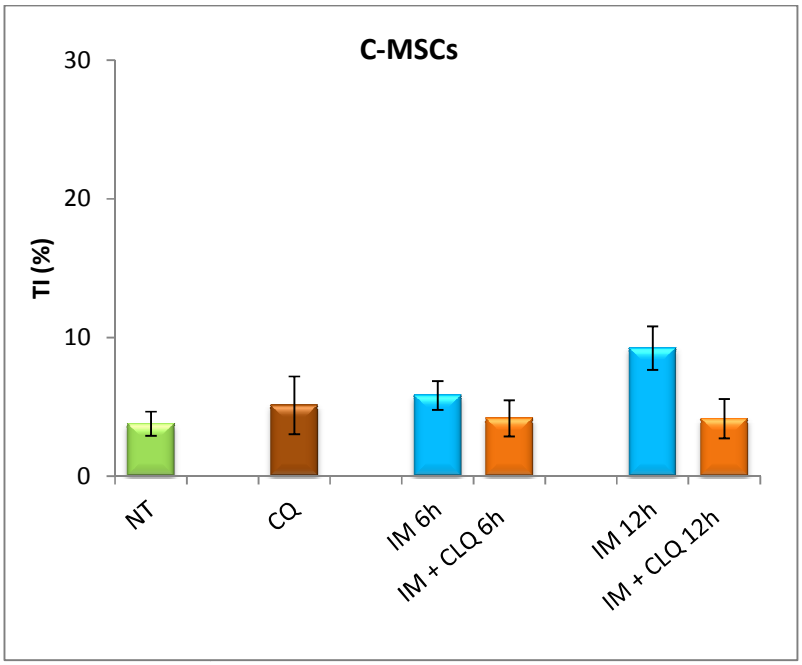


Figure 28: Genotoxicity (Comet assay) in C-MSCs cell line after treatment with Imatinib mesylate (5 µM) and Imatinib mesylate plus chloroquine evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated; CQ= chloroquine.

Evaluation of *in vitro* Doxorubicin effects

To compare the in vitro effects observed after IM treatment with those induced by molecule with known cardiotoxic activity I used doxorubicin, an old generation chemotherapy. The effects induced by Doxorubicin treatment have been evaluated on MCF7, doxorubicin target cell line, and on C-MSCs cell line.

MCF7 cell line:

The cytotoxicity evaluation on MCF7 (Doxorubicin target cell line) treated with Doxorubicin (0.1, 0.5, 1, 10 µM) for 24 and 48 hours shows a reduction of cell concentration for all testes doses (fig. 29). DNA damage induction was evaluated through Comet assay on cells treated with doxorubicin (1-10-100 µM) for 4 and 24 hours; as expected a time and dose dependent genotoxicity is induced in MCF7 cell line ($p<0.001$) (fig. 30).

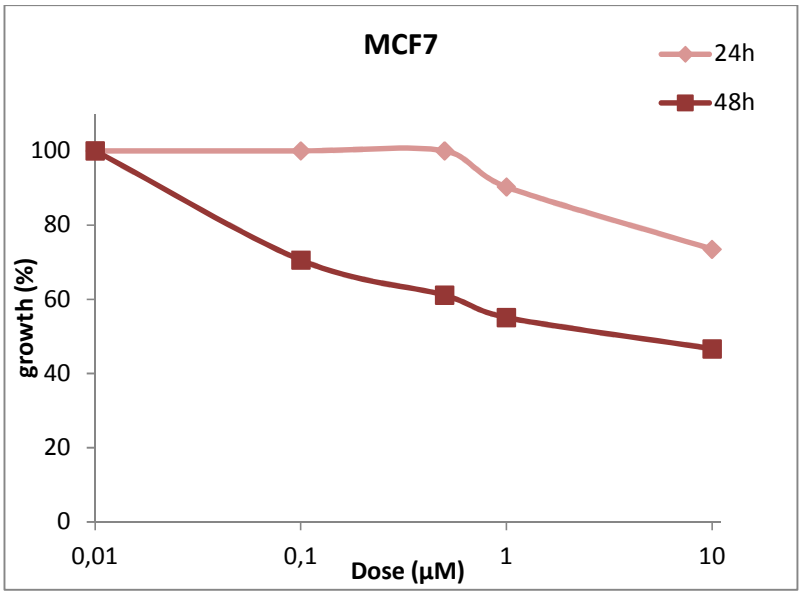


Figure 29: Cytotoxicity (MTS assay) induced on MCF-7 cell line after 24 and 48 h treatment with doxorubicin (0.1, 0.5, 1, 10 µM) rapresented in terms of growth percentage (growth %).

