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6-(Methylsulfonyl) hexyl isothiocyanate as potential chemopreventive agent: molecular and cellular profile in leukaemia cell lines

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ABSTRACT

***Introduction**:* Despite numerous therapeutic interventions (chemotherapy, surgical removal, radiotherapy), cancer is still today the second leading cause of death, after heart disease, in both man and woman; for this reason, new anticancer approaches are needed. A growing interest has been addressed to isothiocytanates (ITC) thanks to their pleiotropism and more recently, the 6-(methylsulfonyl) hexyl isothiocyanate (6-MITC), the main constituent of the rhizome of *Wasabia Japonica,* has stimulated the interest of researchers thanks to its anti-inflammatory and antioxidant properties.

In this contest, aim of the research was to study if 6-MITC is able to modulate the main mechanisms underlying chemopreventive process in leukemic cells lines, verify the selectivity of action toward healthy lymphocytes and the safety of use in terms of mutagenicity.

Materials and Methods: The study was conducted on different cell types. In particular, Jurkat (acute T lymphoblastic leukemia) and HL-60 cells (acute promyelocytic leukaemia) were treated with increasing concentrations of 6-MITC and cell viability, induction of apoptosis, cell cycle analysis, autophagy modulation and stimulation of differentiation were evaluated by flow cytometry. PBL (pheriferal blood lymphocytes), the non-transformed counterparty of leukemia cells, was used to analyse the selectivity of action by studying the same mechanisms previously indicated. Finally, safety of use and antimutagenicity were studied in TK6 cells (human lymphoblasts), as indicated by the OECD guideline no.487, adopting an automated and recently published protocol in flow cytometry, alternative to the classical method in microscopy (cytokinesis-block micronucleus assay-test CBMN).

***Results**:* The achieved results have demonstrated that isothiocyanate modulates many signaling pathways involved in chemopreventive mechanism.

6-MITC induces apoptosis of transformed cells that results statistically significant at 24h in a concentration and time related; in particular, apoptosis is triggered by an extrinsic pathway increasing activated caspase-8 level. 6-MITC is able to limit tumor growth by slowing down the cell cycle of Jurkat cells after 24h of treatment and blocks HL-60 cell cycle by modulating Cyclin D3 expression level after 24 and 72h. After 24h of treatment, a statistically significant increase in the autophagic flux, with a corresponding increase in the levels of BCL-2 and ROS formation, was observed in both leukemia lines.

Furthermore, 6-MITC induces cytodifferentiation of promyelocytic HL-60 into macrophage and granulocytic phenotypes after 72h of treatment.

Essential feature of a good chemopreventive agent is selectivity toward cancer cells and low toxicity towards non-transformed cells. The results obtained with 6-MITC on PBL from healthy donors suggest that the isothiocyante is a good selective cytotoxic agent.

Finally, the analysis of the micronuclei revealed that 6-MITC is not mutagenic, ensuring safe use, and that instead, it is able to counteract the mutagenic activity of the aneuploidogen Vinblastine (VINB), demonstrating another important and interesting chemopreventive activity.

CHAPTER 1
INTRODUCTION

Despite efforts in the medical and scientific fields, cancer is still the second cause of death and it is estimated that, in the next 15 years, the number of new cases of cancer will increase by 70% worldwide (Siegel *et al*., 2011; Durko and Malecka-Panas 2014).

In recent years, we have witnessed a significant improvement in survival, however it is still unsatisfactory. Indeed, the lack of early diagnosis, limited therapies and multiple drug resistance lead to fatal outcomes and high mortality rates. In addition to these issues, the serious toxicity profile and the high costs of many therapies should be considered. Therefore, it is necessary to identify and develop new preventive and therapeutic strategies to counteract the morbidity and mortality associated with cancer.

Epidemiological, preclinical and clinical studies associate a diet rich in phytochemicals with a lower risk of developing chronic degenerative diseases, including cancer (Quideau *et al*., 2011; Durko and Malecka-Panas, 2014). The phytochemicals are compounds naturally present in plants and include an extremely inhomogeneous set of chemicals: flavonoids, alkaloids, tannins, triterpenes, tocopherols, phenols, flavonoids and isothiocyanates (Landete, 2011; Quideau *et al*., 2011; Ismail *et al*., 2012). Thanks to their pleiotropism that includes anti-inflammatory, antioxidant, antiproliferative, proapoptotic properties as well as modulatory effects on subcellular signaling pathways, phytochemicals are therefore an effective option to counteract the incidence and mortality of cancer (Tariq *et al*., 2016).

1.1 Isothiocyanates

Isothiocyanates (ITCs) are the major pharmacological active constituents present in plants belonging to the *Crucifereae* family of the genus *Brassica* and derive from the enzymatic hydrolysis of glucosinolates (Fahey and Stephenson, 1999; Barba *et al.,* 2016). Glucosinolates do not have their own biological activity and, as such, they are only partially absorbed; for this reason, the intervention of the enzyme Mirosinase (Myr) is necessary. Myr is a thermolabile thio-glucuronidase present in plants that is also produced by human intestinal bacterial flora in small quantities. In the plant, the enzyme is kept separate from glucosinolates and is released when a cell wall is damaged, as in the case of food preparation or during chewing. Once released, the enzyme breaks down glucosinolate into β-D glucose, sulfate and an unstable aglycone that spontaneously reorganizes itself, generating isothiocyanates (ITC), thiocyanates, oxazolidin-2-tione or nitrile, depending on the pH, substrate and presence of different ions. In particular, at neutral pH ITCs are formed, highly reactive compounds

that contain a thiocyanate as a functional group (-N=C=S), which acts as a nucleophile, and an alkyl or allyl side chain (Barba *et al*., 2016; Novío *et al*., 2016; Sharma *et al*., 2016) (Figure 1).

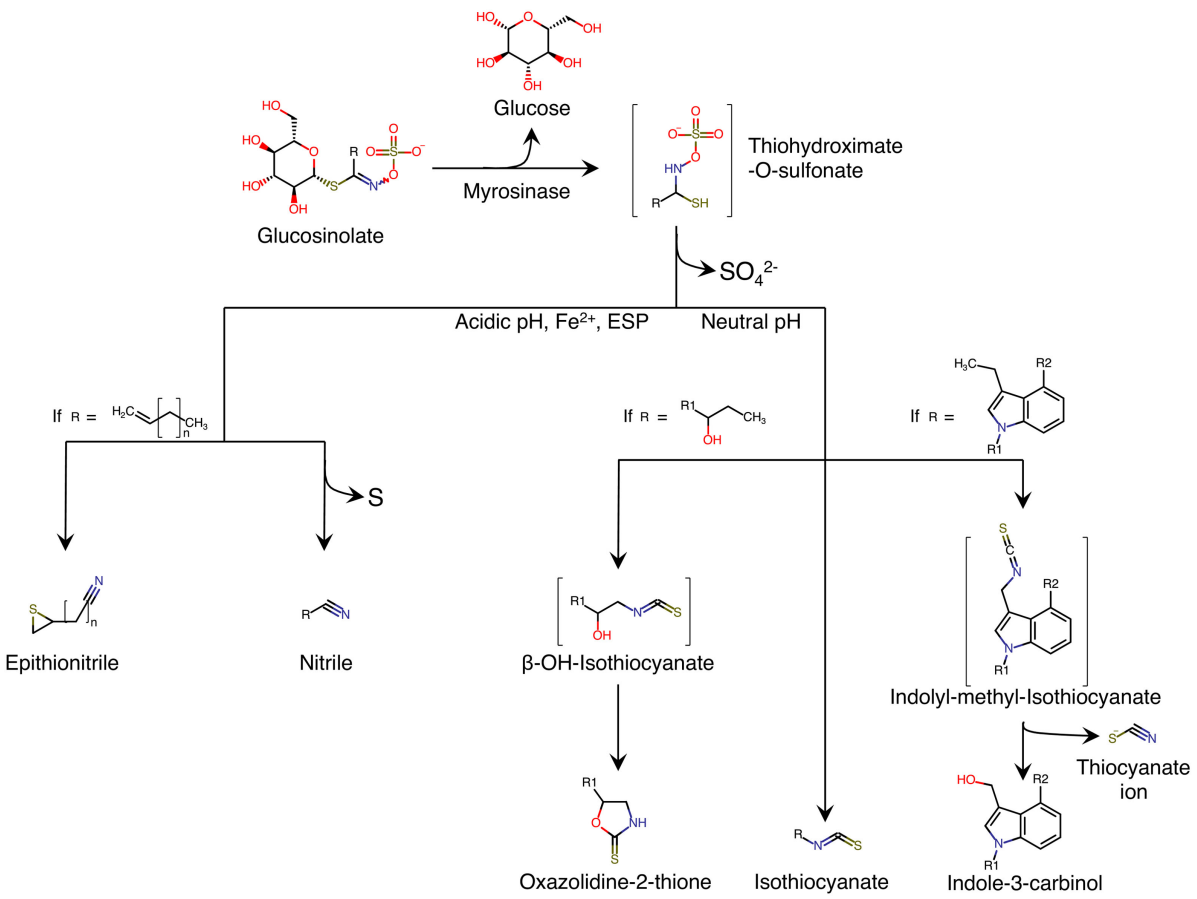


Figure 1: Enzymatic hydrolysis reaction of glucosinolates. Copyright Barba et al., 2016

The ITCs have a bioavailability six times higher than that of glucosinolates, which are not however converted in their totality, but only by 10-30% (Barba *et al*., 2016; Sharma *et al.,* 2016). Once released, the ITCs are rapidly absorbed and appear in the systemic circulation after about 3h, as shown by pharmacokinetic studies. Subsequently, most ITCs are converted into an acetylcysteine derivative which is 50% eliminated in this form via urine in the following 12 hours. The conversion to the N-acetylcysteine derivative occurs through conjugation with glutathione (GSH) through a glutathione S-transferase (GST), followed by hydrolysis of the resulting conjugate in a cysteine derivative and finally by N-acetylation (Mennicke *et al*., 1988; Chung *et al*., 1992;). The proven chemopreventive activity of the ITCs is mainly due to the modulation of the enzymes involved in the activation/detoxification of xenobiotics. In fact, they inhibit and deactivate the various cytochrome P450 isoforms that are implicated in the metabolic activation of many procarcinogens and, instead,

stimulate the expression of enzymes, such as GST, NADPH, quinone reductase (QR), aldo-ketoreductase and gamma glutamylcysteintetase (γ-GSC), involved in the detoxification of numerous carcinogens (Seow et al., 2001). Stimulation of GST enzymes is coordinated at the level of transcription by a regulatory element called the antioxidant response element (ARE). The detoxification signal is transmitted through an erythroid nuclear factor (Nrf2) that, at the nuclear level, binds ARE into the promoter region, with small different Maf proteins, and stimulates transcription (Itoh *et al.,* 1997; Venugopal and Jaiswal, 1998; Huang *et al.,* 2000). The exact role of Maf proteins is unknown, but it seems that Nrf2 dimerizes with them. Recent studies have suggested that Maf proteins are transcriptional repressors and Nrf2 binding removes this repressive activity. The kinases phosphorylate the Maf proteins which thus induce the transcription of ARE-dependent genes.

Furthermore, some ITCs also have another chemopreventive mechanism: the ability to block the growth of tumor cells and selectively stimulate their death through the induction of apoptosis (Hasegawa *et al*., 1999; Dai *et al*., 2016). Numerous laboratory experiments have shown this ability especially for sulforaphane and phenylethyl isothiocyanate (PEITC). PEITC induces apoptosis by activation of the c-JUN N-terminal kinase (Yu *et al*., 1996; Chen *et al*., 1998). PEITC also induces transactivation of the p53 oncosuppressor gene which, by stimulating apoptosis, promotes the elimination of cancer cells (Huang *et al*., 1998; Gupta *et al*., 2014).

Sulforaphane, on the other hand, induces apoptosis in various tumor lines, such as T-lymphoblastoid cells (Fimognari *et al.,* 2009), prostate cells (Singh *et al*., 2004) and melanoma cells (Misiewicz *et al*., 2003; Vanduchova *et al*., 2019).

More recently another ITC, 6-(methylsulfonyl) hesyl isothiocyanate (6-MITC) (Figure 2), present at high concentrations in *Wasabia Japonica* rhizome, has stimulated the interest of researchers.

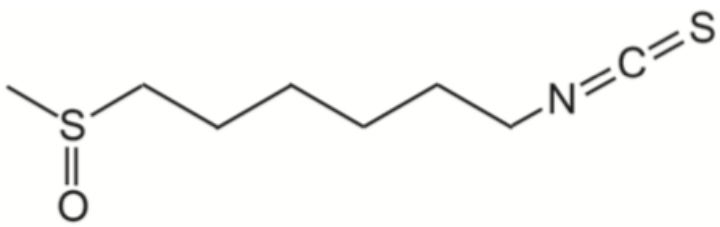


Figure 2: chemical structure of 6-MITC. Copyright Uchida et al., 2012

1.1.1 6-MITC

Wasabia Japonica, also known as wasabi or Japanese radish, is a plant of Japanese origin belonging to the *Brassicaceae* family. From the rhizome of this plant, a green paste with a particularly spicy taste is obtained, used in traditional Japanese cuisine (Figure 3).

Figura 3: Rhizome of Wasabia Japonica. Cpyright by https://wasabi.org/products/namida-100-pure-wasabi-powder/

Wasabi contains numerous isothiocyanates and 6-MITC is the most interesting active ingredient thanks to the demonstrated pharmacological properties and in particular, its antimicrobial (Hirokuni et al., 1998; Ko et al. 2016), anti-inflammatory (Uto *et al.,* 2005; Uto *et al.*, 2012), antiplatelet (Morimitsu *et al*., 2000), antioxidant (Mizuno *et al.,* 2010) and anticancer activities (Hou *et al.,* 2000).

Antimicrobial properties

Bacteria are single-celled microorganisms that can be both pathogenic and resident. The colonization or the greater growth of harmful bacteria in our body determines a pathological condition that can alter the homeostatic balance. The use of natural substances may represent an excellent strategy both to reduce the side effects of antibiotics and to preserve their action. Therefore, researchers focused on the study of different natural substances, with particular attention to isothiocyanates; it was observed that the ITCs act on various bacterial strains including Escherichia Coli, Klebsiella Pnuemoniae, Staphylococcus Aureus, Pseudomonas aeruginosa (Hirokuni *et al.,* 1998; Dufour *et al.,* 2015; Nowick *et al*., 2016). Analyzing the whole class, it emerged that the major antibacterial action occurs with 6-MITC thanks to the presence of a long methylenic chain. The mechanism by which the antibacterial action occurs is not yet completely clear, but it would seem to be attributable to an

interaction of the isothiocyanate with some proteins of the bacterial wall that would prevent the growth and duplication of the microorganism (Ko *et al.,* 2016).

Furthermore, some studies highlight the antibacterial action against *Helicobacter Pylori* by certain foods such as broccoli and wasabi. The mechanism seems to be due to an inhibitory action of isothiocyanates against the urease produced by *Helicobacter Pylori*, a fundamental enzyme for the survival of the bacterium. The authors of the study specify that the antibiotic activity against *Helicobacter pylori* would be attributed to the synergy of various substances, which act with different mechanisms of action. For example, some isothiocyanates that have strong anti-urease activity have been found to be free of bactericidal activity, and the same is true vice versa (Fahey *et al*., 2013).

Anti-inflammatory properties

A group of researchers from the International University of Nagasaki focused their studies on *Wasabia Japonica* and in particular on the anti-inflammatory properties of 6-MITC. The inflammatory process is one of the most important defense systems in our body. In inflammation, macrophages play a central role in the induction of enzymes, cytokines, chemokines and other phlogogenic factors; the overexpression of these factors is implicated in the pathophysiology of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis and inflammatory diseases of the nervous system (Medzhitov, 2008).

Through the studies conducted by Uto and collaborators, it has been shown that 6-MITC is able to block the expression of cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and other inflammatory cytokines, at the transcriptional level, in a murine macrophage cell line (RAW264) and in a human monocytic cell line (U937).

In particular, 6-MITC acts at the level of the MAPK signaling pathway, (main pathway involved in the inflammatory response), blocking the expression of COX-2 through the suppression of all three enzymatic pathways: the MAPK p38 pathway, the pathway of kinases regulated by extracellular signals (ERK) and the N-terminal kinase pathway c-Jun (JKN). 6-MITC also attenuates the expression of iNOS, blocking the activation of activator protein 1 (AP-1) and finally inhibiting the Janus kinase pathway (JAK2) that regulates the expression of inflammatory factors (Uto *et al.,* 2012).

Antiplatelet aggregation property

Already in the 20th century and more thoroughly in the 21st century, some researchers have investigated the potential use of 6-MITC as an antiplatelet agent (Kumagai *et al*., 1994; Morimitsu *et al.*, 2000). Platelet aggregation is a very important process regulated by numerous enzymes that must be perfectly balanced between them. In fact, an imbalance of these can lead to bleeding on the one

hand and on the other hand to the formation of clots that can cause myocardial infarction and/or stroke (Waltenberger *et al.,* 2016).

In view of the increased incidence of cardiovascular diseases, the potential benefits of these natural substances acquire fundamental importance. Studies conducted both in *vitro* and in *vivo* have given encouraging results, also clarifying the mechanism that determines the anti-aggregation action. The structure-activity relationship (SAR) of the 6-MITC was analyzed, which underlined the importance of the oxidized sulfur atom and of the long central chain consisting of 6 methylene groups. It has been seen, in fact, that the antiaggregation action of 6-MITC is not provided by an enzymatic block at the level of the enzymes involved in the coagulation cascade, but by the interaction of the isothiocyanate with thiol groups (SH) of proteins expressed on the surface of the platelets, which lead to the formation of a di-thiocarbamate on the outer membrane. This structural modification prevents the binding of platelets with its agonists (ADP, thrombin, thromboxane) and consequently blocks their activation (Morimitsu *et al*., 2000). Therefore, 6-MITC represents an excellent antiplatelet agent.

Antioxidant properties

Oxidative stress is a pathological condition caused by the breakdown of the physiological balance between production and elimination of oxidizing chemical species; this condition causes significant impairment of the functionality of cells and tissues. It is involved in cell aging (Droge and Schipper, 2007) and is associated with numerous pathologies such as: cardio-circulatory disorders (atherosclerosis, ischemia, stroke) (Blomgren and Hagberg, 2006), cancer (Hayes and McMahon, 2011) and neurodegenerative diseases (Parkinson's and Alzheimer's disease) (Lin and Beal, 2006).

In a study conducted on a rat neuronal primary culture, Mizuno and the research group of the University of Kyoto examined the antioxidant properties of 6-MITC. In particular, they showed that following treatment with 6-MITC, the cytoplasmic complex Keap1-Nrf2 dissociates and Nrf2 translocates into the nucleus, thereby activating the gene transcription, ARE-mediated, of numerous detoxification enzymes, such as γGCS, GSH and heme-oxygenase 1 (HO-1) (Mizuno *et al.,* 2010).

Free radicals are implicated in the etiology of many neurodegenerative pathologies and this study shows how isothiocyanates could provide a drug for their potential prevention and treatment (Mizuno *et al.,* 2010). To support this hypothesis, in a study conducted by Morroni *et al*. the neuroprotective potential of 6-MITC was evaluated in the murine model of Parkinson's disease. The data show that the antiparkinsonian effects induced by 6-MITC can be traced back to an increase in glutathione-dependent antioxidant systems and to a decline in apoptosis at the neuronal level (Morroni *et al.,* 2014).

Antitumor properties

Fuke *et al.* observed that 6-MITC blocks cell proliferation of gastric carcinoma cells (MKN-28) (Fuke *et al.,* 1994), cancer cells of breast tissue (MCF-7), melanomatous cells (LOX-IMVI) (Nomura *et al.,* 2004), and is also able to stop the pulmonary metastatic diffusion in mice of the C57BL/6 strain (Fuke *et al*., 2005). However, the mechanism underlying these activities is not yet fully known. A possible target is the epidermal growth factor receptor (EGF), a tyrosine kinase receptor that plays a key role in the development, proliferation and progression of tumor cells (Kellof *et al.,* 1996). Other hypothetical targets of the action of 6-MITC are represented by the factors that regulate apoptosis (Jarvis *et al.,* 1994; Watanabe *et al.,* 2002) and cell differentiation, also related to the activity of protein kinases (Davido *et al*., 2001). In a study conducted on epidermoid carcinoma cells (A431), the results suggest that the anticancer activity of 6-MITC involves the suppression of the EGF receptor and activates the non-receptorial PTK proteins involved in cell differentiation. The mechanism of suppression/activation of protein kinases is not yet clear; however, it is hypothesized that 6-MITC reacts with the cysteine residues of enzymes thus altering their function (Nomura *et al*., 2009). Finally, a team of researchers from Taiwan have recently conducted a study to assess the possible therapeutic effects of wasabi isothiocyanate on pancreatic cancer. The 6-MITC and two synthetic derivatives I7447 and I7557 were tested on two pancreatic cell lines, PANC-1 and BxPC-3. The study showed that 6-MITC and the two synthetic derivatives are able to reduce the tumor stem cell population (CSC) and the expression of SOX2, an essential transcription factor for self-renewal and cell differentiation (Chen *et al*., 2014).