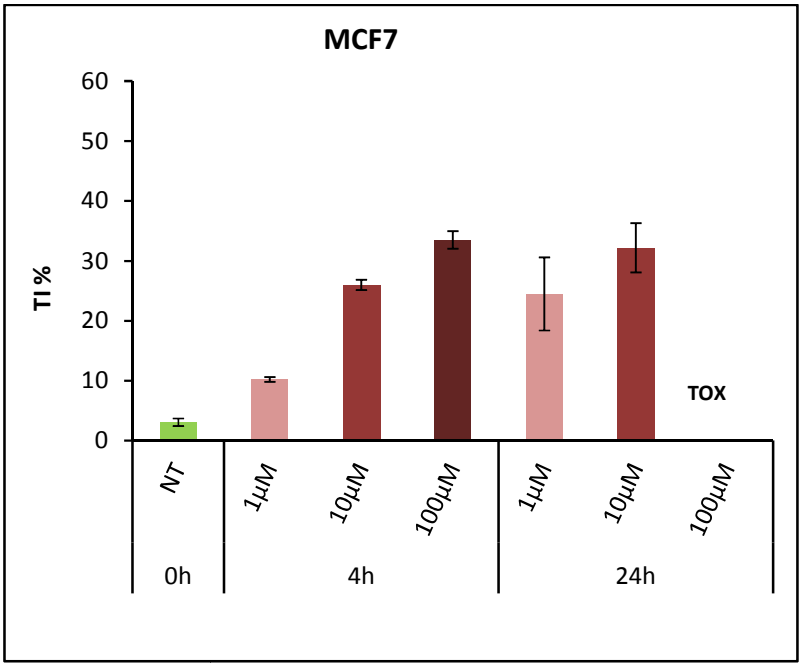


Figure 30: Genotoxicity (Comet assay) in MCF-7 cell line after 4 and 24 h treatment with doxorubicin (1, 10, 100 µM) evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated

Autophagy evaluation through the transfection assay was conducted and it shows an evident activation of autophagic pathway after Doxorubicin (0.5- 1 µM) treatment (fig. 31).

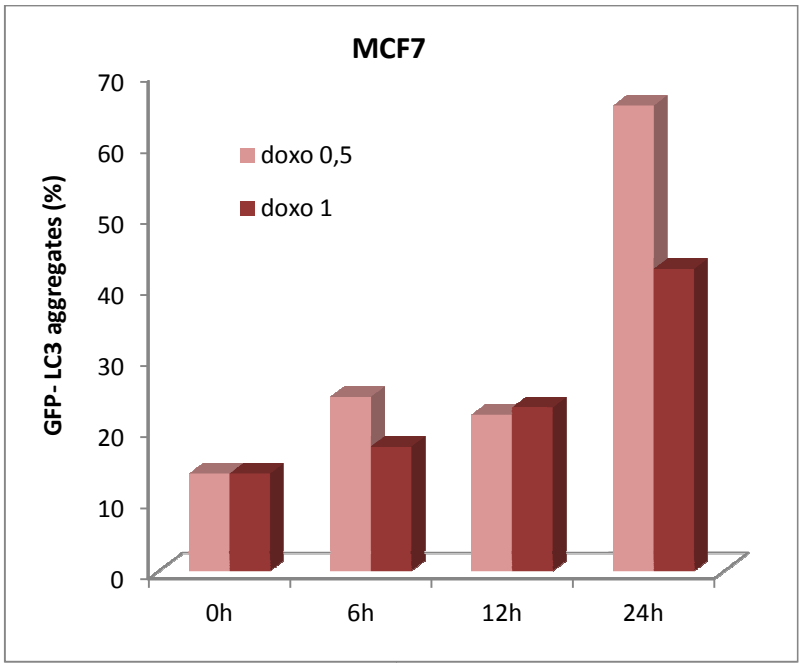
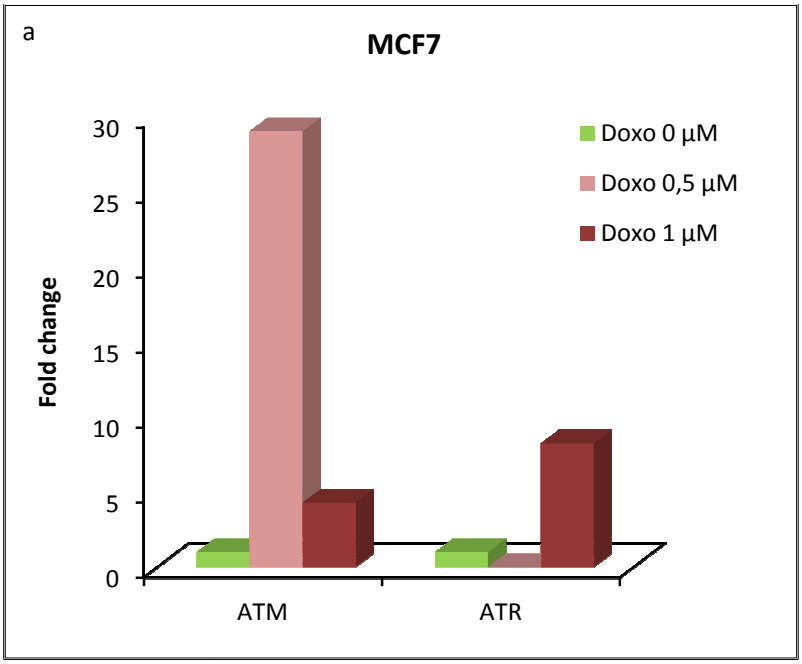