1.2 Chemopreventive mechanisms

The current classification, proposed in 1985 by Wattenberg, classified chemopreventive agents into: inhibitory agents, blockers and suppressors (Wattenberg, 1985). It is however necessary to specify that this is a non-rigid outline, no chemopreventive agent is so specialized that it can intervene on a single target or in only one stage of carcinogenesis. On the contrary, most chemopreventive agents show multiple mechanisms of action that give rise to a pleiotropic response.

In fact, there are many compounds of natural or synthetic origin capable of reversing, blocking or at least slowing down the carcinogenic process, modulating the enzymes responsible for the detoxification/activation process of xenobiotics, or acting directly at the cellular level inducing apoptosis, inhibiting cellular proliferation and stimulating differentiation and autophagy (Manson, 2000).

1.2.1 Apoptosis

One of the main characteristics of cancer cells is the ability to escape the normal mechanisms of regulation of cellular survival/death. Stimulating apoptosis of transformed cells, therefore, represents an important chemotherapeutic strategy with the aim of hindering neoplastic development (Fulda, 2015). Apoptosis, or programmed cell death is a process essential for maintaining cellular homeostasis at tissue level. It is a necessary mechanism in numerous physiological processes including the development and functioning of the immune system, cell turnover, and embryo development; it also represents an important element of defense against the onset of different pathological conditions (Elmore, 2007). From a morphological point of view, during the early stages of the apoptotic process, a reduction in cell volume – accompanied by cytoplasmic and nuclear condensation – is observed. The integrity of the organelles and the membrane is maintained and, at first, also the integrity of the chromatin, which then undergoes fragmentation forming compact, electron-dense organelles, which migrate towards the nuclear periphery giving a cell a typical bean shape. During the terminal phase the plasma membrane surrounds the fragments of nuclear material generating the bubbles, which when detached from the body of the cell give rise to the apoptotic bodies. These are rapidly phagocytosed and digested by macrophages, thus preventing the release of material and enzymes in the surrounding tissues. The cell then rapidly loses volume, detaches from adjacent cells and exposes components on the outer membrane that are normally present in the intracellular side, such as phosphatidylserine or adhesive glycoproteins, and lastly it releases soluble factors that recruit macrophages. A peculiar characteristic of the whole process is therefore the selective elimination of the cell and the absence of inflammation (Hacker, 2000; Elmore 2007; Ouyang *et al.*, 2012). At the molecular level apoptosis is triggered by two main pathways: the

extrinsic (or receptor) pathway and the intrinsic (or mitochondrial) pathway. Although the induction of the two mechanisms is different, both pathways converge with the activation of effector caspases and result in cell death. The extrinsic pathway is induced following the interaction of specific cell death receptors (death receptors), belonging to the tumor necrosis factor (TNFR) family, with the respective ligands (TNF): TNFR1-TNF, FAS (CD95, APO- 1) -FasL, TRAILR1 (DR4) -TRAIL, TRAILR2 (DR5) -TRAIL (Guicciardi and Gors, 2009; Pistritto, 2016). Death receptors are transmembrane proteins characterized by an intracellular domain, called death domain (DD), which plays a crucial role in activating the proteolytic cascade. The ligand-receptor interaction determines a conformational change at DD level leading to functional receptor activation and subsequent recruitment of adaptive proteins such as FADD (FAS Associated protein with Death Domain) and TRADD (Tumor necrosis factor Receptor type 1-Associated DEATH Domain protein). The initiating caspases, following proteolytic activation, are therefore able to activate other proteins, including caspases 3, 6 and 7, (effector caspases) which are indispensable for the execution of programmed cell death (Fulda and Debatin, 2003; Pistritto *et al.,* 2016).

The action of the caspases is to generate preoteolytic cuts where aspartic acid residues are present in cytoplasmic and nuclear substrates. This system activates a cascading mechanism that culminates in the activation of nucleases, enzymes normally present in the cell in an inactive form, and consequent fragmentation of DNA.

In some cells, the extrinsic pathway alone is not sufficient to induce cell death, therefore they use a further step that exploits the caspase 8. Once activated, the initiating caspase is able to cleave and activate the Bid protein (BH3-interacting domain death agonist) generating the t-Bid fragment which, in its truncated form, stimulates the mitochondrial apoptotic pathways causing an interaction between the two pathways (Figure 4) (Pistritto *et al,* 2016).

The intrinsic pathway, on the other hand, is characterized by a variation in the permeability of the mitochondrial membrane without the involvement of transmembrane receptors. It can be activated by numerous signals, such as: deprivation of growth factors, UV radiation, damage to genetic material, oxidative stress, or stress induced by chemotherapeutic agents (Green and Kroemer, 2004; Kroemer *et al*., 2007). A central role is played by the proteins of the B-Cell Lymphoma 2 (Bcl-2) family, which thanks to the presence of transmembrane domains, regulate the permeability of the mitochondrial membrane through the formation of pores, Mithocondrial Outer Membrane Permeabilization, (MOMP). The generation of these pores causes the loss of the transmembrane mitochondrial potential and the release of cytochrome C in the cytosol. This protein recruits the Apoptotic Protease activating factor-1 (Apaf-1) and generates the formation of a multiprotein complex, called Apoptosama, which

binds and activates procaspases 9 (initiating caspases) through the CARD domain. (Figure 4) (Kroemer *et al.,* 2007).

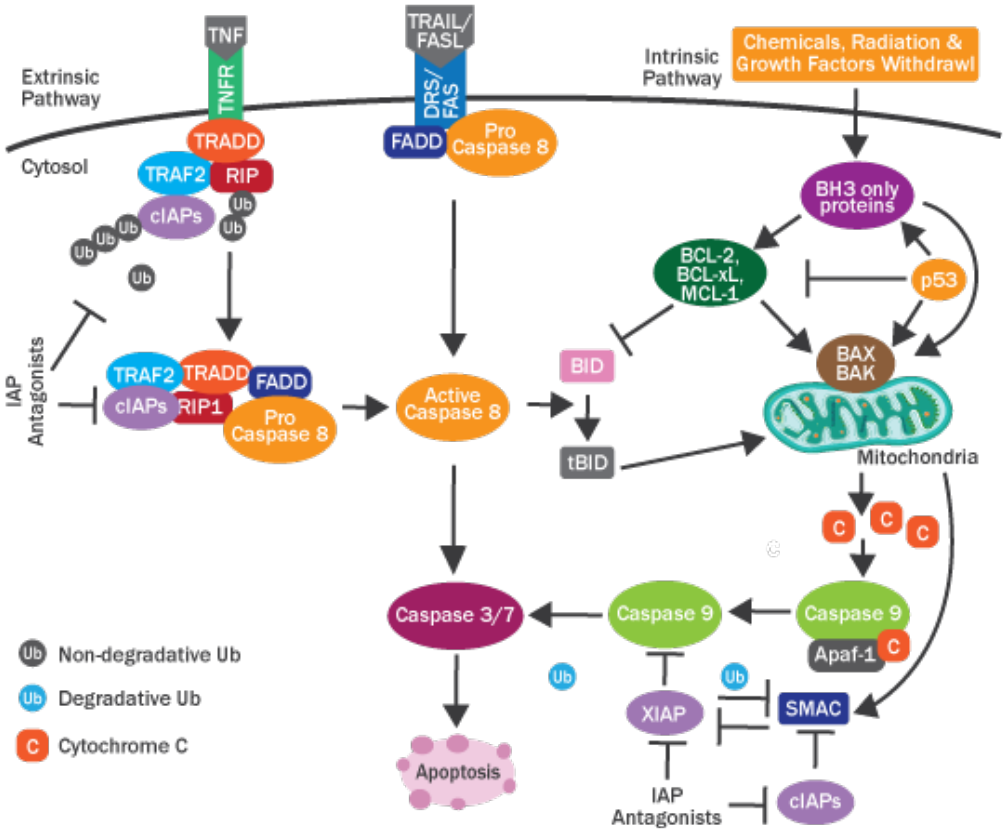


Figure 4: intrinsic and extrinsic pathway. Copyright by https://www.novusbio.com/antibody-news/tag/apoptosis

The proteins of the Bcl-2 family have both pro-apoptotic and anti-apoptotic functions and are classified into three subgroups depending on the number of Bcl-2 Homology domains (BH): BH1, BH2, BH3, BH4. The anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, present all four domains, whereas the pro-apoptotics are divided into: proteins with multiple BH domains such as Bax, Bak, Bok, or with a single domain (BH3 only) such as Bid, Bim and Bad (Figure 5) (Danial, 2007; Youle and Strasser, 2008).

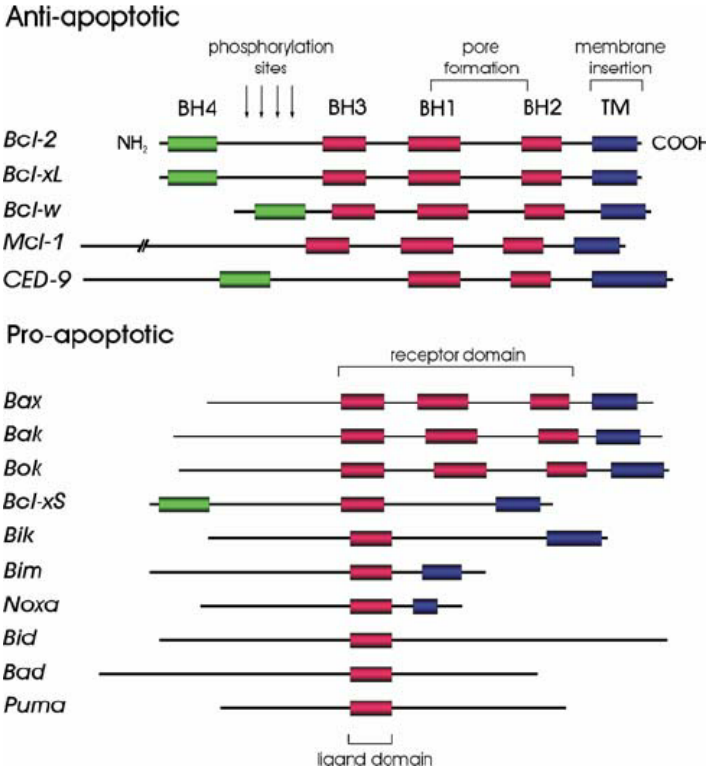


Figure 5: Anti-apoptotic and Pro-apoptotic protein. Copyright by Youle and Strasser, 2008

These proteins are able to interact with each other forming homo or hetero-dimers. Depending on their composition, homo/hetero dimers perform pro-apoptotic or anti-apoptotic functions. For example, the bax/bax homodimers have an pro-apoptotic action, while the bax/bcl-2 heterodimers perform an anti-apoptotic function. Therefore, the mitochondrial pathway is triggered, there is an increase in pro-apoptotic proteins, and consequently an increase in the bax/bax homodimer responsible for the increased permeability of the mitochondrial membrane (Dewson *et al.,* 2008; Brunelle and Letai, 2009).

Finally, a fundamental role in the modulation of the apoptotic process is covered by p53, the guardian of the genome, an important onco-suppressor gene which, following DNA damage, is activated thus regulating the cell cycle and/or stimulating apoptosis (Vousden and Lane 2007). It is therefore evident that p53 stimulation plays a crucial role, not only in preventing neoplastic transformation, but also as a therapeutic response aimed at tumor eradication (Vousden, 2000; Pistritto *et al.,* 2016).

1.2.2 Cell Cycle

The cell cycle or cell division is characterized by a sequence of events, coordinated and dependent on each other, that take place in a cell between one division and another. Cell division is generally divided into two main phases: interphase, which includes the phases G_1, S, and G_2 and Mitosis (M)

(Schafer, 1998; Wenzel and Singh 2018). The G_1 gap is a time of intense biosynthetic activity: the cell increases in size and synthesizes enzymes as well as organelles necessary for DNA duplication. DNA replication takes place in the next phase, specifically called the phase of Synthesis (S), which leads to the formation of two identical copies of DNA to be transmitted to the daughter cells. In contrast, during the G_2 phase, the cell continues to grow and synthesize new material, such as RNA and proteins, indispensable for the mitotic phase. This phase is, in turn, divided into five phases (prophase, prometaphase, metaphase, anaphase and telophase) and ends with cytodieresis, the physical division of the cytoplasm of the mother cell (Schafer, 1998; Wenzel and Singh 2018).
The progression of the cell cycle is regulated by different proteins; a key role is played by the cyclin-dependent kinases (CDK) which, in association with their respective cyclins, coordinate the correct progress of the cell cycle from one phase to another. While CDK levels remain constant, the levels of the cyclin rise and fall during cell cycle progression, activating the CDK periodically. During the G_1 phase the CDK4, CDK6 and CDK2 come into play; in the synthesis phase the CDK2 is activated again while the CDK1 acts both during the G_2 and during mitosis (Table 1) (Vermeulen *et al*., 2003).

CDK	**Cyclin**	**Cell cycle phase activity**
CDK 4	Cyclin D1 D2 D3	G1 phase
CDK 6	Cyclin D1 D2 D3	G1 phase
CDK 2	Cyclin E	G1/S phase
CDK 2	Cyclin A	S phase
CDK 1	Cyclin A	G2/M phase
CDK 1	Cyclin B	Mitosis
CDK 7	Cyclin H	Cak, all cell cycle phase

Table 1: CDK and cyclins involved in cell cycle

Numerous cyclins are then synthesized and degraded to allow the cell cycle to progress correctly. The first cyclin that comes into play is the Cyclin D, which when associated with CDK4/CDK6 controls and promotes entry into phase G_1. Another cyclin that acts in phase G_1 is cyclin E. It is associated with CDK2 and regulates progression in phase G_1 and the entry of cells into phase S. The Cyclin A and CDK2 complex is required instead during phase S and stimulates the beginning of DNA replication. At the end of phase G_2 it is the cyclin A-CDK1 complex that promotes entry into phase M, while synthesis of cyclin B (cyclin B-CDK1) is responsible for the degradation of the nuclear envelope and promotes the beginning of mitosis.

The function of CDK is contrasted by a group of proteins called CDK inhibitors (CKI); they, by binding the kinase alone or the CDK-cyclin complex, regulate its activity. There are two main families of CKI: the INK4 family and the Cip/Kip family. The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), p19 (INK4d) which specifically inactivates CDK4 and CDK6 by binding to the enzyme and consequently preventing interaction with cyclin D. The second family of inhibitors, on the other hand, includes p21, p27, p57 which inhibit the CDK-cyclin complexes. For example, p21 prevents entry into phase S by inhibiting the activity of the CDK4-cyclin D complex. The expression of p21 is strictly controlled by the p53 oncosuppressor and, through this interaction, p53 leads to the arrest of the cell cycle in the G_1 phase (Vermeulen *et al.,* 2003; Lim and Kaldis, 2013; Karimian *et al.,* 2016).

Cell division has several internal control points, which are fundamental for correct DNA duplication. If DNA damage is present, cell cycle repair and arrest mechanisms are activated. If the damage is repaired the cell cycle continues, otherwise the cell is selectively eliminated by activating the apoptotic pathways. The two main checkpoints are between phase G_1 and phase S (G_1/S checkpoint) and between phase G_2 and phase M (G_2 / M checkpoint). In the G_1/S checkpoint the blockade of the cell cycle is p53-dependent: normally the p53 levels are low but, when DNA damage occurs, there is a rapid induction of the p53 activity which in turn stimulates the transcription of different genes, including p21. When DNA damage occurs during the G_2 phase, the cell is able to block the cell cycle both in the presence and absence of p53. The G_2/M checkpoint therefore has the function of checking the integrity of the DNA after replication and prevents the entry of damaged cells into mitosis (Figure 6) (Vermeulen *et al.,* 2003).

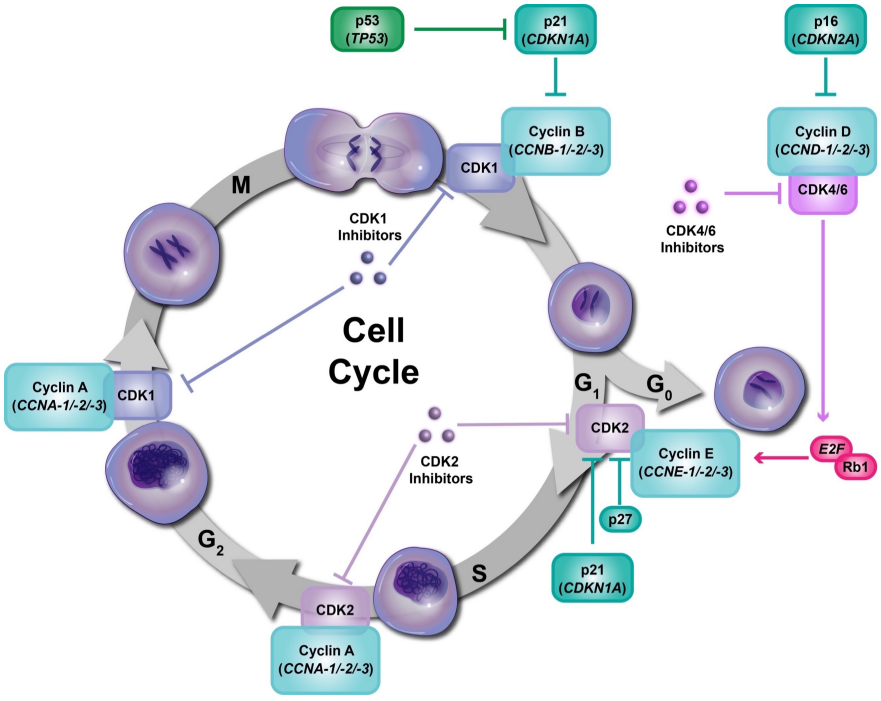


Figure 6: Cell Cycle progression. Copyright by https://www.mycancergenome.org/content /pathways/ cell-cycle-control/

It is therefore evident that the cell cycle is a finely controlled process and consists of a series of events on which correct cell proliferation depends. The alteration of this process leads to abnormalities of the normal cell cycle and therefore to uncontrolled proliferation, one of the main characteristics of neoplasms. Identifying therapeutic compounds capable of inhibiting cell cycle progression, and which act on specific targets, is certainly a winning approach to cancer treatment (Hanahan and Weinberg, 2011; O'Leary *et al.*, 2016).

1.2.3 Cytodifferentiation

Along with resistance to apoptosis and uncontrolled proliferation, tumor cells, and in particular leukemia cells, are characterized by a highly undifferentiated stage. Cell differentiation is a finely regulated process that leads to the formation of cells, tissues or organs with specific morphological, biochemical and functional characteristics. The clearest example of cellular differentiation is hematopoiesis: a process by which hematopoietic stem cells (HSC) differentiate, i.e. mature, transform, into the corpuscular component of the blood (Youssef, 2014). Blood cells have a very short half-life (eg: erythrocytes have an average life of 120 days) and for this reason they must be continually renewed throughout our life, about 10^{12} blood cells are replaced every day.

The HSCs are equipped with two fundamental characteristics: they are both capable of self-renewal by maintaining a pool of stem cells fundamental for continuous turnover and of differentiating

towards the lymphoid line, thus giving rise to B, T and NK (natural killer) lymphocytes as well as towards the myeloid line that includes monocytes, granulocytes (eosinophils, basophils, neutrophils), erythrocytes and platelets (Figure 7) (Laurenti and Gottgens 2018).

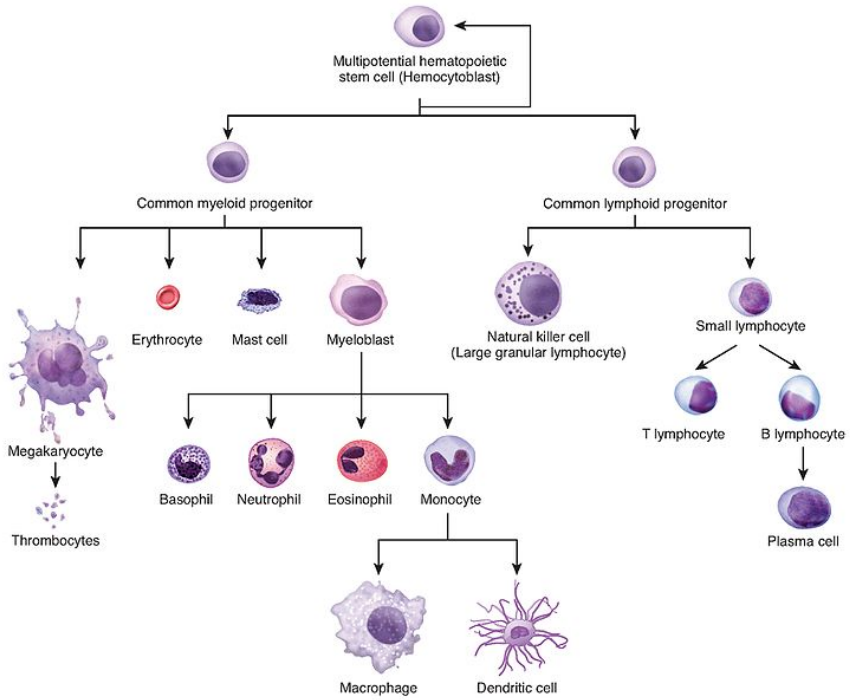


Figure 7: Hematopoiesis. Copyrigh by Hill, 2019

The differentiation of stem cells into mature blood cells is complexly coordinated by a group of hematopoietic cytokines, including: Colony-Stimulating Factors (CSF) – which includes GM-CSF, G-CSF, M-CSF and IL -3, Stem Cell Factor (SCF), Erythropoietin (EPO), Thrombopoietin (TPO), Tumor Necrosis Factor alpha (TNF- α) and Interferon (IFN) (Metcalf, 2008). They bind with high affinity to specific receptors, triggering a series of signal transduction pathways [protein Kinase C (PKC) pathway, mitogen-activated protein kinase (MAPK) pathway, Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway] which lead to the activation and/or repression of genes responsible for controlling cell proliferation and differentiation (Metcalf, 2008). Hematopoiesis is the result of a delicate balance between cell proliferation and differentiation on the one hand and apoptosis and senescence induction on the other; it is therefore clear how the alteration of this delicate homeostatic balance can lead to different hematological pathologies including leukemia (Figure 8) (Leung *et al.,* 2005).