Figure 31: Autophagy induction in MCF7 cell line evaluated through a transfection assay, presented in terms of cells with GFP-LC3 aggregates on 100 cells (GFP- LC3 aggregates %).

The evaluation of gene expression shows the activation of genes involved in DNA damage response and in cell surviving or death pathways, like apoptosis or autophagy (fig. 32a- b).

Gene	MCF7	
	ΔΔCt (1 µM)	**ΔΔCt (5 µM)**
ATM	-4,86	-2,10
ATR	0,83	-3,04
LC3	-1,56	-1,53
Beclin1	-2,99	-2,43
mTOR	-1,82	-1,44

Table 4: mRNA expression of genes involved in DNA damage response and in autophagy in K562 cell line and in C-MSCs cell line after 0.5- 1 µM Doxorubicin treatment.
ΔΔCt values = ΔCt of treated − ΔCt of control; ΔCt = (Ct of sample − Ct ActB)



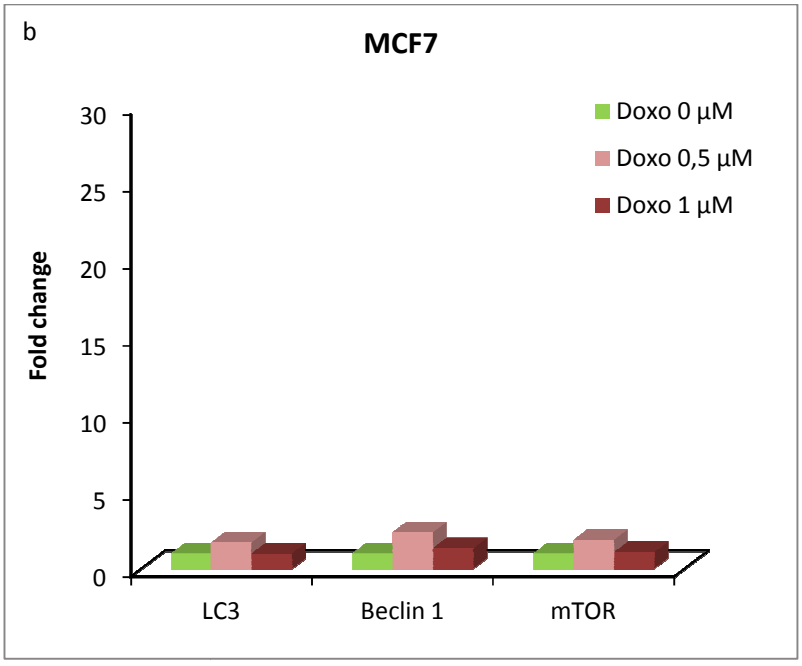


Figure 32: evaluation of expression of genes involved in DNA damage cell response (a) and in autophagic pathways (b) after doxorubicin (0.5 - 1 µM) treatment.

C-MSCs cell line:

Doxorubicin (1, 5, 50 µM) treatment induces a reduction of cell number in culture in C-MSCs cell line in a dose and time dependent manner (fig. 33). As in MCF7 cell line also in C-MSCs cell line is observable genotoxicity induction after Doxorubicin (1, 10, 100 µM) treatment ($p<0.001$) (fig. 34).