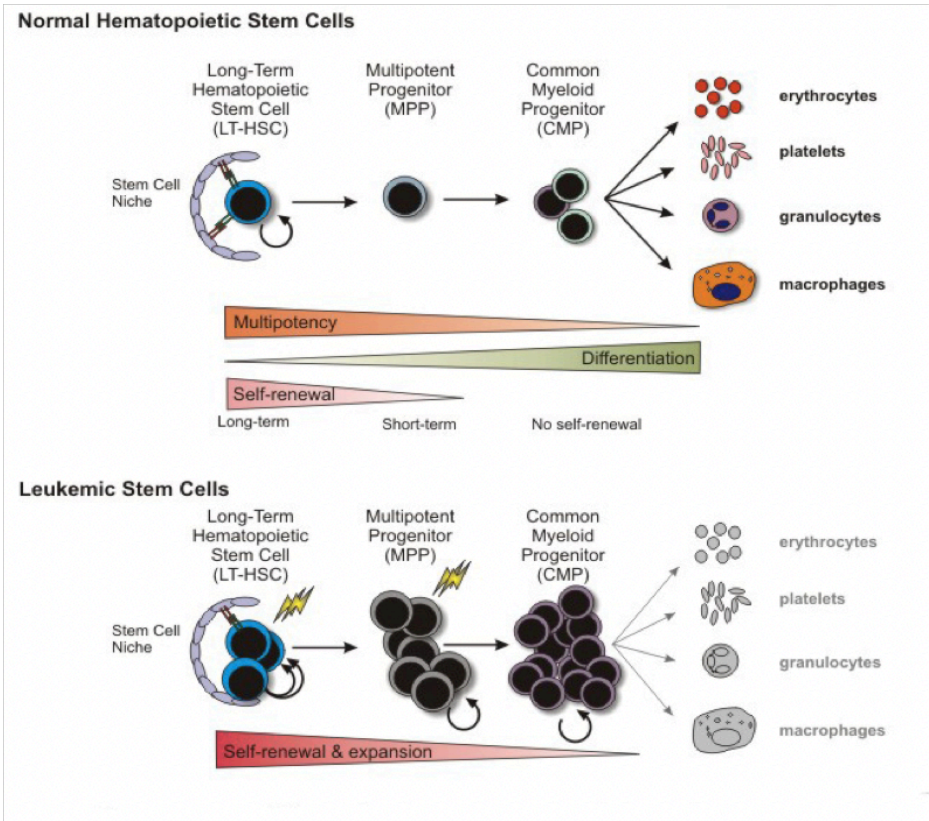


Figure 8: Alteration of the hematopoietic process: leukemia. Copyrigh byt https://www.rug.nl/research/internal-medicine/hematologie/researchlines/res3?lang=en

Between the end of the 1970s and the beginning of the 1980s, the publication of scientific studies on the use of natural or synthetic substances in order to stimulate malignant cells to differentiate has revealed a new possible strategy to combat leukemia. From here, the concept of "differentiation therapy" is born, whose purpose is precisely to overcome the differentiation block, typical of leukemia cells, and stimulate their maturation (Sachs,1978; Huberman and Callaham 1979). All-trans retinoic acid (ATRA) is one of the most successful in this field and especially in the treatment of acute promyelocytic leukemia (APL), one of the most aggressive subtypes. ATRA, by binding to the retinoic acid receptor (RAR α), inhibits cell proliferation and simultaneously removes the maturation block of the promyelocytes inducing their differentiation. Treatment with ATRA alone leads to complete remission, but of short duration; most patients suffer relapse, refractoriness to treatment and in some cases, they develop a cardio-respiratory syndrome called "ATRA syndrome", characterized by fever, dyspnea, pulmonary infiltrates, pleural and pericardial effusions, episodic phenomena of hypotension and acute renal damage (Montesinos and Sanz, 2011). With the discovery in the 1980s of retinoic acid, numerous studies have been carried out in order to emulate its success on the one hand and overcome its toxic effects on the other; several studies have proposed multiple molecules

and the most well-known include TPA (12-O-tetradecanoylphorbol-13-acetate), Vitamin D3 and derivatives of retinoic acid which, however, have not achieved the same success (Nowak *et al*., 2009) At the end of the 1990s a group of Chinese researchers announced the results obtained using arsenic trioxide (ATO or As2O3) in the treatment of leukemia. ATO performs a dual action: in low concentrations it induces differentiation, while in high concentrations it stimulates apoptosis causing the death of leukemia cells (Chen *et al.,* 1997). Currently, as demonstrated by the phase 3 study APL0406, the therapy of choice for the treatment of APL foresees the association of ATRA and ATO that, in synergy, stimulate the cells to cellular differentiation notably reducing the percentage of relapses, due to the association of ATRA and classic chemotherapy (anthracycline, Ara-C) (Platzbecker, 2017; Schultze *et al.,* 2018).

The study and synthesis of new molecules capable of stimulating differentiation without exhibiting high adverse effects may represent, in the future, essential therapy against hematological tumors. In fact, despite the successes of classic therapies in the treatment of pathology (chemotherapy, radiotherapy, transplantation), many patients still die due to relapses, therefore, a more effective and less cytotoxic approach is certainly required (Nowak *et al.,* 2009; Laurenti and Gottgens, 2018) (Figure 9).

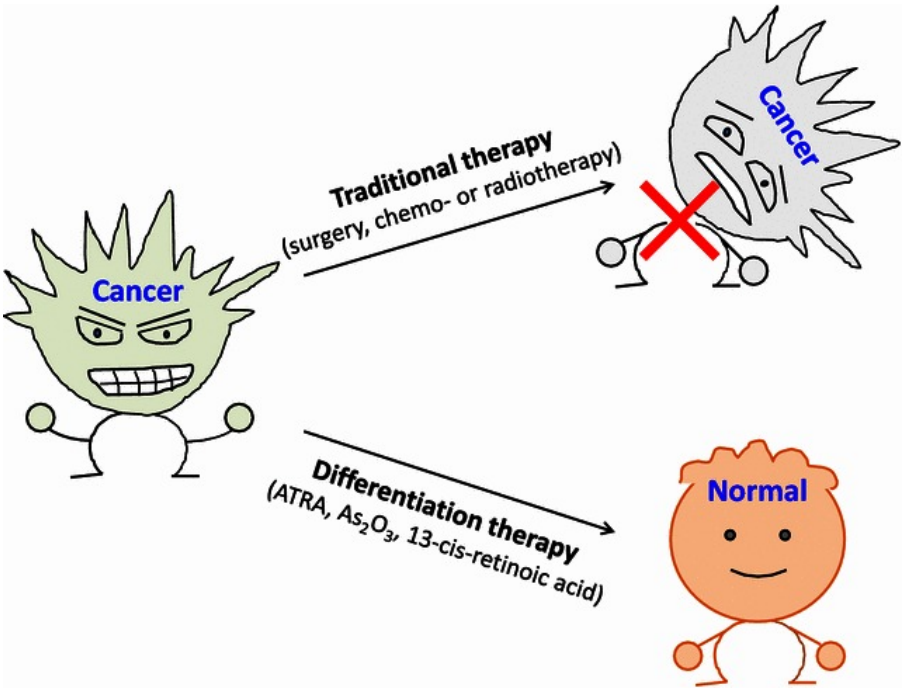


Figure 9: Traditional therapy vs Differentiation therapy. Copyright by Yan and Liu, 2016

1.2.4 Autophagy

The term autophagy, from the Greek *autòs* "oneself" and *phagéin* "to eat", is a homeostatic, catabolic process highly conserved in all eukaryotes (Glick *et al*., 2010). Three main types of autophagy have been characterized in mammalian cells: Microautophagy, Chaperone-Mediated Autophagy (CMA) and Macroautophagy; they are morphologically different processes, but all three end with the degradation and recycling of cellular components within lysosomes (Yang and Klionsky, 2010; Parzych and Klionsky, 2014). Macroautophagy is certainly the most studied mechanism and the one referred to when speaking more generally about autophagy. It is a finely regulated process, in fact, in normal conditions, it is found at very low basal levels in order to prevent the gradual accumulation of damaged proteins and organelles, which over time would be toxic to the cell (Mizushima and Komatsu, 2011). In stressful situations, however, such as nutrient and energy deficiency or hypoxic conditions, autophagy is induced to provide an alternative resource of metabolic substrates, indispensable for cellular survival. Furthermore, autophagy performs multiple functions: in fact, it is involved in embryonic development, in cellular differentiation and proliferation, in aging, in innate immunity, and defends the organism from infection by viruses and bacteria by intracellular degradation (Yormitsu and Klionsky, 2005; Parzych and Klionsky, 2014).

It is therefore mainly a cytoprotective mechanism; however, dysfunctions of the autophagic system are associated with numerous pathologies such as neurodegenerative diseases, cardiomyopathies, cellular aging, metabolic dysfunctions and cancer (Wirawan *et al*., 2012; Parzych and Klionsky, 2014).

Macroautophagy is characterized by the formation of an autophagosome, a double membrane structure that sequesters cytoplasmic proteins, mitochondria, endoplasmic reticulum and ribosomes for the degradation, lysosome mediated, of the material to be recycled (Levine and Klionsky, 2004; Radogna *et al*., 2015). It is generally divided into 4 phases: initiation, nucleation, maturation/fusion and degradation. In the initiation phase, a crucial role is played by the mammalian Target of Rapamycin Complex 1(mTORc1). Under basal conditions, such as abundance of nutrients, mTORc1 is activated and, by phosphorylation of the Unc-51-like kinases 1(ULK1) complex (composed by ULK-ATG13-FIP200-ATG101), inhibits the autophagic process. Nutrient deprivation, on the other hand, leads to the activation of the AMP-activated protein kinase (AMPK) which, in turn, stimulates the initiation of autophagy both indirectly – inhibiting the mTORc1 complex – and directly, activating the ULK1 complex (Mizushima *et al*., 2011). In the next phase, the nucleation phase, another important regulatory complex comes into play: the PI3K-III complex (PhosphatidylInositol-3-Kinase Class-III (PI3K-III) complex composed of the proteins VPS34, VPS15, ATG14 and Beclin1. This complex is mainly regulated at Beclin1 level and the main regulators are: the anti-apoptotic protein

Bcl-2 (negative regulator of Beclin1) and AMBRA1 (positive regulator of Beclin1). The dual function of the Bcl-2 protein in inhibiting the apoptotic and autophagic pathways is a clear example of how the two mechanisms are closely related (Parzych and Klionsky, 2014). The PI3K-III complex is the main actor in phagophore formation; in fact, through the recruitment of ATG proteins, it is responsible for the lengthening and maturation of the autophagosome. In mammals, various sites of phagophore formation have been observed, including the plasma membrane, mitochondria, endoplasmic reticulum, Golgi apparatus, and the nucleus. The maturation process then, requires the intervention of two ubiquitin-like conjugation systems (UBL). ATG12-ATG5-ATG16L is the first UBL system that comes into play: it is associated with the vesicle membrane in the training phase and dissociates only once the autophagosome is completed. The second ubiquitin-like system involved is the microtubule-associated protein light chain 3 (LC3) system (Parzych and Klionsky, 2014). The LC3 protein is cleaved by the ATG4 protease to form the LC3-I fragment, which then combines with phosphatidylethanolamine to generate LC3-II. Phosphatidylethanolamine acts as a lipid anchor to allow the insertion of LC3-II at the level of the autophagosome membrane. The LC3 protein is commonly used as an autophagic marker, as its presence is correlated with the number of autophagosomes. The last step involves the fusion of the autophagosome with the lysosome and the degradation of the material to be recycled by lysosomal enzymes (Figure 10) (Radogna *et al*., 2015).

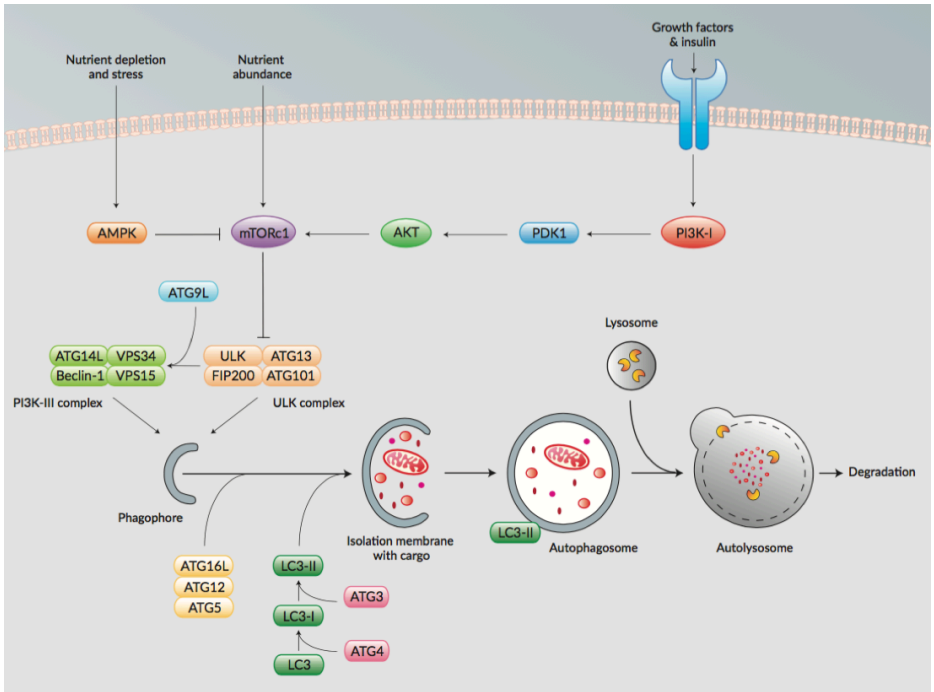


Figure 10: Autophagic mechanism. Copyright by https://www.invivogen.com/review-autophagy

In recent years, thanks to the awarding of the Nobel Prize for Medicine to Yoshinori Oshumi in 2016, the study of autophagy is becoming more and more interesting. In fact, thanks to the discoveries of this Japanese scientist, it was possible to define the mechanism underlying the autophagic process and the numerous pathologies related to it, including cancer (Ohsumi, 2014). The maintenance of cellular homeostasis, through autophagy, is essential to prevent cancer; however, this mechanism represents "a double-edged sword" since it not only suppresses, but is also capable of promoting cellular survival in the tumor environment. This paradox can be clarified by analyzing the role that autophagy plays during tumor progression (Jin *et al*., 2017). In the early stages of tumor transformation, autophagy represents a defense mechanism towards neoplastic growth. In fact, through the elimination of damaged mitochondria it prevents the accumulation of ROS, which are now known to represents the main source of oxidative stress. Consequently, the lack of the process at an early stage leads to chronic oxidative stress, accumulation of damaged mitochondria, tissue damage and inflammation, events that jointly promote tumor initiation. In later stages, however, autophagy represents an adaptive response of cancer cells. They use this mechanism in response to cellular stress and/or increased metabolic demands related to rapid cell proliferation, which can promote tumor growth and therapeutic resistance. In this case therefore, inhibiting autophagy could prove to be an important therapeutic strategy (Figure 11) (Poillet-Perez *et al.,* 2015).

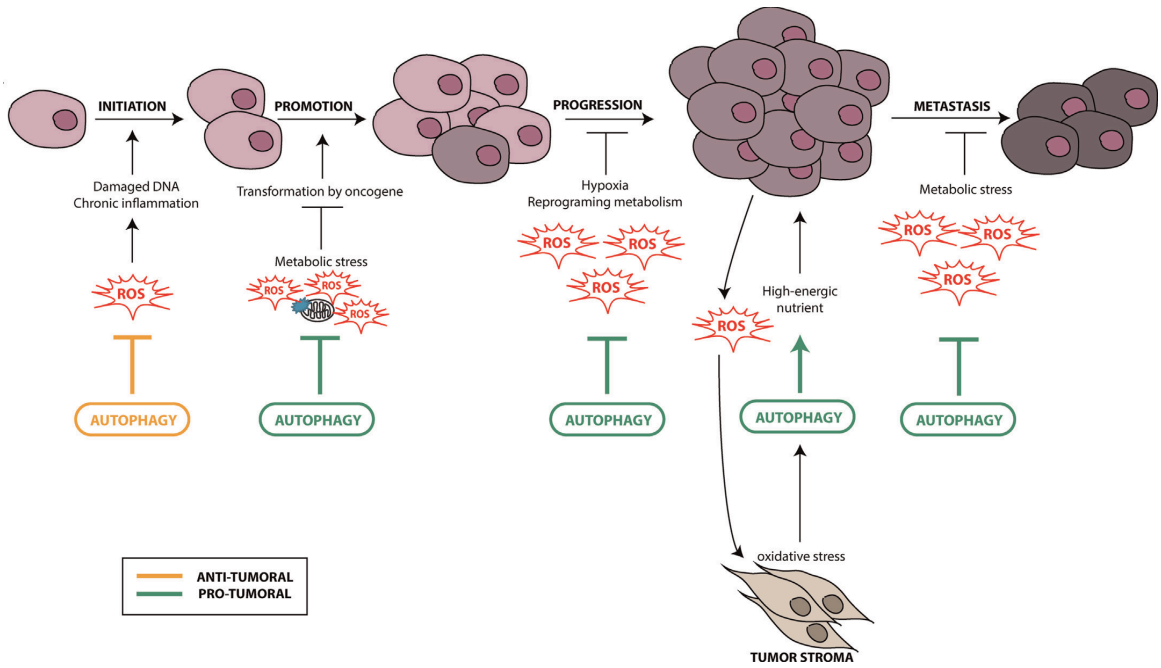


Figure 11: Autophagy and cancer. Copyright by Poillet-Perez et al., 2015

1.2.5 Antimutagenesis

Genetic material is constantly subject to damage by exogenous agents (chemical or physical factors) or endogenous processes, such as spontaneous damage to DNA. The term mutagenicity was introduced in 1901 by Hugo de Vries and indicates those modifications of the genetic material of a living organism that, if compatible with life, are transmitted to subsequent generations (Maurici *et al.*, 2005; Eastmond *et al*., 2009). The term "genotoxicity", on the other hand, represents a broader concept of mutagenicity and describes the ability of a substance to induce modifications within the DNA sequence. The genotoxic effects on DNA are not always related to mutations, in fact, not all genotoxic agents are mutagenic (Maurici *et al.,* 2005; Eastmond *et al*., 2009).

Mutations can occur at the level of germ cells or somatic cells with very different consequences. Germ line mutations affect the gametes and therefore can be transmitted to the offspring; somatic mutations, on the other hand, affect an individual's somatic cells and are transmitted to the progeny of the cell affected in origin, generating a "genetic mosaic" that will contain both wild cells and mutated cells. Somatic mutation is a phenomenon of fundamental biological importance in that it plays a crucial role in the etiology of numerous degenerative diseases, including cancer. Mutagens are generally divided into three categories: direct mutagens, able to interact directly with DNA; promutagens, compounds that in themselves are not reactive but must be converted to carry out their action; and indirect mutagens, substances that cause modifications of the genetic material while not reacting directly or as metabolic derivatives (Basu, 2018).

In recent years, the study and identification of compounds capable of counteracting the effects of mutagenic agents is creating great interest (Słoczyńska *et al*., 2010). In 1952 Novick and Szilard coined the term "antimutagen" to indicate those compounds, both natural and synthetic, capable of reducing the rate or frequency of induced or spontaneous mutations (Novick and Szilard, 1952). Based on their mechanism of action, they are classified as: Antimutagens with antioxidant activity, Antimutagens that inhibit the activation of mutagens, Antimutagens as blocking agents (Kada *et al.,* 1982; De Flora, 1992; Słoczyńska *et al*., 2014).

Many mutagens, such as Mitomycin-C, act through the generation of ROS. The use of substances with antioxidant activity, therefore, represents an important strategy in order to remove the reactive species generated before they react with DNA. In a study conducted by Unal and research assistants, lipoic acid, a known antioxidant agent, was shown to have an antimutagenic effect against Mitomycin-C (Tian *et al*., 2012; Unal *et al*., 2013).