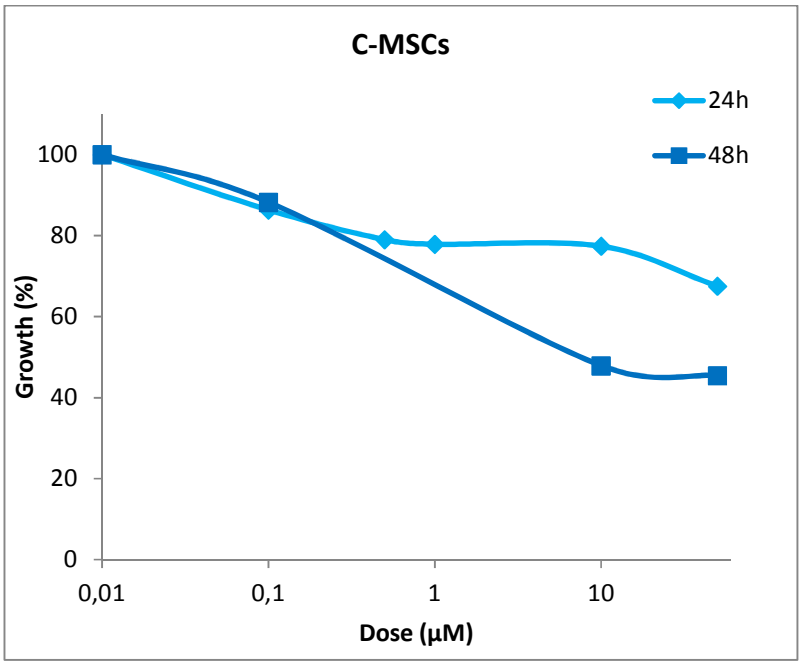


Figure 33: Cytotoxicity (MTS assay) induced on C-MSCs cell line after 24 and 48 h treatment with doxorubicin (0.1, 0.5, 1, 10, 50 µM) represented in terms of growth percentage (growth %).

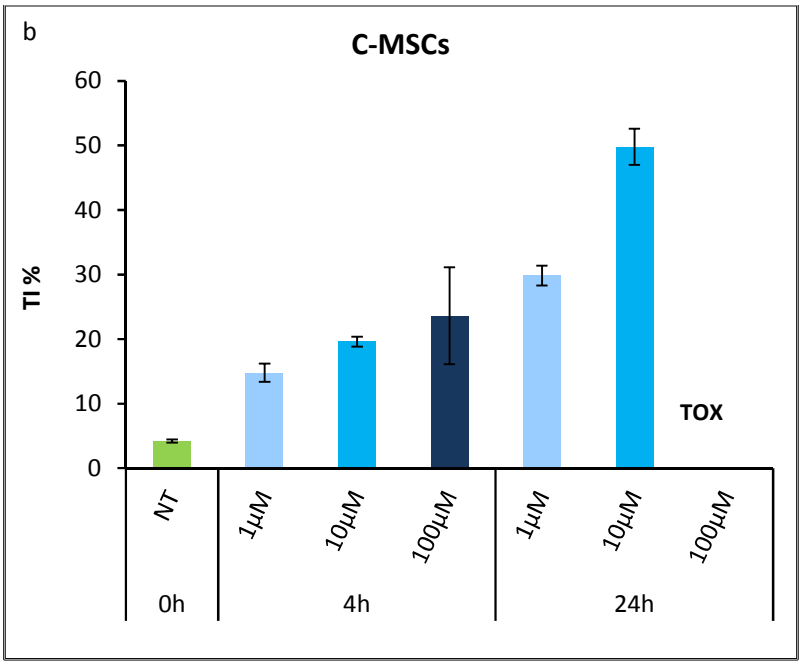


Figure 34: Genotoxicity (Comet assay) in C-MSCs cell line after 4 and 24 h treatment with doxorubicin (1, 10, 100 µM) evaluated in terms of percentage of DNA in comet tail (tail intensity : TI%); NT= untreated

Discussion and Conclusions

Imatinib mesylate, inhibitor of specific tyrosine kinases like BCR-ABL and c-kit, has revolutionized treatment of chronic myeloid leukemia and gastro- intestinal stromal tumors patients. IM and other drugs belonging to the family of targeted therapies have changed our thinking about cancer, which can now be viewed as a group of diseases that, even if not curable, can be managed for years, similar to many other chronic diseases.

Despite of its high selectivity, side effects have been found in non- target tissues. In particular IM treatment seems to be related with adverse effects on cardiovascular system.

Objective of this thesis was to analyze *in vitro* response of different cellular types to IM treatment. Since IM side effects could be due not only to damages in terminally differentiated cells [Kerkela R, et al. 2006] but also to a pauperization of cardiac stem cells, I have used for my study two cardiac stem cell lines, rat cardiac progenitor cells (CPCs) and human cardiac mesenchimal stem cells (C-MSCs). Furthermore a chronic myeloid leukemia cell line (K562) expressing the specific target protein BCR-ABL has been used as positive control. Different endpoints indicating the presence of a cellular stress have been evaluated: cytotoxicity through MTS assay, genotoxicity through alkaline Comet assay, reactive oxygen species (ROS) production through a fluorescent assay, autophagy activation through a transfection assay, and changes in gene expression were evaluated by quantitative RT-PCR.