Other antimutagens, on the other hand, exert their activity by inhibiting the enzymes responsible for the biotransformation of promutagens to mutagens. Isothiocyanates, for example, act on phase I

enzymes (cytochrome P450 1A1 and 1A2) thus preventing the metabolic activation of the promutagen (Hamilton and Teel, 1995).

Finally, the blocking agents interact directly with the mutagenic compound, preventing it from reaching the target sites. The extract of Acanthopanax divaricatus eliminates the mutagenic compounds from the cells, before the induction of damage to the genetic material, thus acting as a blocking compound (Hong *et al.*, 2011).

The subdivision in categories however, must not be understood too rigidly because, due to their complexity, the substances can act through multiple mechanisms of action (Figure 12).

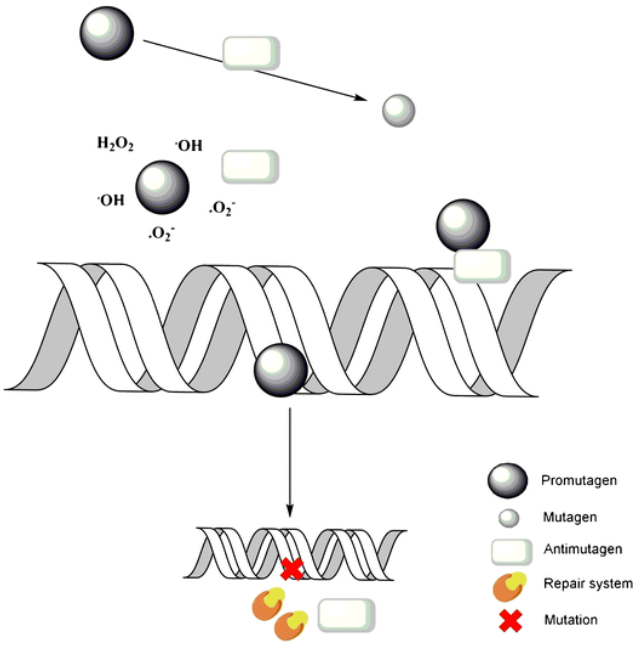


Figure 12: mechanism of action of mutagens. Copyright by Słoczyńska et al., 2014

In the field of drug discovery, the study of mutagenicity represents a fundamental step, inasmuch as any candidate compound for use as a therapeutic agent must not show mutagenic activity. To this end, the Organization for Economic Cooperation and Development (OECD) has identified numerous tests, both in *vitro* and in *vivo*, in order to identify the potential mutagenic and carcinogenic risks of a substance before it is placed on the market (Combes *et al.*, 2007). In August 2017, the OECD published the last overview indicating the eliminated, revised and recently validated tests (OECD 487, 2016) (Table 2).

TG	Title	Adopted	Revised	Deleted	Reference
	Recently Revised				
473	*In vitro* mammalian chromosomal aberration test	1983	1997 / 2014		OECD, 2014a
474	Mammalian erythrocyte micronucleus test	1983	1997 / 2014		OECD, 2014b
475	Mammalian bone marrow chromosomal aberration test	1984	1997 / 2014		OECD, 2014c
476	*In vitro* mammalian cell gene mutation test using the *Hprt* and *xprt* genes	1984	1997 / 2015		OECD, 2015a
478	Rodent dominant lethal test	1984	2015		OECD, 2015b
483	Mammalian spermatogonial chromosomal aberration test	1986	1997/2015		OECD, 2015c
	Recently Adopted				
487	*In vitro* mammalian cell micronucleus test	2010	2014		OECD, 2014d
488	Transgenic rodent somatic and germ cell gene mutation assays	2011	2013		OECD, 2013
489	*In vivo* mammalian alkaline comet assay	2014			OECD, 2014e
490	*In vitro* gene mutation assays using the *TK* gene	2015			OECD, 2014d
	Archived/Deleted				
472	Genetic toxicology: *Escherichia coli*, Reverse Assay	1983		1997	
477	Sex-linked recessive lethal test in *Drosophila melanogaster*	1984		2014	
479	*In vitro* sister chromatid exchange assay in mammalian cells	1986		2014	
480	*Saccharomyces cerevisiae,* gene mutation assay	1986		2014	
481	*Saccharomyces cerevisiae,* mitotic recombination assay	1986		2014	
482	DNA damage and repair, unscheduled DNA synthesis in mammalian cells *in vitro*	1986		2014	
484	Mouse spot test	1986		2014	
	Retained, but not revised				
471	Bacterial reverse mutation assay	1983	1997		OECD, 1997a
485	Mouse heritable translocation assay	1986			OECD, 1986
486	Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo	1997			OECD, 1997b

[1]After the revision, TG 476 is only used for the mammalian cell gene mutation test using the *Hprt* or *xprt* locus

Table 2: Overview OECD Guidline. Copyright by ENV/JM/MONO(2016)33/REV1

Micronucleus test -OECD 487

The micronucleus test is a mutagenesis test that allows research scientist to evaluate chromosomal damage by detecting the presence of micronuclei in the cytoplasm of cells in interphase (OECD 487, 2016). Micronuclei (MN) are small accessory nuclei that originate from the loss of entire chromosomes or fragments of chromosomes during mitosis and remain quite distinct from the main nucleus of the cell (Figure 13) (Fenech *et al*., 2003).

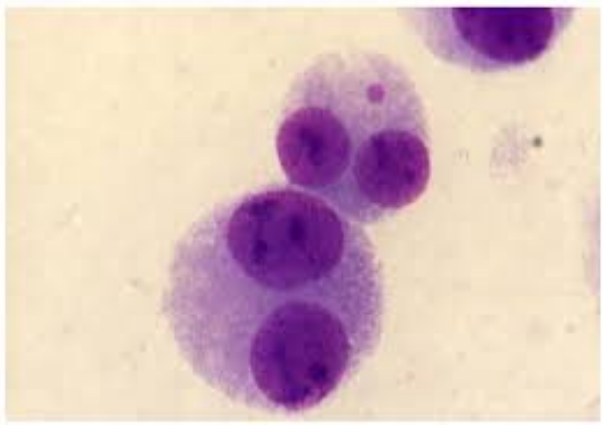


Figure 13: Micronuclei in binucleate human lymphocytes, observed under an optical microscope.

The micronucleus has long been used as an important biomarker of chromosomal damage, genomic instability and cancer risk (Holland *et al*., 2008; Iarmacovai *et al*., 2008).

The analysis of MN is, therefore, a valid indicator of genetic damage that offers the advantage of simultaneously quantifying structural chromosomal alterations (clastogenicity) and numerical anomalies of the karyotype (aneuploidy) (Fenech *et al*., 2003).

There are various causes that can lead to the formation of MN: exposure to environmental pollutants, ionizing radiation, cytotoxic drugs, as well as incorrect eating habits and damage caused by oxidizing agents such as free radicals (Konopacka, 2003; Lehucher-Michel *et al*., 1995). Other causes may be the presence of chronic inflammation (Nair *et al*., 1991; Suruda *et al*., 1993), contact or ingestion of heavy metals (Goud *et al*., 2004; Sailaja *et al.,* 2006), genetic pathologies (Rosin and Anwar, 1992; Thomas *et al*., 2008), infections (Rosin and Anwar, 1992; Cortes-Gutierrez *et al*., 2010) and nutritional deficiencies (Fenech, 2005).

The mechanisms responsible for the formation of MN are the breaking of a chromosome (the agent that causes the damage is called clastogen) or the altered formation and activity of the mitotic spindle (aneuploidogenic agent) (Figure 14).

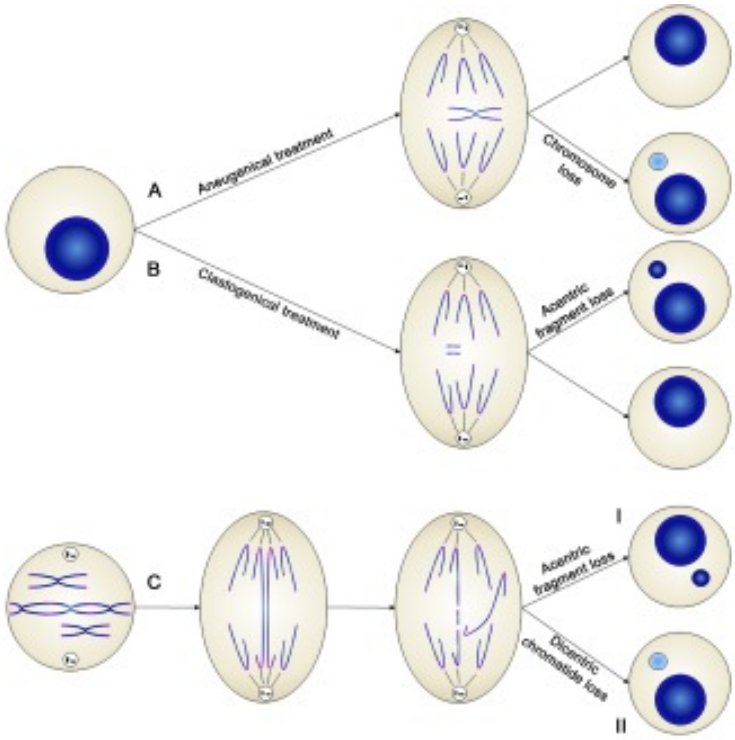


Figure 14: Micronucleus formation mechanism. Copyright by Terradas et al., 2010

The formation of a micronucleus can also occur spontaneously as a result of physiological events taking place in the cell. One of these processes is the double-stranded breakage of DNA, a physiological phenomenon that occurs when the cell passes from the G_0 phase to the G_1 phase of the cell cycle; this indicates therefore that the formation of micronuclei can be considered a process that normally occurs in a healthy cell, obviously with a much lower probability than cells exposed to mutagenic agents (Bartkova *et al.*, 2005; Pickering and Kowalik, 2006).

Exposure to mutagenic agents can, on the other hand, cause the onset of micronuclei by different mechanisms. For example, the loss of a chromosome or a chromatid can be generated by damage to the mitotic spindle, manifested by the loss of the binding capacity of the microtubule to the kinetochore. Merotelic orientations of chromosomes may be caused by an incorrect binding of the microtubule to the kinetochore, an event that can lead to the formation of micronuclei (Cimini and Degrassi, 2005; Mateuca *et al*., 2006). Other causes of micronucleus formation can be traced to centromere damage, or to kinetochore damage, which leads to the loss of chromosomes and the consequent origin of aneuploid cells. Moreover, the mutation of some genes that control the cell cycle, and more specifically genes that control the formation or destruction of the mitotic spindle, or phenomena such as slow DNA replication, peripheral localization of nucleus and centromere

hypomethylation are factors which predispose cells to micronuclei formation (Albertini *et al.,* 2000; Fenech, 2005; King, 2008).

The micronuclei that originate instead from the loss of chromosomal fragments can be created by breaking the double strand of DNA, by transforming the break of a single strand into a double-strand break after cell division or by inhibition of DNA synthesis. The fragmentation of a chromosome can also derive from the disruption of the cytoplasmic bridges formed due to a chromosomal rearrangement during the anaphase, as happens for example for dicentric chromosomes, due to gene amplification phenomena when the amplified genetic material is recognized by the cell, covered by a nuclear membrane, and expelled from the cell itself (Norppa and Falck, 2003), or by the union of sister chromatids (Mateuca *et al.,* 2006).

The life of the micronucleus, on the other hand, is not yet fully known. Many studies have tried to describe the possible events affecting the micronucleus after its formation (Leach and Jackson-Cook, 2004) and there are several hypotheses:

- maintenance of cell cytoplasm MN as an extranuclear entity after the cell has completed several cell cycles;

-possible incorporation of MN in the main nucleus (when it becomes indistinguishable from the remaining genetic material, it can resume normal biological activity);

- expulsion of MN from the cell (this occurs when the DNA of the micronucleus is not functional or is unable to replicate due to the lack of essential cytoplasmic components) (Terradas *et al.,* 2010).

The micronucleus can also undergo apoptosis. There are two possible ways: apoptosis can affect the whole cell including the micronucleus, or the event only involves the micronucleus/i (Decordier *et al*., 2002; King, 2008).

Since the micronucleus can only be expressed by cells that have gone through a phase of mitosis, it is necessary to be able to identify those cells that have divided at least once; for this reason, the ideal cell cycle stage to detect them is the binuclear one (Fenech and Morley, 1986). Their frequency, therefore, which is observed in a cell population in active replication, is influenced by the rate of proliferation and environmental conditions (Martinez-Expòsito *et al*, 1994; Al Sabti and Metcalfe, 1995).

The MN test cannot therefore be conducted effectively in cells that are not in active replication, or in replicating cell populations but in which the kinetics and proliferation rate is not yet well understood or controlled (Fenech, 2000).

The method that has so far been most successful, thanks to its simplicity, is the micronucleus test with blockade of the cytodieresis (CBMN) (Fenech and Morley, 1986).

In CBMN, cytodieresis is prevented in cells that have completed nuclear division by using cytochalasin-B, an inhibitor of actin polymerization necessary for the formation of the ring of microfilaments that restricts the cytoplasm between the nuclei of daughter cells during mitosis (Carter, 1967). With this technique, micronuclei are detected in those cells that have completed nuclear division, that is binucleate cells (Fenech, 2000).

Morphologically, the micronuclei are identical to but smaller than nuclei. Their diameter, in fact, generally varies between 1/3rd and 1/16th of the normal diameter of the main nuclei, whereas the area goes from 1/9th to 1/256th of the area of one of the two main nuclei in a binucleate cell.

The micronuclei are not refractive; they can therefore be easily observed by staining them with dye; normally they have the same color gradation as the main nuclei, but sometimes this can be more intense. Moreover, they are not linked or connected in any way to the main nuclei: they can appear close to the point of touching them, without, however, overlapping them. (Fenech, 2000).

The characteristics of this test, and in particular its greater rapidity and the less technical preparation required compared to the chromosomal aberration test, have favored its wide diffusion as an effective and relatively simple method for evaluating both the clastogenic and aneuploidogenic potential of a vast number of substances (Nesslany and Marzin, 1999). Given the growing enthusiasm for this method, efforts are also increasing to automate the analysis phase, in order to overcome some limitations, such as the subjectivity of reading results and long analysis times (Nüsse and Marx, 1997; Lenzi *et al*., 2018).

CHAPTER 2

RESEARCH AIM

The aim of my research project was to study the potential of 6-MITC as a chemopreventive agent in leukemia cell lines.

At the end of 1970, Dr Sporn coined the scientific term "chemoprevention" to define the ability to prevent, halt or reverse the cancer process with the use of synthetic or natural compounds (Sporn and Suh, 2002; Sporn and Lyby, 2005). The development and progression of cancer is associated with a series of events, including dysregulation of tumor suppressor genes, cellular differentiation, uncontrolled proliferation, dysfunction of apoptotic genes and alteration of the autophagic mechanism (Hanaahan and Weinberg, 2011). Therefore, acting on these processes to restore regular mechanisms is one of the most important strategies for fighting cancer (Raicht *et al*., 1980; Hosseini *et al*., 2017). Phytochemicals derived from edible and medicinal plants have been extensively studied for cancer chemoprevention because of their efficacy in modulating the enzymes involved in xenobiotic activation/detoxification, inhibiting cell proliferation and/or inducing apoptosis, differentiation and autophagy of neoplastic cells (Nowak *et al*., 2009; Goozee *et al*., 2016). A large number of pure compounds and extracts has also been tested in various experimental models due to their long history of human exposure, their high tolerability and low toxicity. In fact, a promising chemopreventive agent should act selectively on cancer cells and cause low toxicity in non-transformed cells (Fimognari *et al.,* 2005; Fimognari *et al*., 2009).

In recent years, growing interest has been focused on ITCs and more recently on 6-MITC, which is found in high concentrations in *Wasabia Japonica* rhizome (Zhang, 2012; Kumar *et al*., 2015). *Wasabia Japonica* is becoming increasingly important in the scientific field due to the presence of several ITCs including 2, 4, 6 and 8-MITCs. Among them, 6-MITC has the higher concentration and proves to be the most interesting bioactive compound. Numerous scientific and epidemiological studies have confirmed, for example, its significant anti-inflammatory (Uto *et al*., 2005; Uto *et al*., 2007; Uto *et al*., 2012) and antioxidant (Hasegawa *et al.,* 1999; Morimitus *et al*., 2000; Trio *et al.,* 2016) properties, suggesting an hypothetical use in chemoprevention. However, its use implies an exhaustive and correct characterization also from a toxicological point of view, which demonstrates the overcoming of numerous and important tests for safety purposes, first and foremost in terms of mutagenicity.

The research was carried out sequentially. In the first instance, antiproliferative, pro-apoptotic and autophagy effects were analysed in Jurkat and HL-60 cell lines. HL-60 are an acute human promyelocytic leukemia cell lines and thus represent an ideal model for the study of

cytodifferentiation. For this reason, the ability of isothiocyanate to induce differentiation towards the macrophage and/or granulocytic phenotype was also studied. In addition to evaluating its cytostatic and cytotoxic effects on transformed cells, its selectivity for non-transformed human peripheral blood lymphocytes was also tested using the same endpoints.

There are two main apoptotic pathways, the extrinsic pathway and the intrinsic pathway; the latter is regulated by the activation or deactivation of BCL-2 family genes (Gerl and Vanux, 2005). For this reason, possible molecular mechanisms, such as the intrinsic and extrinsic apoptosis pathways, apoptotic markers regulating certain tumourigenesis and the cell proliferation mechanisms, such as p53, BAX/BCL-2 ratio, cytochrome c, cyclin E2 and cyclin D3 levels, were analysed (Lenzi *et al*., 2018).

Furthermore, despite apoptosis and autophagy constitute two distinct cellular pathways, the proteins that control their regulation and execution are closely related (Booth *et al*., 2014). Therefore, it is extremely interesting to evaluate the modulation of the autophagic process and the related pathways, paying particular attention to the ROS-apoptosis-autophagy interconnection**.**

In the second phase of the research, the mutagenicity of 6-MITC was evaluated through the MN test, using TK6 cell lines as indicated by the OECD guideline no. 487 (OECD 487, 2016). First the cytotoxic potential of 6-MITC was evaluated on TK6 cells in terms of viability, apoptosis and cellular necrosis, in order to select the concentrations to be used in the following studies. Subsequently, the frequency of micronuclei was measured in order to exclude any mutagenic capacity and the results were compared with those obtained in control cultures.

Considering the obtained results, the research continued with the evaluation of the possible antimutagenic capacity of the 6-MITC, another important chemopreventive mechanism. For this purpose, TK6 cells were treated, in absence or presence of 6-MITC in a co-treatment regimen, with two known mutagens: the clastogen Mitomycin C and the aneuploidogen Vinblastine, as reported in the OECD guideline no. 487 (OECD 487, 2016).

Mitomycin C is an antitumor antibiotic isolated from the bacterium *Streptomyces Caespitosus* and a toxic substance with a complex mechanism of action. It is, in fact, able to give monoalkylation or dialkylation products, in particular for alkylation of the amino group in position 2 of guanine, and to form cross-linked covalent bonds between the complementary strands of DNA. This interaction prevents the separation of the complementary DNA strand, inhibiting replication and inducing a DNA rupture. Furthermore, it generates ROS ($O_2^{\cdot -}$, H_2O_2, OH) (Tomasz, 1995).

Vinblastine is a drug belonging to the category of Antineoplastics and specifically Alkaloids of vinca and analogues; it is contained in the leaves of the Vinca Rosea (*Catharanthus Roseus*) and acts at the level of the cellular mitosis, preventing the polymerization of the tubulin and consequently inhibiting

the aggregation of the microtubules during the formation of the mitotic spindle. In this way it slows down or blocks the proliferation of cancer cells (Navarro *et al.,* 1989).

Mutagenic and antimutagenic activity, in particular, was measured in terms of increase/decrease of micronuclei frequency with a flow cytometric analysis and by adopting an automated and recently published protocol (Lenzi *et al.,* 2018). The Micronucleus Test with the cytochalasin B technique (classical method), in fact, has some limitations, such as the high subjectivity of microscopic reading, the small number of cells analysed for the purpose of adequate and robust statistical analysis, and the long times that it requires. Hence the need to develop a new method capable of making the analysis faster, more accurate and more objective (Fenech 2000; Lenzi *et al.,* 2018).

CHAPTER 3
MATERIALS AND METHODS

Reagents

Bovine Serum Albumin (BSA), Anti-β-tubulin, Digitonin, 2',7'- dichlorodihydrofluorescein diacetate (H_2DCF-DA), 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), diidroetidio (DHE), Dihydrorhodamine 123 (DHR123), Ethanol, Foetal Bovine Serum (FBS), Formaldehyde, Histopaque-1077, Hoechst 33258 solution, L-Glutamine (L-GLU), Lysis solution (components: Sodium Chloride, Potassium Chloride, Sodium Hydrogen Phosphate, Potassium Dihydrogen Phosphate, Ethylenediamminetetraceticacid (EDTA), Nonidet, water bpc grade), Methanol, Mitochondria Peroxy Yellow 1 (MitoPY1), Mitomycin C (MMC), MitoSOX Red Mitochondrial Superoxide Indicator (MitoSOX), Monochloromobimane (MCB), Penicillin-Streptomycin solution (PS), Phosphate Buffer Saline (PBS), Phytohaemagglutinin (PHA), Propidium Iodide (PI), Roswell Park Memorial Institute (RPMI) 1640 medium, Triton X-100, Vinblastine (VINB) were purchased from Sigma-Aldrich, St Louis, MO.; CYTO-ID Autophagy Detection Kit 2.0 was purchased from Enzo Life Scien, Farmingdale, New York, USA; Guava Caspase-8 FAM Kit, Guava Cell-cycle Reagent, Guava MitoPotential Kit, GuavaNexin Reagent, Guava ViaCount Reagent were purchased from Merck Millipore, Darmstadt, GER.; Anti-CD-14 (glycosylphosphatidylinositol (GPI)-Linked), anti-CD-15 (3-fucosyl-N-acetyllactosamine (3-FAL), anti-cyclin D3 were purchased from BioLegend, San Diego, CA.; Anti-cytochrome C, Anti-p53, Anti-BAX were purchased from BD Biosciences, San Jose, CA.; Anti-BCL-2, Anti-cyclin E2 were purchased from Abcam, Cambridge, UK.; Mini–PROTEANTGX Precast Protein Gels, Clarity Western ECL reagent were purchased from BIO-RAD, Hercules, CA,USA; Nitrocellulose membrane was purchased from GE Healthcare, Buckinghamshire, UK; RNase A, SYTOX Green, 7-aminoactinomycin D (7-AAD) (purchased from Thermo Fisher Scientific, Waltham, MA).

6-MITC

6-MITC was purchased from Abcam, Cambridge, UK. The purity of ITC was >98%. The ITC was dissolved in DMSO up to 97.39 mM stock solution and stored in the dark at -20°C. Increasing concentrations of 6-MITC from 0 to 128μM were tested. DMSO concentration was always in the range 0.05–1% in all the experimental conditions.

Cell cultures

Jurkat

Jurkat cells (acute T lymphoblastic leukaemia) were purchased by Zooprophylactic Institute from Lombardia and Emilia Romagna (Brescia) and were grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% FBS, 1% PS, and 1% L-GLU. To maintain exponential growth, the cultures were divided every third day in fresh medium.

The cell density of Jurkat did not exceed the critical value of $3x10^6$ cells/ml of medium and, for every 6-MITC treatment concentration and time, were seeded at $3.75x10^5$ cells/ml.

HL-60

HL-60 cells (acute promyelocytic leukaemia) were purchased by by Zooprophylactic Institute from Lombardia and Emilia Romagna (Brescia) and were grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 20% FBS, 1% PS, and 1% L-GLU. To maintain exponential growth, the cultures were divided every third day in fresh medium.

To reduce their spontaneous differentiation, the HL-60 cells were never allowed to exceed a density of $1.0x10^6$ cells/ml and, for every 6-MITC treatment concentration and time, were seeded at $1.25x10^5$ cells/ml.

PBL

Authorization to the use of human blood samples (Buffy coat), for research purposes, has been obtained from AUSL Bologna IT, S. Orsola-Malpighi Hospital - PROT GEN n° 0051937, and informed consent was obtained by AUSL Bologna IT, S. Orsola-Malpighi Hospital from donors for the use of their blood for scientific research purposes.

PBL were isolated using density gradient centrifugation with Histopaque-1077 from the whole peripheral blood of 5 AVIS (Italian Voluntary Blood Donors Association) donors. The donors had the following characteristics: under the age of 35, healthy, non-smoker and with no known exposure to genotoxic chemicals or radiation. The PBL were cultured at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 1% PS, 15% FBS, 1% L-GLU, and 0.5% PHA. The cell density of PBL did not exceed the critical value of $1x10^6$ cells/ml of medium and, for every 6-MITC treatment concentration and time, were seeded at $1.5x10^5$ cells/ml.

TK6

TK6 cells (human lymphoblasts) were purchased by Sigma-Aldrich (St. Louis, Missouri, USA) and were grown at 37° C and 5% CO2 in RPMI-1640 supplemented with 10% FBS, 1% L-GLU and 1% PS. To maintain exponential growth, the cultures were divided every third day in fresh medium.

The cell density of TK6 did not exceed the critical value of $9x10^5$ cells/ml of medium and, for every 6-MITC treatment concentration and time, were seeded at $2.0x10^5$ cells/ml.

Treatment

Jurkat and HL-60 cells were treated with 0, 2, 4,8, 16, 32, 64 μM of 6-MITC and incubated at 37°C and 5% CO_2 for 24, 48, 72h.

After isolation, the PBL were cultured for 44h in complete medium and in the presence of PHA, then treated with 0, 2, 4, 8, 16, 32, 64, 128 μM of 6-MITC and incubated at 37°C and 5% $CO_2$for 24h.

TK6 cells were treated with 0, 1, 2, 4, 8, 16, 32, 64 μM of 6-MITC and incubated at 37°C and 5% CO_2 for 3h (short term treatment) followed by 23h of recovery in fresh medium or 26h consecutive (long term treatment), corresponding to two replication cycles.

Flow cytometry (FCM)

FCM analyses were performed using a Guava easyCyte 5HT flow cytometer equipped with a class IIIb laser operating at 488 nm (Merk Millipore, Darmstadt, Germany), a Gallios 3L 10C flow cytometer equipped with three lasers operating at 488 nm, 633 nm and 405 nm and a Beckman Coulter Cytomic FC500 flow cytometer equipped with two lasers operating at 488 nm and 633 nm (Beckman Culter, Indianapolis, United States)

Guava ViaCount Assay

The cellular density and percentage of viable cells were assessed by FCM and analysed using Guava ViaCount software. After treatment, Guava ViaCount Reagent was added to the cells to discriminate viable from dead cells; the reagent contains the dye propidium iodide (PI), which is only able to penetrate the altered membrane of necrotic cells, bind covalently to the DNA and emit red fluorescence. In contrast, cells with an integral membrane are impermeable to PI and, thus, emit low red fluorescence. The obtained results were expressed as total cells/mL and as the percentage of live cells in treated cultures compared to that in the control cultures.

In parallel, for genotoxicity studies, the number of cells seeded at time 0 and that measured at the end of the treatment time, was used to check the correct replication in the controls cultures and compare it to that measured in the treated culture using the Relative Population Doubling (RPD).

$$\mathbf{RPD} = \frac{\text{(No. of Population doublings in treated cultures)}}{\text{(No. of Population doublings in control cultures)}} \times 100$$

Figure 15: mathematical formula of Relative Population Doubling

In fact, the OECD guideline no. 487 recommend proceeding to assess the genotoxicity of a xenobiotic, only if the highest concentration tested show cytotoxicity and cytostasis at most equal to 55 ± 5 %.

Guava Nexin Assay

The percentage of apoptotic cells was assessed by FCM and analysed using Guava Nexin software. After treatment, Guava Nexin Reagent was added to the cells: the reagent contains two dyes, 7-aminoactinomycin D (7-AAD) and Annexin-V-PE. As previously described for PI, 7-AAD allows the discrimination between live and dead cells, while Annexin-V-PE allows the identification of apoptotic cells by binding to phosphatidylserine and emitting yellow fluorescence. More specifically, live cells are negative to both 7-AAD and Annexin-V-PE, apoptotic cells are 7-AAD negative and Annexin-V-PE positive, and necrotic cells are positive to both 7-AAD and Annexin-V-PE. The obtained results were expressed as the percentage of apoptotic cells in treated cultures compared to that in the control cultures.

Guava Caspase-8 Assay

The percentage of cells with activated caspase-8 was assessed by FCM and analysed using Guava Caspase software. Guava Caspase-8 Reagent was added to the cells: the reagent contains two dyes, FLICA (an inhibitor of caspase-8) linked to FAM, and 7-AAD. As previously described, 7-AAD allows the discrimination between live and dead cells, while FLICA is cell permeable. Once inside the cell, FLICA binds covalently to the activated caspase-8 and emits green fluorescence. More specifically, live cells are negative to both 7-AAD and FLICA, cells with activated caspase-8 are 7-AAD negative and FLICA positive, and necrotic cells are positive to both 7-AAD and FLICA. The obtained results were expressed as the percentage of cells with activated caspase-8 in treated cultures compared to that in the control cultures.

Guava MitoPotential Assay

The percentage of apoptotic cells with an altered mitochondrial membrane potential was assessed by FCM and analysed using Guava MitoPotential software. Cells were stained with the Guava MitoPotential Reagent that contains two dyes, JC-1 and 7-AAD. 7-AAD allows the discrimination between live and dead cells, as previously described, while JC-1 is a cell-permeant cationic dye that fluoresces either green or orange depending upon the mitochondrial membrane potential. More specifically, live cells (polarised cells) are 7-AAD negative and orange JC-1 positive, apoptotic cells (depolarised cells) are 7-AAD negative and green JC-1 positive, and necrotic cells are 7-AAD positive and green JC-1 positive. The obtained results were expressed as the percentage of apoptotic

cells with an altered mitochondrial membrane potential in treated cultures compared to that in the control cultures.

Guava Cell Cycle Assay

The percentage of cells in each stage of the cell cycle was assessed by FCM and analysed using Guava Cell Cycle software. After treatment, cells were fixed and permeabilised with ice-cold 70% ethanol and washed with PBS. The cultures were then suspended in Guava Cell Cycle Reagent that contains the dye PI. PI is able to penetrate the membrane of cells, bind covalently to DNA and emit red fluorescence. More specifically, cells initially in the G_0/G_1 phase begin to synthesise DNA in the S phase, until complete duplication in the G_2/M phase. For this reason, cells in theG_2/M phase have a double fluorescence compared to those in the G_0/G_1 phase, while cells in the S phase have an intermediate fluorescence. The obtained results were expressed as the percentage of cells in each of the different stages of the cell cycle in treated cultures compared to those in the control cultures.

Analysis of Cytodifferentiation

The percentage of CD-14 or CD-15 positive cells was assessed by FCM and analysed using Guava InCyte software. Upon conclusion of the treatment time, cells were washed with ice-cold PBS. Cells were then incubated with Anti-CD-14-FITC or Anti-CD-15-FITC and washed. The obtained results were expressed as the percentage of CD-14 and CD-15 positive cells in treated cultures compared to those in the control cultures.

Analysis of Cytochrome C Release

The mean fluorescence intensity value of cytochrome c present was analysed by FCM using Guava InCyte software. Cells were permeabilised with Digitonin (100cg/mL) and fixed in a 4% formaldehyde solution in PBS. Cells were then washed in PBS 1X, incubated in an incubation buffer (0.5g BSA in 100mL PBS 1X) and then incubated overnight at 4°C with anti-cytocrome c monoclonal antibody. At the end of incubation, cells were washed in PBS1X and incubated at room temperature with fluorescein isothiocyanate-labelled secondary antibody. The obtained results were expressed as the mean fluorescence intensity value of cells in treated cultures compared to that in the control cultures. Non-specific binding was excluded by isotype control.

Analysis of Cell Cycle and Apoptotic Protein

The mean fluorescence intensity value of proteins present was analysed by FCM using Guava InCyte software. Cells were fixed in a 4% formaldehyde solution in PBS and permeabilised with 90% cold

methanol. Cells were then incubated with Anti-p53-PE, Anti-BCL-2-FITC, Anti-cyclin E2-PE, Anti-BAX and Anti-cyclin D3 antibodies. The cells (except those stained with BAX and cyclin D3) were washed and analysed. Cells stained with Anti-BAX and Anti-cyclin D3 were washed and incubated with anti-mouse IgG-FITC secondary antibody. The obtained results were expressed as the mean fluorescence intensity value of cells in treated cultures compared to that in the control cultures. Non-specific binding was excluded by isotype control.

Analysis of apoptosis by fluorescence microscopy

Apoptosis-associated nuclear condensation and fragmentation were evaluated in untreated and treated cells by fluorescence microscopy at 100x magnification. $1x10^6$ cells were loaded into cytospin chambers and centrifuged ad 450 rpm for 10 minutes. Cells were then fixed in formaldehyde 3.7%, washed in PBS pH 7.2, permeabilized in 0.15% triton X-100 and nuclei were stained with 0.5 μM Hoechst 33258 as reported by Henry et al. (Henry *et al.,* 2013).

Analysis of Cell Cycle and Apoptotic Protein by Western Immunoblotting (WB)

Jurkat and HL-60 cell lysates was obtained as previously reported by Angeloni *et al.* (Angeloni *et al.,* 2011). Samples were denatured prior to separation on 4%–20% Mini- PROTEAN TGX Precast Protein Gels. The proteins were transferred to a nitrocellulose membrane at 110 V for 90 min in Tris-glycine buffer. Membranes were then incubated in a blocking buffer containing 5% (w/v) BSA and incubated with anti-p53, anti-Cyclin D3 and anti-β-tubulin, as internal normalizers, overnight at 4°C on an orbital shacker. The results were visualized by chemiluminescence using Clarity Western ECL reagent according to the manufacturer's protocol (BIO-RAD). Semiquantitative analysis of specific immunolabeled bands was performed using Image Lab 6.0 (BIO-RAD).

Analysis of Autophagy by FCM

The percentage of autophagic cells was assessed by FCM and analysed using Kaluza software.

After treatment, aliquots containing $1X10^5$ to $1X10^6$ cells were centrifugated at 1000 rpm for 5 minutes and washed. Subsequently, 200 μL of fresh medium and 200 μL of CYTO-ID Green stain solution were added to each sample, mixed well and then, the cells were incubated for 30 minutes at 37°C in the dark. At the end of incubation, cells were washed and the pellet was resuspended in 500 μL of Assay Buffer and 1 μL of DAPI (cell permeable fluorescent dye that binds to DNA and discriminates between live and dead cells.) Rapamycin 500 nM and Cloroquine (CQ) 10 μM were used as positive control.

The 488 nm-excitable green fluorescent detection reagent becomes brightly fluorescent in vesicles produced during autophagy and provides a rapid approach to monitoring autophagic activity at the cellular level. The obtained results were expressed as the percentage of autophagic cells in treated cultures compared to that in the control cultures.

Analysis of Oxidative Stress by FCM

The mean fluorescence intensity value of fluorescent probes (DCF, MitoPY, HE, MitoSOX, DHR123, MCB) was analysed by FCM using Kaluza software.

H_2DCF-DA is a cell-permeant probe which is cleaved by intracellular esterase forming the anion, H_2DCF^-. This dye shows low green fluorescence in its reduced state, but is highly fluorescent in its oxidized state (DCF). DCF is activated by various ROS and in particular by hydrogen peroxide.

MitoPY1 is a fluorescent probe that selectively tracks to the mitochondria and responds to local fluxes of H_2O_2 by a turn- on fluorescence enhancement. This dye uses a triphenyl- phosphonium targeting group and a boronate-based molecular switch to selectively respond to H_2O_2 over competing ROS within the mitochondria. MitoPY1 is used to accurately measure mitochondrial H_2O_2 levels in living cells; when excited, the oxidized form displays enhanced green fluorescence.

DHE is a cell-permeant redox probe selectively oxidized and hydroxylated by superoxide to a specific product, 2-OH-ethidium, emitting orange fluorescence when bound to DNA.

MitoSOX is a fluorogenic probe specifically targeted to mitochondria in live cells. Mito-SOX is readily oxidized by superoxide but not by other ROS- or reactive nitrogen species-generating systems to a fluorescent product with orange fluorescence emission.

DHR123 is an uncharged and no fluorescent substrate indicator that diffuses passively across membranes where it is oxidized to cationic rhodamine 123, which localizes in the mitochondria and exhibits green fluorescence. DHR123 is activated by various ROS, including ONOO and superoxide.

MCB is essentially non-fluorescent until conjugated, readily reacts with several low molecular weight thiols, including glutathione, N-acetylcysteine, mercaptopurine, peptides and plasma thiols. The glutathione conjugate of monochlorobimane has absorption/emission maxima ~394/490 nm. Reduced glutathione (GSH) is a major thiol bound to proteins. Protein thiols including GSH play an important role in determining the redox status of cells. Therefore, detection of GSH levels is a useful indication of redox potential and a cell's ability to prevent oxidative stress.

Briefly, in aliquots containing $5x10^4$ to $2x\ 10^4$ cells were added the fluorochrome and the cells were incubated for 30 minutes at 37°C in the dark. 5 minutes before the incubation time expired 1 μL of DAPI or PI was added. The final assay concentration for each fluorochrome was as follows: H_2DCF-

DA 2,5 µg/mL; MitoPY1 5 µM; DHE 2,5 µg/mL; MitoSOX 0,25 µM**,** DHR1230,04 µg/mL; MCB 1 µM.

Analysis of Genotoxicity by FCM

The analysis of the micronuclei frequency was performed using an automated protocol by Lenzi et al. (Lenzi *et al*., 2018). Briefly, after treatment time, aliquots of $7x10^5$ cells were collected and stained with 7-AAD and SYTOX Green. The discrimination between nuclei and micronuclei was performed on the basis of the different size analyzed by FSC, and the different intensity of green fluorescence. For each sample the micronuclei frequency was measured on 10,000 nuclei derived from viable and proliferating cells on the basis of different red fluorescence. The results were expressed as increase frequency of micronuclei in treated cultures compared to that present in the control cultures.

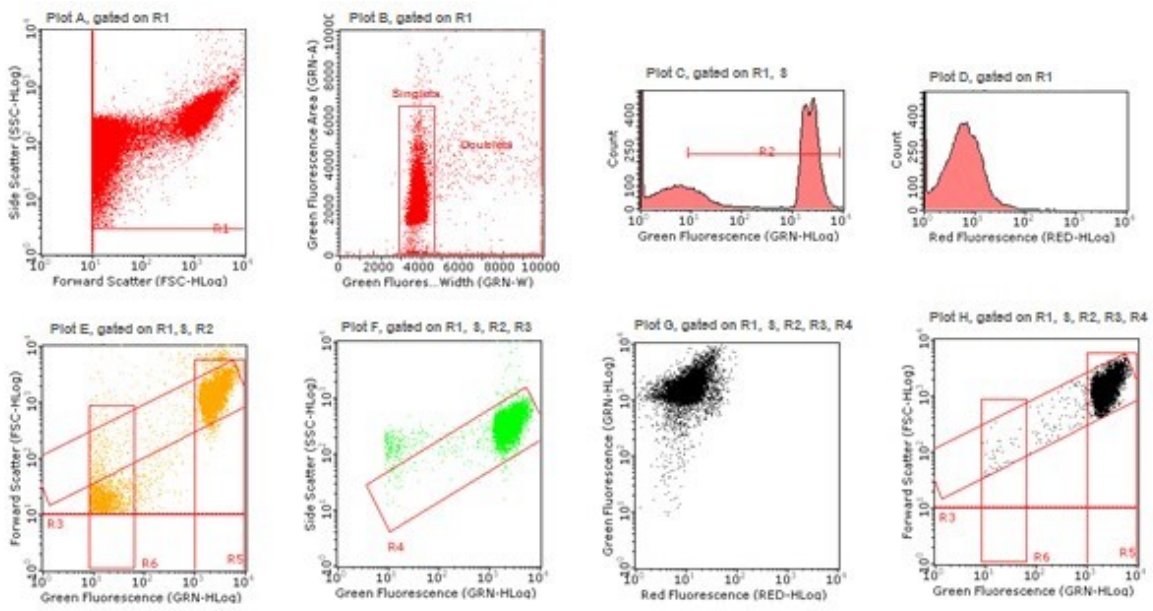


Figure 16: Image of bivariate plots of nuclei (gate R5) and micronuclei (gate R6), double stained and analyzed by Guava Incyte software. With gate R1the events to be considered are selected on the basis of Side Scatter-log (SSC) and FSC-log, which were then divided into nuclei and micronuclei based on FSC and green fluorescence

Statistical Analysis

All results are expressed as mean ± standard error mean (SEM) of at least five independent experiments. For the statistical analysis of apoptosis, apoptosis pathways, autophagy, cell cycle and micronucleus we used the Analysis of Variance for paired data (repeated ANOVA), followed by Bonferroni or Dunnet as the post-test. For statistical analyses of cytodifferentiation, protein levels and Western Blotting densitometry we used the t-test for paired data. All the statistical analyses were performed using Prism Software 6.

CHAPTER 4
RESULTS

Determination of the 6-MITC concentrations used in subsequent experiments on Jurkat cells, HL-60 cells and PBL/Guava ViaCount Assay

The research began with a preliminary study of the cytotoxic and cytostatic potential of 6-MITC by way of a Guava ViaCount assay to determine the concentrations of ITC to be used in subsequent experiments. To this end, the Jurkat and HL-60 cells were treated for 24h, 48h and 72h with 6-MITC at concentrations of 0 to 64µM while the PBL cells were treated for 24h. The results obtained demonstrated that necrosis of the cancer cells was restrained up to 16µM at 24h and up to 8µM at 48h and 72h. Moreover, the viability of the healthy cells at 24h remained greater than 50% at all concentrations tested (data not shown).

Effect of 6-MITC on viability, apoptosis and necrosis of Jurkat cells, HL-60 cells and PBL/Guava Nexin Assay

On the basis of the results previously obtained, a Guava Nexin assay was conducted to measure the percentage of live, apoptotic and necrotic cells following the 6-MITC 0-16µM treatment for 24h, 48h and 72h of Jurkat cells (Table 3), the 6-MITC 0-32µM treatment for 24h, 48h and 72h of HL-60 cells (Table 4), and, in parallel, the 6-MITC 0-128µM treatment for 24h of PBL (Table 5).