Two different concentrations of IM have been used to evaluate the toxicity induced by treatments: 5 µM comparable with plasmatic drug dose in patients after *in vivo* treatment; 50 µM to induce a response that mimic drug accumulation due to a prolonged treatment.

As expected IM treatment, with both the tested doses, induces an high cytotoxicity in the target cell line K562 (fig. 16). An high cytotoxicity is detectable at 50µM in both cardiac cell lines (fig. 17a- b); while at 5µM, rat CPCs show a mild growth inhibition (fig. 17a) and human C-MSCs show a proliferation rate comparable to the untreated cells rate (fig. 17b). Even if lower than in target cell line, cytotoxicity observed in cardiac stem cells can't be explained by the inhibition of tyrosine kinase BCR- ABL, IM primary action; suggesting that the adverse effects of IM could be due to inhibition of aspecific tyrosine kinase. It is known that IM can interact, even if with minor affinity, with other proteins like c-kit that is fundamental for cardiac stem cells (CSC) and cardiac resident stem cells (CRSC) differentiation in cardiomyocytes and for CSC migration from bone marrow to hearth. Alterations in stem cell migration and maturation could prejudice their capability to replace damaged tissue and repair lesions [Ayach et al., 2006; Cheng e Force, 2010].

The high cytotoxicity levels led me to investigate cell death pathway involved. The first studies of adverse IM effects on cardiac tissues underlined a reduction of left ventricle mass not associated with an increase of apoptosis levels; furthermore they have observed the lack of fibrotic area meaning the absence of necrotic cell death [Kerkela et al., 2006; Leipner et al., 2008; Terabe et al., 2009]. These assumptions led me to investigate alternative cell death pathways, like autophagy. In the last year it is emerging the fundamental role of autophagic pathway in cardiac tissue for its capacity to eliminate damaged cell material or the entire cell. Our data seem to confirm the intervention of this cell pathway in response to IM treatment with both tested concentrations (fig. 21, 22a- b). Interestingly autophagy basal levels in cardiac cells are higher than in K562, meaning that autophagy could have an important function in cells committed to cardiac compartment (fig. 22a- b). Autophagy activation has been confirmed through quantitative RT- PCR that shows induction of genes involved in autophagic pathway like LC3, Beclin1, mTOR and Bcl2. Investigations performed through Alkaline version of Comet Assay show the induction of an increment of DNA migration after IM treatment in all cell lines tested; in particular K562 treatment with both IM concentrations induces a significant genotoxicity during the early times of treatment that seems to decrease over time. The reduction of genotoxicity could be due to repair of initial DNA damage or most likely to death of damaged cells (as confirmed also by cytotoxicity data) (fig. 18) and the consequent selection of a resistant population. 5 µM IM seems to induce a lower damage in cardiac stem cells than in K562, and this damage disappears after 24h treatment. On the other hand the higher dose induces in these cell lines an acute genotoxicity that increases over time.

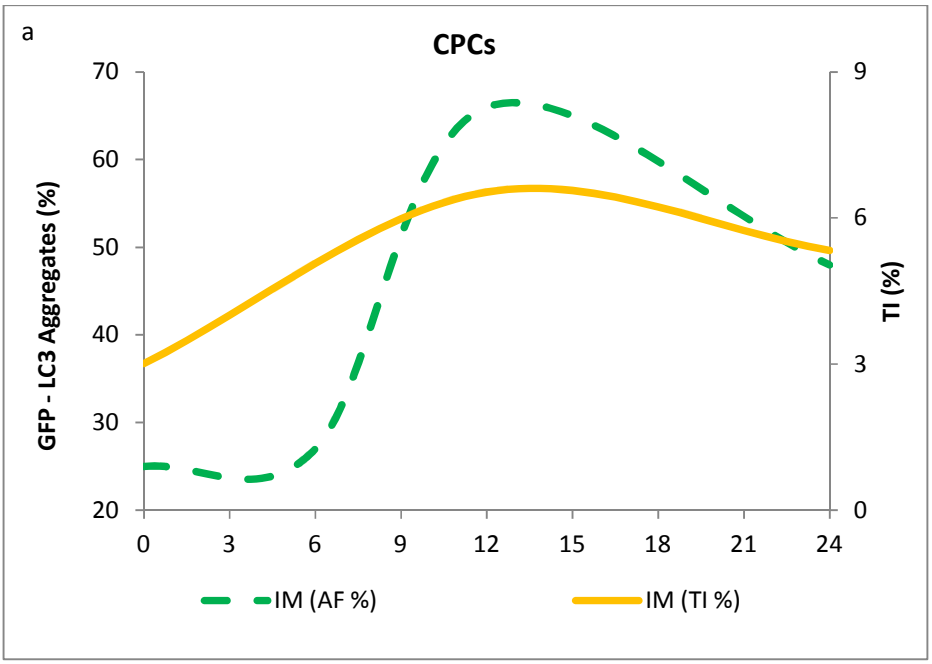
DNA damage can induces a series of cellular response to face insult: the first step is the activation of damage checkpoints, cell cycle arrest and induction of repair machinery and as last chance cell death pathways are activated to eliminate highly damaged cells; every cellular events is associated with modifications in transcriptome and proteome profile [Rodriguez-Rocha et al., 2011].

The presence of DNA damage is confirmed also through quantitative real time PCR (RT-PCR) that shows the activation of transcription of genes that encoding ATM and ATR, two kinases which are activated by DNA double-strand breaks (DSBs) and single-stranded DNA respectively. These proteins are able to induce a cell cycle arrest to allow DNA damage repair [Smith et al. 2010]. IM treatment induces the contemporary induction of ATM and ATR in K562 cell line, in c-MSCs cell line only ATR is expressed; this differential transcription of genes involved in DNA damage response could indicate the induction of a different kind of damage in cells after treatment. Recent studies have shown that the induction of ATM transcription could occur not only in presence of DNA damage but also as result of a direct inhibition of ATM kinase; Khalil and collaborators

have demonstrated that pharmacological inhibition of ATM protein induces an autoregulatory induction of ATM transcription [Khalil et al. 2012].

The Induction of genotoxicity from a molecule that acts as tyrosine kinase inhibitor was totally unexpected, but the presence of DNA fragmentation already after few hours of treatment led me to speculate about and to confirm the induction of oxidative damage as suggested by Czechowska and collaborators (2005). In their study they have detected the induction of DNA damage on K562 cell line after IM treatment; the use of a modified version of comet assay, which involves the use of endonuclease III (enzyme with glycosilase an liase activity during the early phases of Base Excision Repair (BER) that allows to detect the presence of oxidized bases), showed an increase of DNA migration, meaning the presence of oxidative damage [Czechowska et al., 2005]. The evaluation of Reactive Oxygen Species (ROS) that I have performed through a fluorescent assay shows a relevant induction of oxidative stress confirming this hypothesis. Interestingly cardiac stem cells seem to have an higher resistance against oxidative stress as shown by the different impact of Menadione (molecule that is known to induce production of reactive oxygen species [Bladen et al., 2012]) on this cell line than on K562 cell line.

Recent studies have demonstrated a link between DNA damage and autophagy activation [Rodriguez-Rocha et al., 2011]. Data reported in this thesis seem to confirm this hypothesis: a contemporary induction of these two cell responses are detectable after IM treatment (fig. 35a- b).



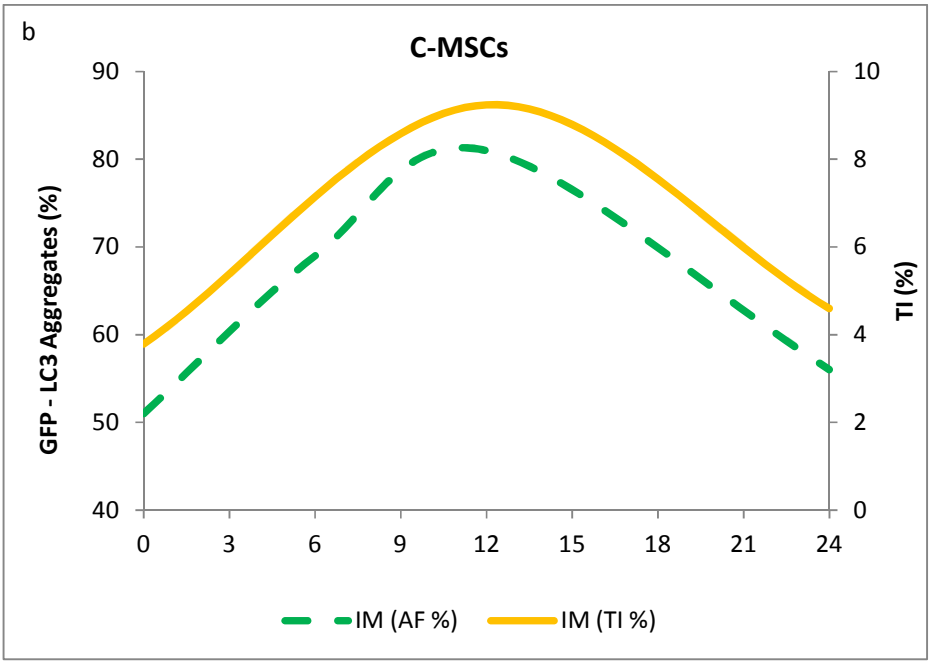


Figure 35: Comparison of autophagic and genotoxic levels in CPCs (a) and C-MSCs (b) after IM treatment; IM= Imatinib mesylate; TI= tail intensity %; AF= cells with active autophagic pathaway.