Jurkat		**24h**			**48h**			**72h**	
	viable cells	**apoptotic cells**	**necrotic cells**	**viable cells**	**apoptotic cells**	**necrotic cells**	**viable cells**	**apoptotic cells**	**necrotic cells**
0 µM	89.7±0.9	7.6±0.6	2.3±0.3	91.4±1.4	6.1±0.8	2.3±0.6	90.0±0.4	4.6±0.5	4.3±0.1
2 µM	79.7±3.3	16.1±2.9	3.9±0.3	85.8±1.6	12.8±0.6	3.3±0.8	85.8±1.3	7.7±0.7	6.2±1.2
4 µM	77.3±1.6	16.2±1.1	6.4±0.6	79.8±1.6	15.4±1.6	3.3±0.9	77.6±0.7	13.2±0.9	9.0±1.5
8 µM	49.6±2.5	25.4±0.9	24.1±2.5	45±4.3	35±2.3	18.3±3.9	48.8±1.8	31.6±3.5	17.4±2.6
16 µM	27.8±1.0	30.3±0.8	42.0±0.8						

Table 3*: percentage of viable, apoptotic and necrotic Jurkat cells treated with 6-MITC for 24h, 48h and 72h. Data are presented as mean ± SEM of five independent experiments.*

HL-60		24h			48h			72h	
	viable cells	apoptotic cells	necrotic cells	viable cells	apoptotic cells	necrotic cells	viable cells	apoptotic cells	necrotic cells
0µM	93.7±02	5.3±0.1	1.1±0.1	94.3±0.5	4.7±0.3	1.0±0.4	94.6±0.2	3.8±0.2	1.3±0.1
2µM	93.9±0.1	5.2±0.1	0.9±0.1	93.9±0.1	4.9±0.1	1.3±0.1	95.9±0.1	3.1±0.1	1.0±0.2
4µM	91.9±0.4	7.1±0.3	1.0±0.2	91.6±0.6	6.8±0.4	1.6±0.2	92.1±8.9	6.3±0.5	1.4±0.2
8µM	83.0±0.4	12.7±0.2	3.3±0.2	66.5±1.5	22.6±1.0	10.5±0.8	58.5±1.7	31.5±2.0	10.0±0.4
16µM	46.8±0.9	12.6±0.5	39.2±0.8						
32µM	33.3±0.5	15.8±0.4	51.1±0.6						

Table 4*: percentage of viable, apoptotic and necrotic HL-60 cells treated with 6-MITC for 24h, 48h and 72h. Data are presented as mean ± SEM of five independent experiments.*

PBL		24h	
	viable cells	apoptotic cells	necrotic cells
0µM	77.9±0.2	10.6±0.4	10.9±0.7
2µM	76.8±0.9	12.3±0.3	11.2±1.0
4µM	68.9±1.0	14.3±0.4	15.9±0.3
8µM	67.7±1.2	14.7±0.3	15.7±1.6
16µM	67.6±0.3	17.0±0.3	15.4±0.2
32µM	68.1±1.3	15.9±0.6	15.3±0.8
64µM	51.4±0.4	12.2±0.4	36.4±0.6
128µM	40.0 ±0.9	11.8±1.0	47.2±0.7

Table 5*: percentage of viable, apoptotic and necrotic PBL cells treated with 6-MITC for 24h, 48h and 72h. Data are presented as mean ± SEM of five independent experiments.*

Viability

The percentage of live cells measured at 24h and normalised to the viability in control cultures (considered to be 100%), was used to obtain the dose-response curve. The IC_{50} value calculated by interpolation was 8.65µM for Jurkat cells, 16µM for HL-60 cells and 86.1µM for PBL, respectively (Figure 17 A, B, C). The analysis at 48h and 72h allowed confirmation that an acceptable percentage of live Jurkat and HL-60 cells remained at up to 8µM, even after longer treatment times (Table 3 and Table 4).

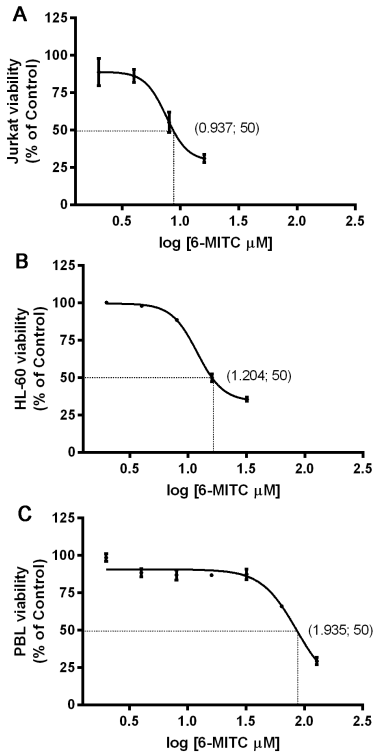


Figure 17**: **Effect of 6-MITC on viability of Jurkat cells, HL-60 cells and PBL

IC_{50} obtained by curve fitting of viable cells after 24h treatment with 6-MITC for Jurkat cells (A), HL-60 cells (B) and PBL (C). Data are presented as mean ± SEM of five independent experiments.

Apoptosis

Already at 24h of treatment, Annexin V-PE/7-AAD double staining highlighted a statistically significant increase in Jurkat cells at all concentrations tested. More specifically, with respect to the control cultures at 2µM and 4µM, a population doubling was detected (16.1% vs 7.6% and 16.2% vs 7.6%), while a 3 and 4 times increase was detected at 8µM and 16µM, respectively (25.4% vs 7.6%

and 30.3% vs 7.6%) (Table 3 and Figure 18A, B). A similar pro-apoptotic effect was observed on the HL-60 cells. In fact, the percentage of apoptotic cells increased in a statistically significant manner at a concentration of 4μM (7.1% vs 5.2% in controls) and at 8μM (12.7% vs 5.2% in controls), while doubling at a concentration of 16μM (12.6% vs 5.2% in controls) (Table 4 and Figure 18A, B). The induction of apoptosis mediated by 6-MITC on tumour cells was both concentration- and time-related. Indeed, a larger increase in the fraction of apoptotic cells was recorded after 48h of treatment than at 24h, while - in Jurkat cells - a 3-times increase was recorded at 4μM (15.4% vs 6.1% in controls) and a 6-times increase at 8μM (35.0% vs 6.1% in controls) (Table 3 and Figure 18c), and - in HL-60 cells - a 5-times increase was recorded at 8μM (22.6% vs 4.8% in controls) (Table 4 and Figure 18C). In addition, after 72h a further 7-times increase of apoptotic cells was recorded in Jurkat cells (31.6% vs 4.6% in controls) (Table 3 and Figure 18D) and an 8-times increase recorded in HL-60 cells at the highest concentration tested (31.5% vs 3.9% in controls) (Table 4 and Figure 18D). To further confirm the 6-MITC's pro-apoptotic effect, nuclear condensation and fragmentation were evaluated by fluorescence microscopy (Figure 19).

In order to support the hypothesised selectivity of 6-MITC's action, we proceeded to similarly analyse its pro-apoptotic potential in PBL. The results showed a statistically significant increase in the percentage of apoptotic cells that only started from a concentration of 16μM (17.0% vs 10.6% in controls) and remained constant at 32μM (15.9% vs 10.6% in controls). At the highest concentration tested, 64μM, a reduction in apoptotic cells was observed in favour of the necrotic cell fraction, which nonetheless remained below 50% (Table 5 and Figure 18A, B). Comparing the results obtained in the different cell lines, it is evident that 6-MITC induces much stronger cytotoxicity on cancer cells than on healthy cells, through stimulation of an apoptotic mechanism (Figure 18A, B, C, D).

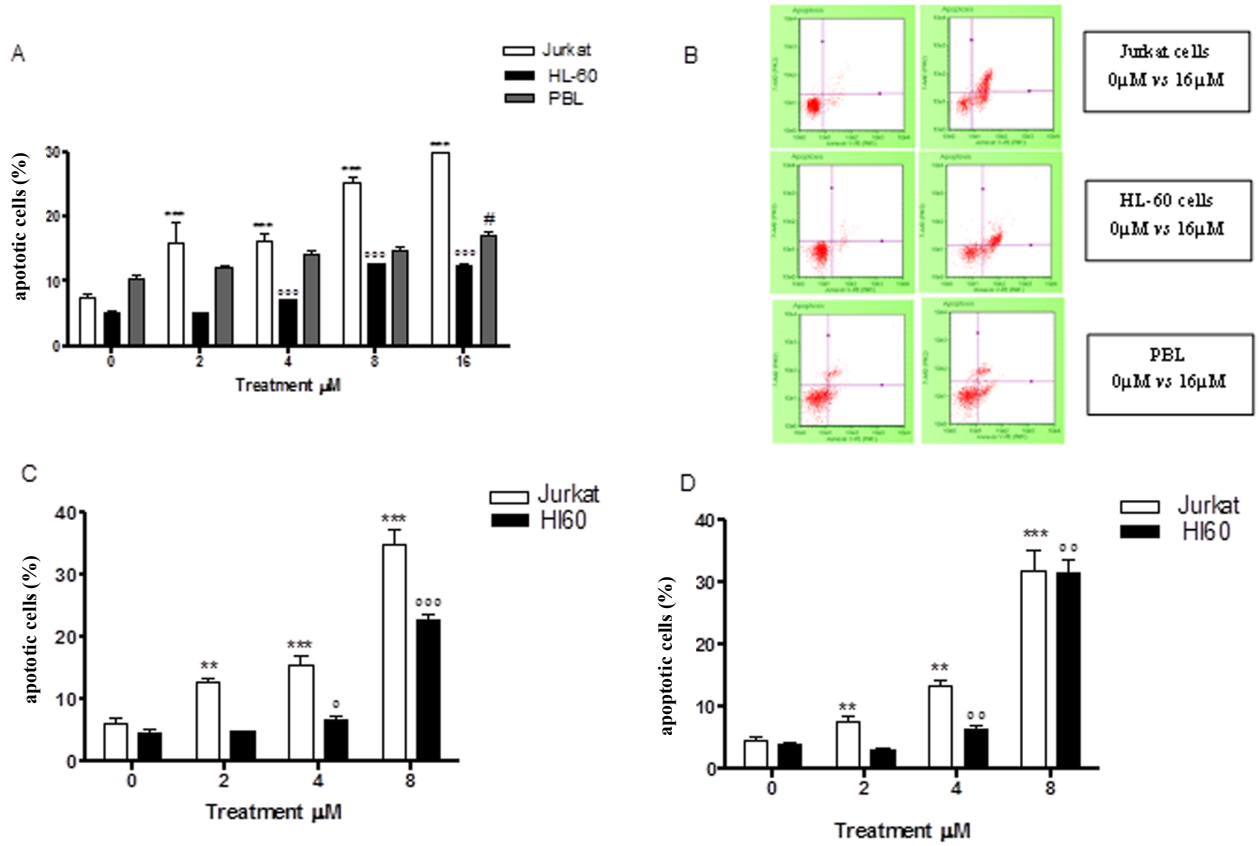


Figure 18**: **Effect of 6-MITC on apoptosis of Jurkat cells, HL-60 cells and PBL

Fraction of apoptotic Jurkat, HL-60 and PBL cells treated with 6-MITC for 24h (A) and representative dot plot of apoptosis analysis at 24h treatment (B), fraction of apoptotic Jurkat and HL-60 cells treated with 6-MITC for 48h (C) and 72h (D).

Apoptosis was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

***p<0.001 vs control of Jurkat; ***p<0.001 vs control of Jurkat;*

°° p<0.01 vs control of HL-60; °°° p<0.001 vs control of HL-60; # p<0.05 vs control of PBL.

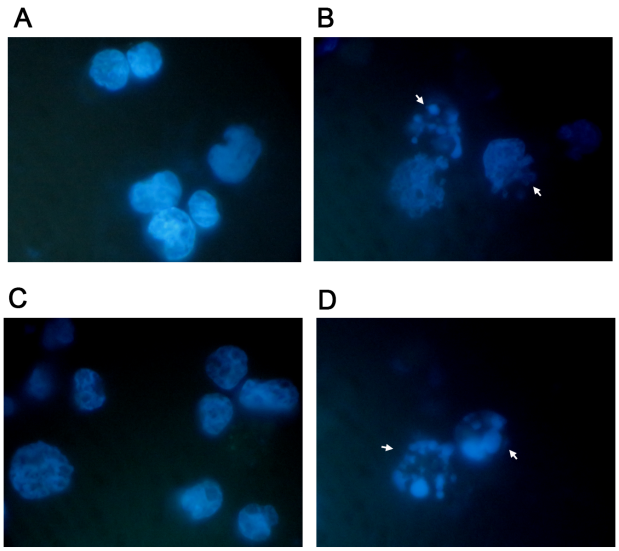


Figure 19: Apoptosis-associated nuclear condensation and fragmentation on Jurkat cells and HL-60 cells

Jurkat (A, B) and HL-60 (B, D) cells after 72h treatment with 6-MITC 0μM (A, C) and 8 μM (B, D) were stained with Hoechst 33258 and evaluated by fluorescence microscopy at 100× magnification as described in Methods. White arrows indicate condensed and/or fragmented nuclei as a marker of apoptosis.

Necrosis

With regard to necrosis, it is important to underline that the results obtained in Jurkat cells further support the hypothesis of pro-apoptotic activity. In fact, at the 8μM concentration, increasing treatment time resulted in a decrease in the percentage of necrotic cells from 10 to 8 to 4 times, while in HL-60 cells the percentage increased up to 10 times after 48h and then remained steady at 72h (Table 3 and Table 4).

Evaluation of pro-apoptotic pathway triggered by 6-MITC on Jurkat cells and HL-60 cells/Guava Caspase-8 and Guava MitoPotential Assay

In order to assess whether the 6-MITC-induced apoptosis was triggered by the extrinsic or the intrinsic pathway, tumour cells were treated for 24h, 48h, and 72h at concentrations of 4μM and 8μM (<IC_{50} obtained in Jurkat and HL-60 cells).

The levels of cells with activated caspase-8 revealed that the apoptosis induced by 6-MITC was mediated exclusively by extrinsic pathway activation in both cell lines, while the intrinsic pathway did not seem at all involved. In fact, the percentage increase in apoptotic cells with respect to the

control cultures measured by the Guava Caspase-8 Assay in Jurkat cells was statistically significant at both concentrations tested at 24h (9.5% and 24.0% vs 6.5%), 48h (14.0% and 31.5% vs 4.5%) and 72h (17.5% and 37.0% vs 5.5%) (Figure 20A, B, C, D). This percentage increase in the treated versus control cultures was, moreover, fully comparable to that measured in the previously conducted Guava Nexin Assay. For example, after 72h treatment with 8μM the increase was 7 times (Figure 18D and 20C), with a similar result recorded in HL-60 cells. The percentage of activated caspase-8 cells was also statistically higher at both concentrations tested at 24h (7.9% and 12.7% vs 4.5% in controls), 48h (7.5% and 27.5% vs 5% in controls) and 72h (8.5% and 48.5% vs 5.5% in controls) (Figure 21A, B, C, D). Moreover, this percentage was fully comparable to that measured in the previously conducted Guava Nexin Assay, being 8 times higher in the treated versus control cultures after 72h at 8μM (Figure 18D and 21C). In contrast, the fraction of cells with mitochondrial potential depolarization in treated cultures matched that in control cultures, in both Jurkat and HL-60 cells (Figure 20A, B, C, D; Figure 21A, B, C, D).

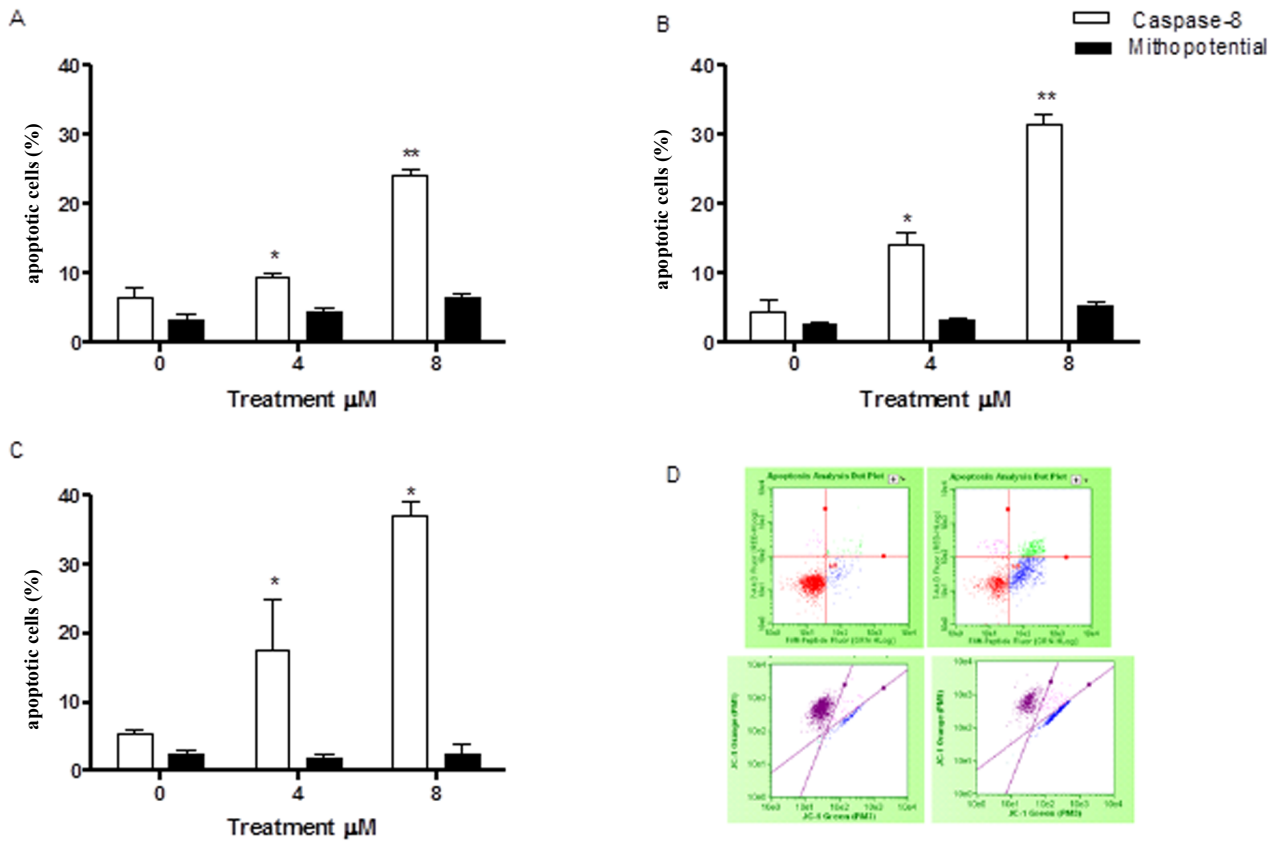


Figure 20**: **Evaluation of pro-apoptotic pathway triggered by 6-MITC on Jurkat cells

Fraction of apoptotic Jurkat cells with active caspase-8 or with altered mitochondrial membrane potential after 24h (A), 48h (B), 72h (C) and representative dot plot of caspase-8 (up) and mitochondrial membrane potential (down) analysis at 72h treatment at 0µM (left) and 8µM (right) (D). Active caspase-8 and altered mitochondrial membrane potential was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

**p<0.05 vs control; **p<0.01 vs control.*

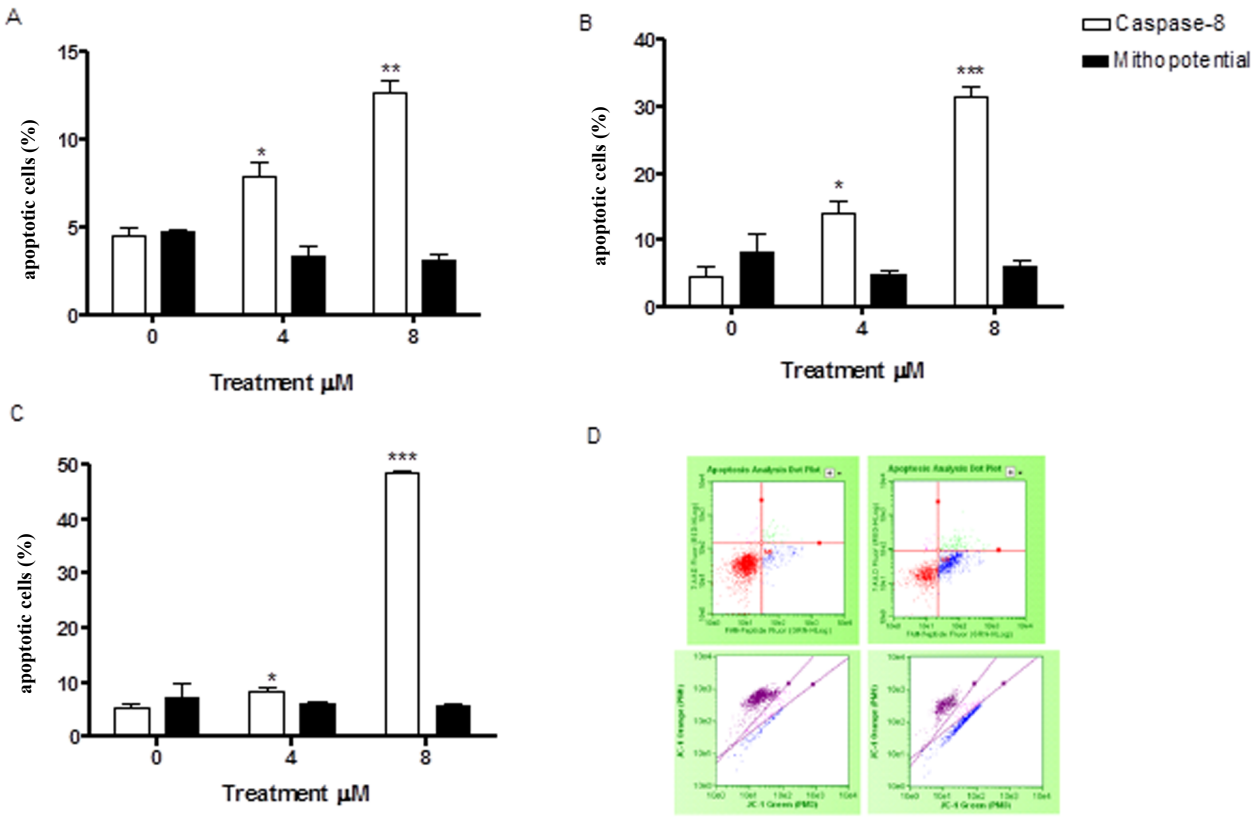


Figure 21**: **Evaluation of pro-apoptotic pathway triggered by 6-MITC on HL-60 cells

Fraction of apoptotic HL-60 cells with active caspase-8 or with altered mitochondrial membrane potential after 24h (A), 48h (B), 72h (C) and representative dot plot of caspase-8 (up) and mitochondrial membrane potential (down) analysis at 72h treatment at 0µM (left) and 8µM (right) (D). Active caspase-8 and altered mitochondrial membrane potential was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

**p<0.05 vs control; **p<0.01 vs control; *** p<0.001 vs control.*

Effect of 6-MITC on cell cycle progression of Jurkat cells, HL-60 cells and PBL/Guava Cell cycle Assay

In order to assess whether the apoptosis induced by 6-MITC was an independent event or subsequent to a cell cycle slowdown/block, Jurkat and HL-60 cells were treated with 4µM and 8µM concentrations for 24h, 48h and 72h. PI staining allowed us to highlight the percentage distribution of cells in the different phases of the cell cycle. In particular, after 24h, Jurkat cells demonstrated a statistically significant percentage reduction of cells in the S phase at both 4µM (11.62% vs 17.4% in controls) and 8µM (8.8% vs 17.4% in controls) (Figure 22A, B).