The growing evidence of the importance that autophagy could have in cellular response to chemotherapy, as pro-survival or pro-death mechanism, led me to deepen the role of autophagy in IM treatment. Furthermore to find a possible link between autophagy and cytotoxic and genotoxic effects, co- treatments with autophagy regulators (chloroquine as autophagy inhibitor or lithium chloride as autophagy inducer) have been conducted. Co- treatments with IM 5 μM and autophagy regulators were performed; CQ/HCQ (autophagy inhibitor) and lithium chloride (autophagy inducer) are drugs used for many years in treatment of human diseases, so their absorption kinetics and collateral effects are known. This aspect drives me to use these two molecules during my study.

The inhibition of autophagy in K562 cell line induces an increase of cytotoxicity observed after IM treatment; on the other hand autophagy activation favors cell surviving (fig. 25), confirming the role of autophagy as resistance machinery in chemotherapy [Salomoni P, et al. 2009]. In both cardiac cell lines autophagy modulation with inducer or inhibitor produces an arrest of cell proliferation (fig. 26a- b). This behavior suggests that autophagy is strongly regulated and every variation could compromise cell response to drug treatment. As in all finally differentiated cells, autophagy has a primary role in cardiac cells. In non dividing cardiac cells cellular wastes can't be redistributed in daughter cells and a reduction of autophagy levels induces a organelles/proteins accumulation that contributes to induce cardiac diseases. Moreover cardiomyocytes are long life cells and for this reason they are susceptible to aging that can hit cellular structures like mitochondria and lysosomes, in this contest autophagy represents a key pathway to allow a rapid turn-over of cellular components. It is known that cardiac cells use autophagic pathway as mechanism needful to cell homeostasis maintenance during condition of pharmacological stress too [Martinet W, et al. 2007]. Comet assays have been performed after autophagy inhibition in human cell lines (K562 and C-MSCs) to evaluate DNA damage variation. In K562 cell line a decrease in DNA damage is observed after autophagy inhibition. This behavior is probably due to death of damaged cells and to the selection of resistant cells as confirmed through cytotoxicity data obtained through MTS assay (fig. 25) . In C-MSCs cell lines a reduction of DNA migration is evident after autophagy inhibition; interestingly the highest DNA damage reduction coincides with the autophagy peak observed in these cells after IM treatment (fig. 36).

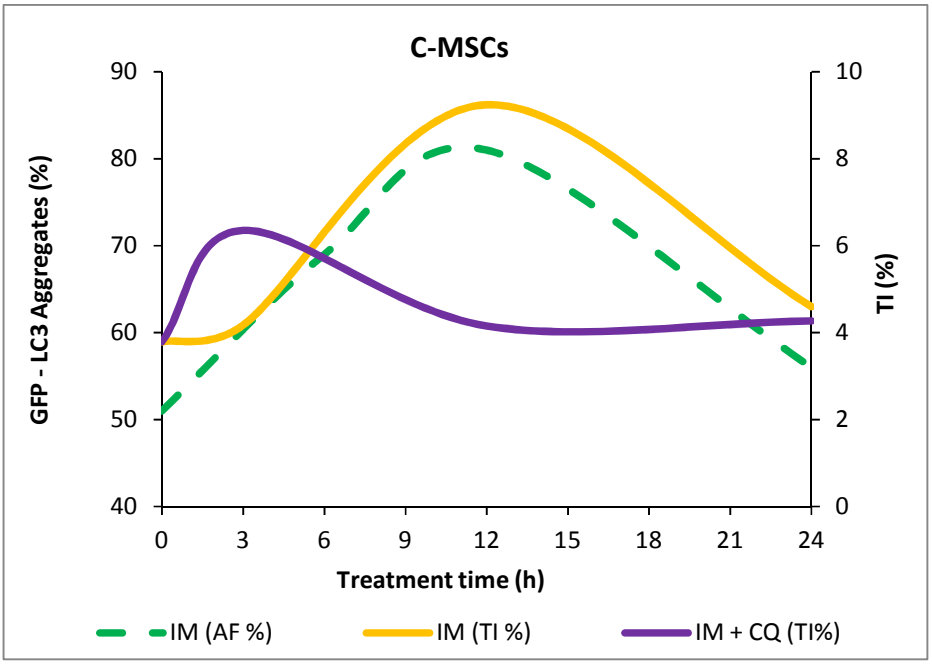


Figure 36: Comparison of autophagic and genotoxic levels in C-MSCs after IM treatment with or without chloroquine; IM= Imatinib mesylate; IM + CQ= IM- chloroquine co-treatments; TI= tail intensity %.