After 48h of treatment, a statistically significant percentage reduction of cells in the S phase was only observed at the higher concentration tested (11.2% vs 13.7%) (Figure 22C), while after 72h, no effect on the cell cycle was observed (Figure 22D).

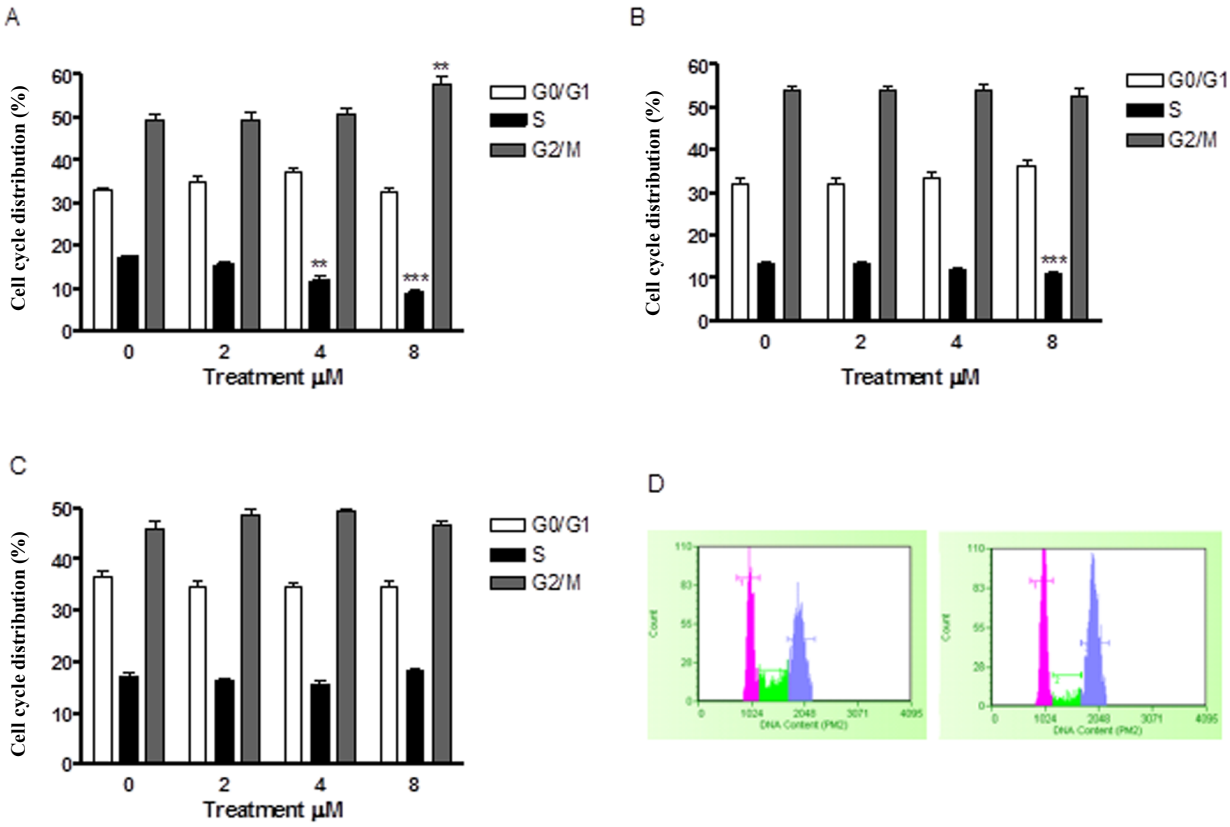


Figure 22**: **Effect of 6-MITC on cell cycle progression of Jurkat cells

Fraction of Jurkat cells in the different phases of the cell cycle after 24h (A) and representative histograms of cell cycle analysis at 24h treatment at 0µM (left) and 8µM (right) (B) 48h (C), 72h (D). Cellular distribution in the different phases was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

***p<0.01 vs control; ***p<0.001 vs control.*

In HL-60 cells, 6-MITC induced no effect after 24h of treatment (Figure 23A), while at 48h and 72h a statistically significant increase of cells in the G_0/G_1 phase was observed at 4µM (70.2% and 69.4% vs 48.2 %) and 8µM (72.7% and 69.4% vs 48.2%) with respect to the control cultures but with no difference in percentage terms between the two treatment times (Figure 23B, C, D). At the same time,

a statistically significant 6-MITC- induced decrease in the S phase was observed at 48h and 72h at concentrations of 4µM (16.4% and 16.5% vs 33.4% in controls) and 8µM (17% and 14.6% vs 33.4% in controls) (Figure 23B, C, D).

These results suggest that 6-MITC has the ability to slow down the Jurkat cell cycle and to induce a true block of the HL-60 cell cycle in G_0/G_1, in both cases leading to a subsequent decrease in S phase cells.

Similarly, the potential effects of ITC on the cell cycle of healthy lymphocytes were analysed, with no activity being observed (Figure 24A, B).

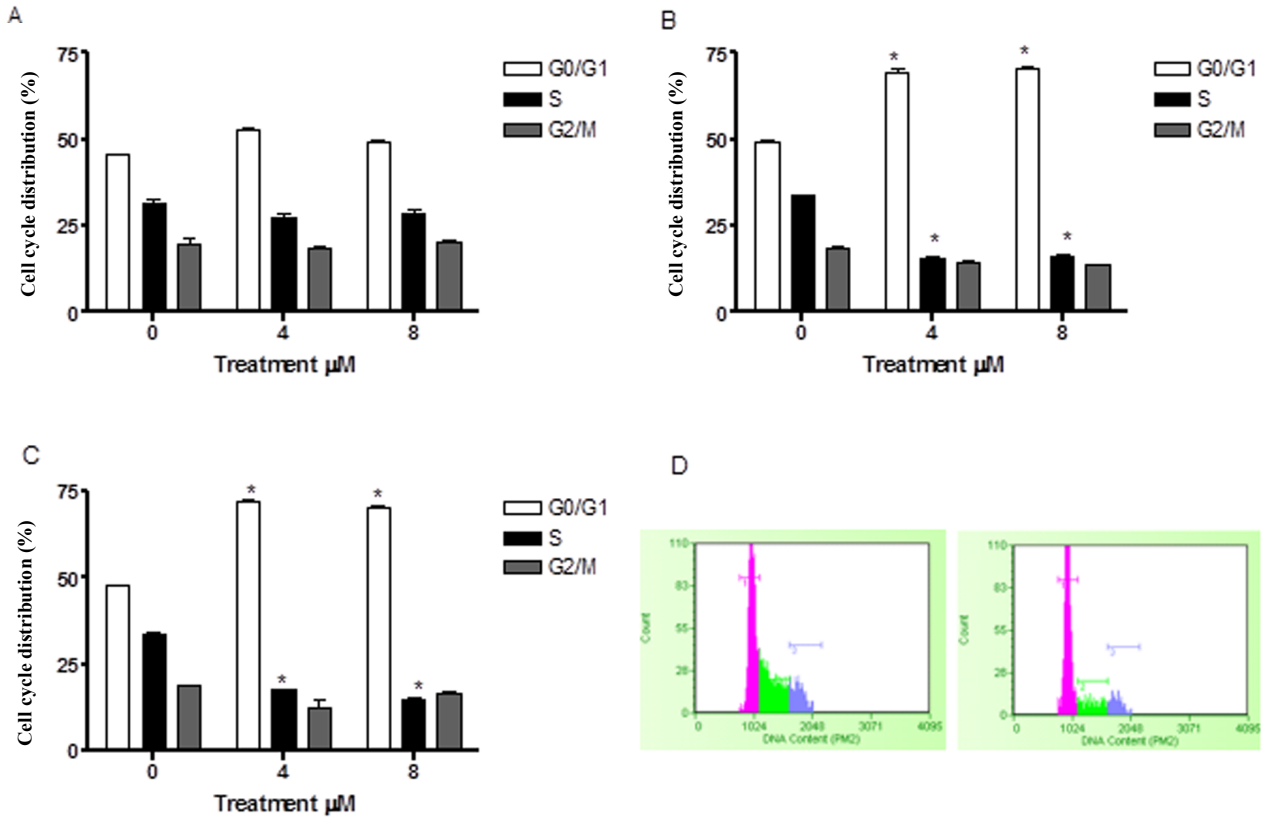


Figure 23**: **Effect of 6-MITC on cell cycle progression of HL-60 cells

Fraction of HL-60 cells in the different phases of the cell cycle after 24h (A), 48h (B), 72h (C) and representative histograms of cell cycle analysis at 72h treatment at 0µM (left) and 8µM (right) (D). Cellular distribution in the different phases was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

**p<0.05 vs control.*

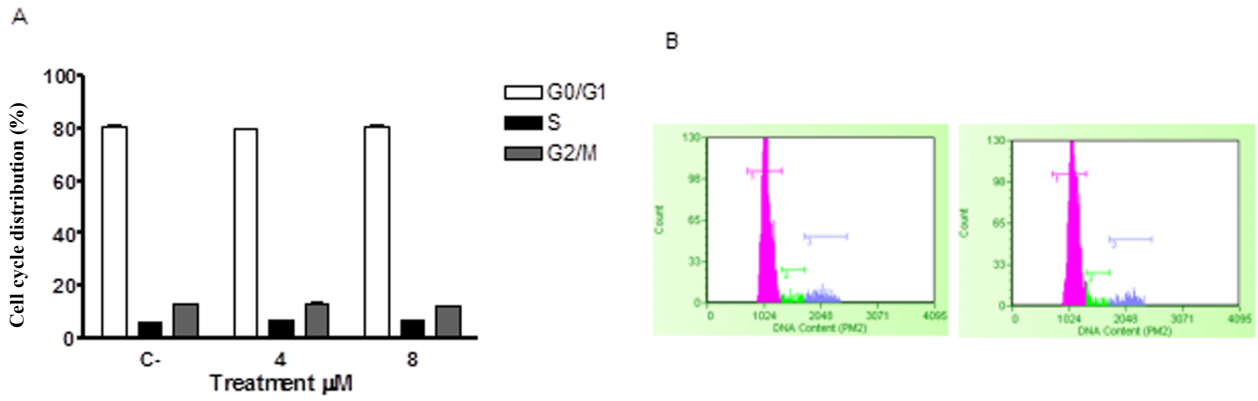


Figure 24**: **Effect of 6-MITC on cell cycle progression of PBL

Fraction of PBL cells in the different phases of the cell cycle after 24 (A) and representative histograms of cell cycle analysis at 24h treatment at 0µM (left) and 8µM (right) (B). Cellular distribution in the different phases was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Bonferroni post-test.

Effect of 6-MITC on differentiation of HL-60 cells/Analysis of Cytodifferentiation

We evaluated 6-MITC's ability to induce cytodifferentiation in HL-60 cells following 24h, 48h and 72h treatment at concentrations of 4µM and 8µM. The results obtained showed that isothiocyanate induces no effect at either concentration after 24h and 48h of treatment (data not shown); however, after 72h of treatment at the higher concentration, it induced a statistically significant increase in differentiated cells of both macrophage and granulocyte phenotypes, more specifically, a 3-times increase in CD-15 positive cells and a 2-times increase in CD-14 positive cells (Figure 25A, B).

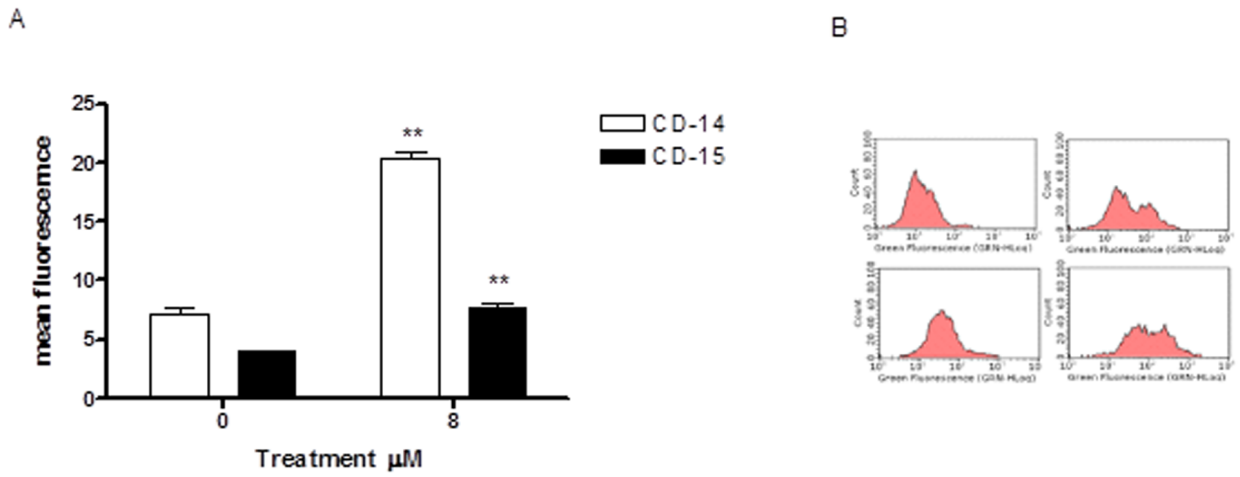


Figure 25**: **Effect of 6-MITC on differentiation of HL-60 cells

CD-14 and CD-15 mean fluorescence intensity after 72h treatment (A) and representative dot plot of CD-14 (up) and CD-15 (down) after 72h treatment at 0µM (left) and 8µM (right) (B). CD-14 and CD-15 levels were evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using the t-test for paired data.

***p<0.001 vs control.*

Effect of 6-MITC on apoptotic and cell cycle proteins on Jurkat cells and HL-60 cells/Analysis of cytochrome C Release and cell cycle and apoptotic proteins by FCM and by WB

In order to assess whether the pro-apoptotic and cytostatic ability demonstrated by 6-MITC in Jurkat cells involved modulation of p53 protein, its levels were analysed following treatment at 8µM concentration for 6h, 24h, 48h and 72h.

As shown in Table 6, the levels of p53 remained unchanged in treated cultures compared to control cultures at all treatment times (Table 6). WB analyses confirmed that 6-MITC's treatment did not affect p53 protein expression level (Figure 26).

Jurkat		6h		24h		48h
	0 µM	8µM	0 µM	8µM	0 µM	8µM
p53	214.7±1.2	238.2±0.9	341.9±1.7	400.9±0.2	590.7±1.6	608.2±1.4
		24h		48h		72h
	0	8µM	0	8µM	0	8µM
BAX/BCL-2 ratio	1.1	0.8	0.9	0.7	0.9	0.4
Cytochrome	33.7±0.8	35.8±1.3	32.3±1.3	34.5±0.5	35.9±0.6	29.8±0.8

Table 6*: p53, BAX/BCL-2 ratio, cytochrome c mean fluorescence intensity of Jurkat cells after 24h, 48h and 72h treatment. Protein levels were evaluated by FCM as described in Methods. Data are presented as mean ± SEM of five independent experiments and analysed using the t-test for paired data.*

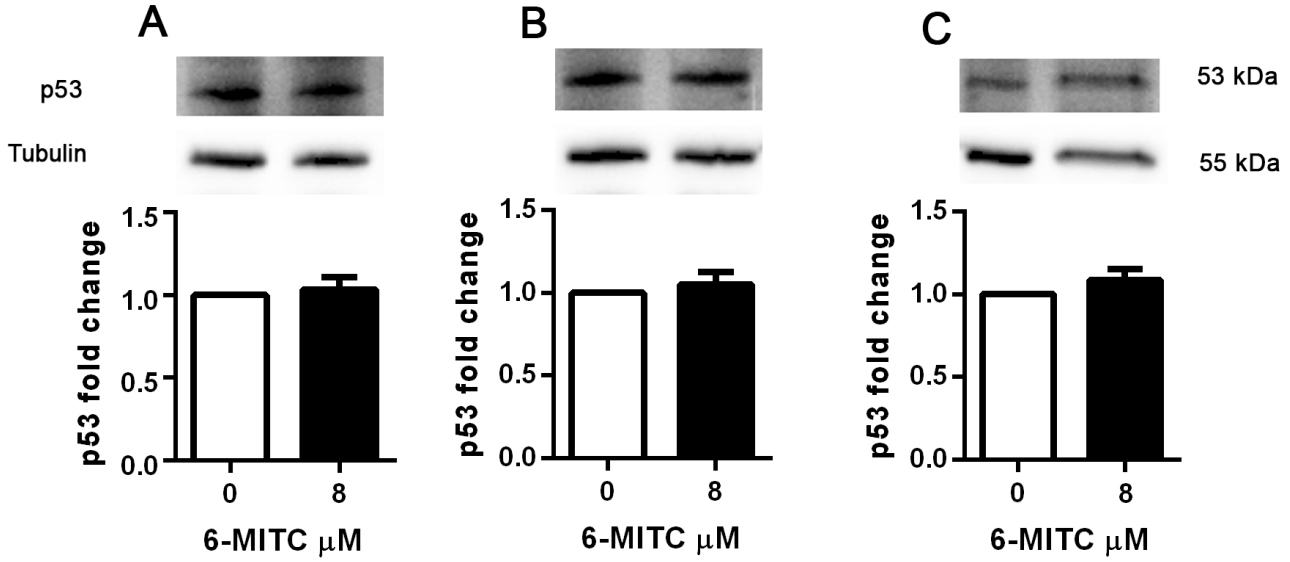


Figure 26: Effect of 6-MITC on p53 protein level in Jurkat cells

Effect of 6-MITC's 8µM treatment on p53 protein expression level in Jurkat cells. Cells were treated for 24h (A), 48h (B) and 72h (C) with 6-MITC 8µM. Cell lysates were immunoblotted with anti-p53 antibodies as reported in Methods. Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts, presented as means ± SEM, were normalized to the intensity of ß-tubulin and represented as fold increase vs control. Data were analysed using the t-test for paired data.

**p<0.05 vs control.*

Moreover, in order to check the exclusive involvement of the extrinsic pathway, we also analysed any modulation of the BAX, BCL-2 and cytochrome c proteins in Jurkat and HL-60 cells. The BAX and cytochrome c levels in the treated samples proved perfectly comparable to those of the controls in both cell lines (Table 6 and Table 7). These data corroborate the hypothesis that the pro-apoptotic effect of 6-MITC does not correlate with a loss of mitochondrial transmembrane potential. In contrast, BCL-2 levels increased in the treated cultures with respect to the control cultures, resulting in the BAX/BCL-2 ratio dropping in both cell lines (Table 6 and Table 7).