This observation could confirm autophagy intervention in DNA damage repair. Also gene expression analysis confirm the co-expression of this two cell events, a contemporary expression of genes involved in authophagic pathway (LC3 e Beclin1) and enzymes that have a role in DNA damage surveillance (ATM and ATR) is observed (fig. 37). Robert and collaborators have demonstrated the involvement of autophagy in degradation of components that takes part in DNA repair. This degradation through autophagy seems to depend on post-translational modifications as acetylation, modification that has been observed in many enzymes involved in repair systems [Robert T, et al. 2011].

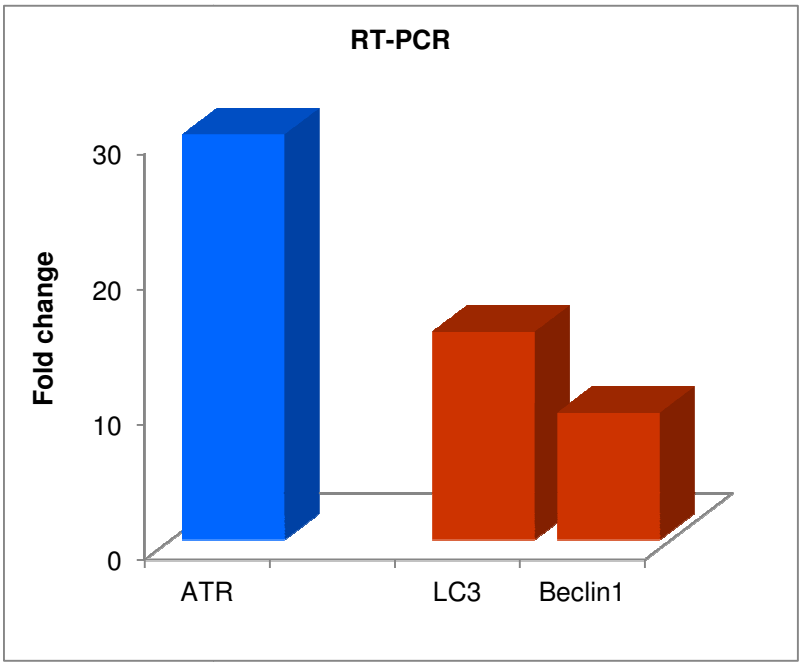


Figure 37: gene expression of ATR and autophagic genes LC3 and Beclin1 in cardiac mesenchimal stem cell (C-MSCs) after IM treatment (5µM).

Ultimately, a comparison between Imatinib mesylate and Doxorubicin, as old generation chemotherapic with known cardiotoxic activity, has been conducted. With both drugs used toxicity has been observed in C-MSCs cell line, as well as in target cells. In particular Doxorubicin seems to induce an high toxicity, in terms of cell death and DNA damage induction, in a comparable manner in both target cell line, MCF-7, and in non target cell line, C-MSCs. Conversely IM induces a differential response in the two different cell lines. An high toxicity is observable in target cell line, K562 with all the tested doses; conversely in C-MSCs cell line toxicity is detectable only after treatment with the higher dose. These observations allow to confirm the higher specificity of IM respect to Doxorubicin.

Data reported in this thesis show how treatment with IM could imply a complex cellular response not only in target cell lines but also in non target ones. Induction of cell death, genotoxicity, oxidative stress and autophagy activation have been observed in all cell lines tested. The higher effects in cardiac stem cell lines are detectable after acute treatment, but these data have not to be underestimated because cell events found with the highest IM dose may occur *in vivo* as a consequence of drug accumulation in cardiac compartment after prolonged treatment.

Furthermore autophagy seems to represent in K562 a defence mechanism during IM treatment; in this cell line in fact autophagy inhibition during IM treatment induces a

reduction in cell viability. Interestingly the use of chloroquine as autophagy inhibitor during IM treatment seems to reduce toxicity observed in cardiac stem cell lines. For this reason autophagy inhibition through chloroquine could represent a good approach in the development of new combined antitumor therapies able to decrease toxicity induced in non- target cell lines without affecting the drug efficacy.

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