HL-60		**24h**		**48h**		**72h**
	0 µM	**8µM**	**0 µM**	**8µM**	**0 µM**	**8µM**
BAX/BCL-2 ratio	1.1	1.2	0.9	0.5	0.7	0.3
Cytochrome	32.8±1.4	34.6±0.5	33.9±0.7	36.1±0.8	34.1±1.1	32.5±1.4

Table7*: BAX/BCL-2 ratio, cytochrome c mean fluorescence intensity of HL-60 cells after 24h, 48h and 72h treatment. Protein levels were evaluated by FCM as described in Methods. Data are presented as mean ± SEM of five independent experiments and analysed using the t-test for paired data.*

Since 6-MITC was found to arrest the HL-60 cell cycle in the G_0/G_1 phase, causing a subsequent decrease of cells in the S phase, we also evaluated its effects in this cell line on the expression of cyclin E2 and the cyclin D3, two proteins involved in the G_1/S transition phase. As shown in Figure 27, the anti-cyclin E2-PE mean fluorescence intensity remained relatively constant after treatment with 6-MITC at 8µM for 24h, 48h and 72h, while the anti-cyclin D3-FITC mean fluorescence intensity increased at each treatment time, reaching statistical significance at 48h and 72h (Figure 27A, B, C, D). WB analyses of Cyclin D3 protein expression level confirmed this trend (Figure 28).

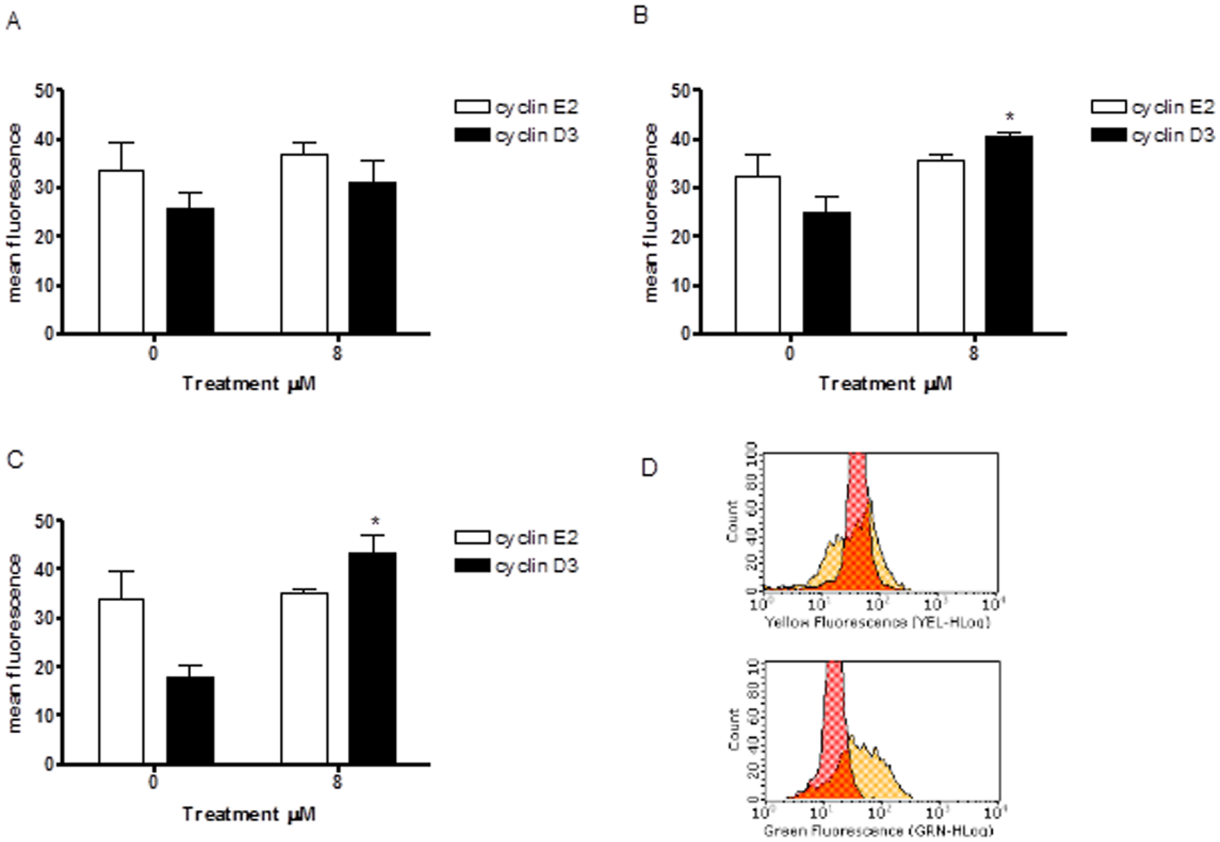


Figure 27**: **Effect of 6-MITC on cyclin E2 and D3 on HL-60 cells

Cyclin E2 and cyclin D3 mean fluorescence intensity after 24h (A), 48h (B), 72h (C) 6-MITC's treatment and representative dot plot of cyclin E2 (up) and cyclin D3 (down) mean fluorescence intensity after 72h treatment (D). Cyclin E2 and cyclin D3 levels were evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using the t-test for paired data.

**p<0.05 vs control.*

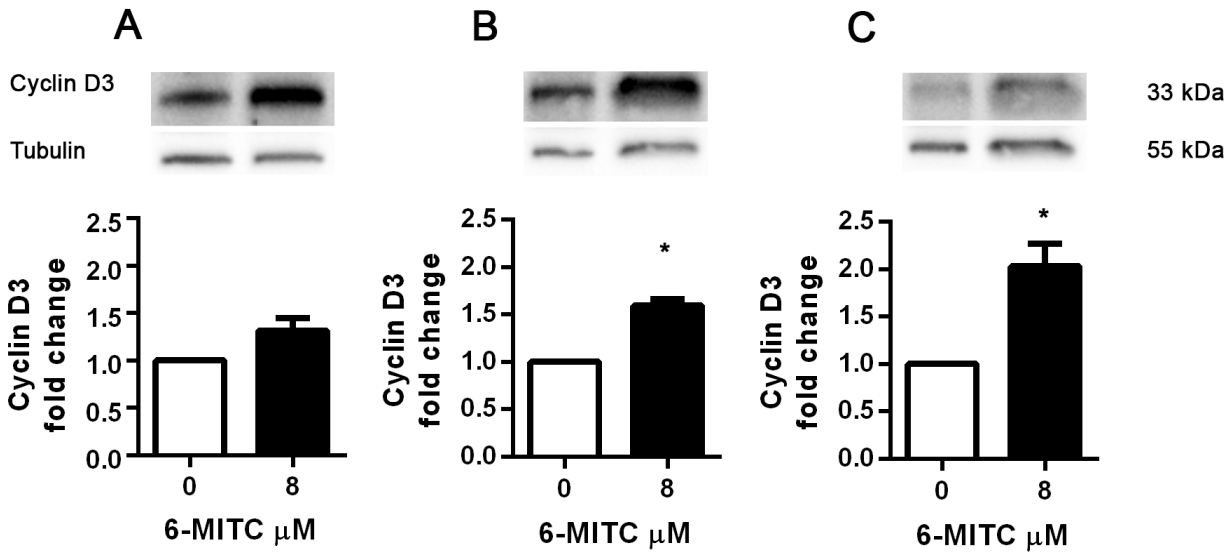


Figure 28: Effect of 6-MITC on cyclin D3 on HL-60 cells

Effect of 6-MITC 8μM treatment on cyclin D3 protein expression level in HL-60 cells. Cells were treated for 24h (A), 48h (B) and 72h (C) with 6-MITC 8μM. Cell lysates were immunoblotted with anti-cyclin D3 antibodies as reported in Methods. Results of scanning densitometry analysis performed on three independent autoradiographs are presented. Relative amounts, presented as means ± SEM, were normalized to the intensity of ß-tubulin and reported as fold increase vs control. Data were analysed using the t-test for paired data.

**p<0.05 vs control.*

The results described above have led to the publication of a work entitled: 6-(Methylsulfonyl) hexyl isothiocyanate as potential chemopreventive agent: molecular and cellular profile in leukaemia cell lines (Lenzi M., Cocchi V., Hrelia P. Oncotarget 2017).

Effect of 6-MITC on Autophagy on Jurkat cells and HL-60 cells/Analysis of autophagic flux by FCM

In order to verify if the 6-MITC is able to modulate the autophagic process, Jurkat and HL-60 cells were treated with the concentrations 4, 8 μM and 8, 16 μM respectively for 24h.

Simultaneously, in view of the fact that in the tumor cell lines the rapid formation and degradation of vesicles by lysosomes leads to a low signal, leukemia cell lines were also treated with 6-MITC in association with CQ (lysosomal inhibitor), to better characterize the autophagic flux. The combination CQ and rapamycin (mTOR inhibitor, therefore stimulator of autophagy) was used as a positive control.

As shown in figure 29, the 6-MITC induces a statistically significant increase in the percentage of autophagic vacuoles only at 8 μM (24.7% vs 2.9% in control), while at 4 μM the percentage of vacuoles is comparable the negative control. Instead, co-treatment with CQ showed a net increase in the percentage of autophagic vacuoles at both concentrations tested. In particular, an increase of 3.6 and 3 times in the cultures treated with 6-MITC and CQ simultaneously, with respect to treatment with isothiocyanate alone, was observed (39.8% vs 11.1% 6-MITC 4μM and 76.8% vs 24.7% 6-MITC 8μM) (Figure 29A, B).

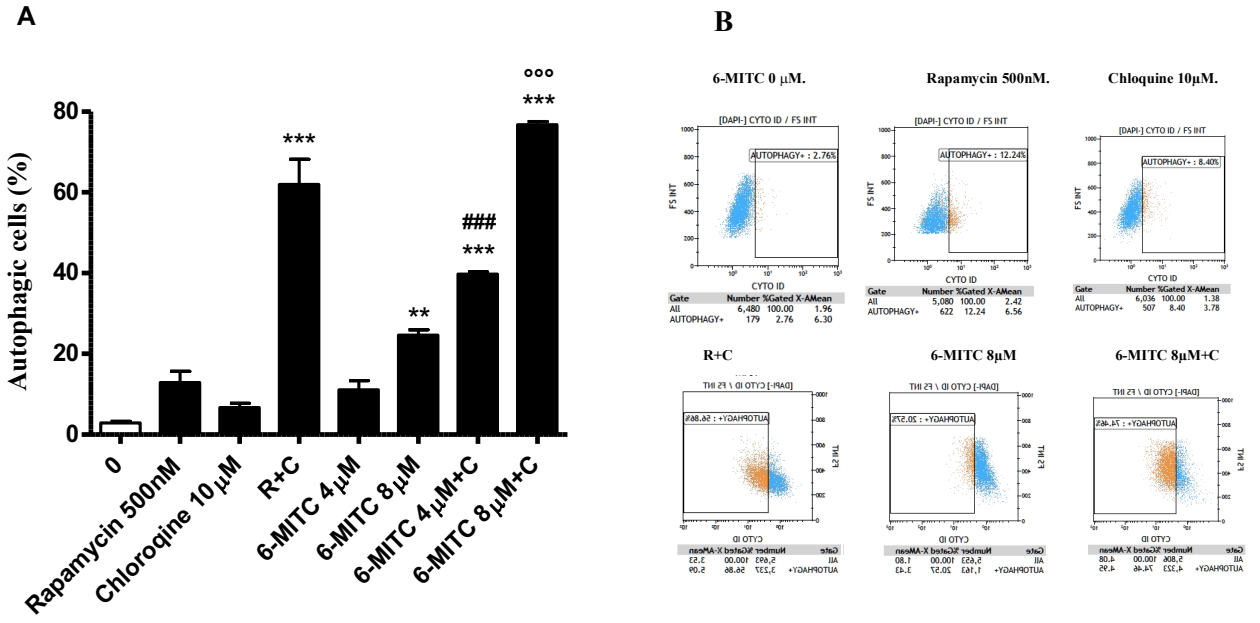


Figure 29: Effect of 6-MITC on autophagic flux in Jurkat cells

Fraction of autophagic vacuoles on Jurkat cells after 24h (A), and representative dot plot (B). Autophagy was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test and using the t-test for paired data.

***p<0.01 vs control; *** p<0.001 vs control;*

p<0.001 vs 6-MITC 4μM; °°°p<0.001 vs 6-MITC 8μM

Conversely, treatment with 6-MITC in HL-60 cells showed no significant change in the percentage of autophagic vacuoles at both concentrations tested (8 and 16 μM) but, also in this case, a statistically significant increase in the percentage of autophagic vacuoles was observed when 6-MITC was associated with CQ. More specifically, a 3 and 6 times increase was detect when isotiocyanate was

associated with CQ with respect to the cultures treated with 6-MITC alone (25.4% vs 7.6% 6-MITC 8μM and 78.56% vs 12.3% 6-MITC 16μM) (Figure 30A, B)

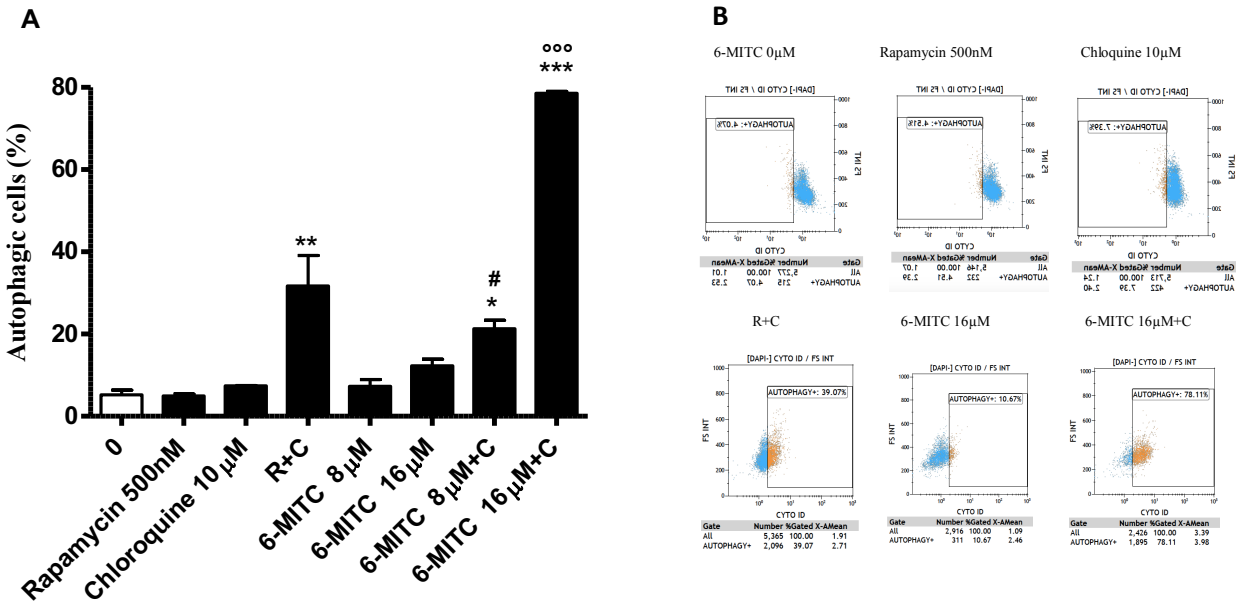


Figure 30: Effect of 6-MITC on autophagic flux in Jurkat cells

Fraction of autophagic vacuoles on HL-60 cells after 24h (A), and representative dot plot (B). Autophagy was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test and using the t-test for paired data.

***p<0.01 vs control; *** p<0.001 vs control*

 # p<0.05 vs 6-MITC 8 μM; °°° p<0.001 vs 6-MITC 16μM

Effect of 6-MITC on Oxidative Stress Biomarkers on Jurkat cells and HL-60 cells/Analysis of DCF, MitoPY, HE, MitoSOX, DHR123, MCB by FCM

To characterize the mechanism behind 6-MITC capability to induce autophagy in leukaemia cells its potential modulatory effect on some oxidative stress biomarkers was investigated in both cell lines. Jurkat and HL-60 cells were treated with concentrations ≤ IC_{50} for 24h.

As shown in Figure 31, in Jurkat cells a statistically significant increase only for DCF biomarker was observed (Figure 31A, G).

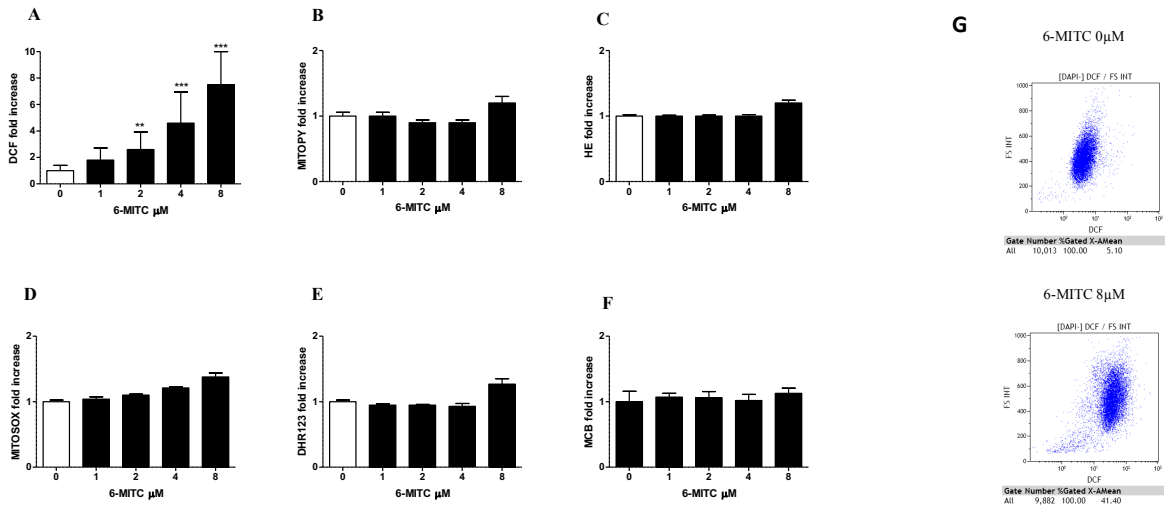


Figure 31: Effect of 6-MITC on oxidative stress biomarkers in Jurkat cells

Effect of 6-MITC on Oxidative Stress Biomarkers on Jurkat cells after 24h (A), and representative dot plot of DCF (B). Oxidative Stress Biomarkers was evaluated by FCM as described in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

***p<0.01 vs control; *** p<0.001 vs control*

On HL60 cells, instead, a statistically significant increase in the levels of DCF and MITOPY biomarkers was observed (Figure 32 A, B, G)

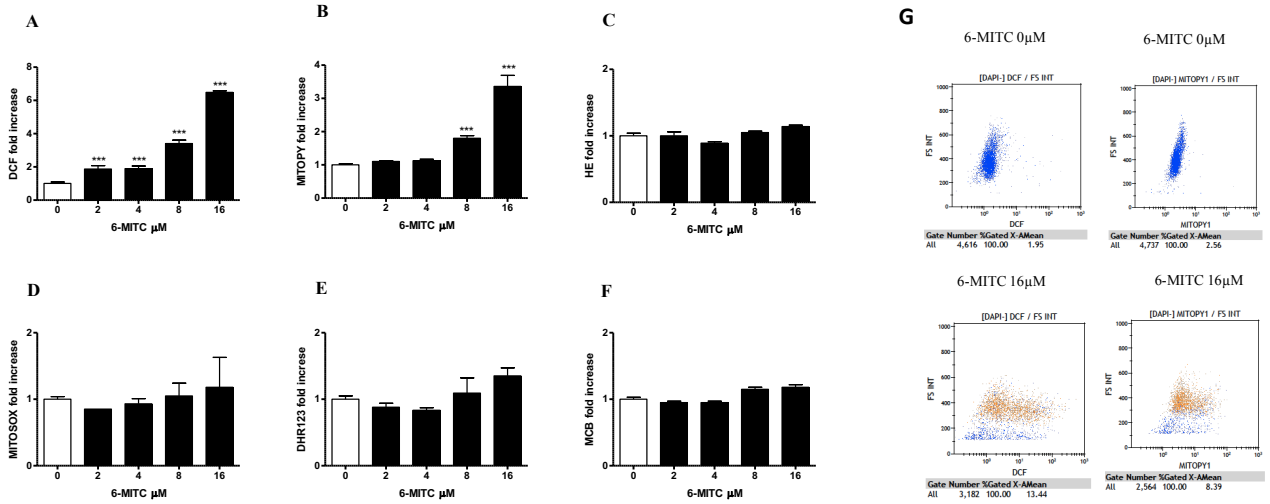


Figure32: Effect of 6-MITC on oxidative stress biomarkers in HL-60 cells

Effect of 6-MITC on Oxidative Stress Biomarkers on HL-60 cells after 24h (A), and representative dot plot of DCF and MITOPY (B). Oxidative Stress Biomarkers was evaluated by FCM as described

in Methods. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.
**** p<0.001 vs control*

Development of a protocol for the automation of the micronucleus test, by FCM analysis

The *in vitro* mammalian micronucleus test (CBMN) actually is performed with Cytochalasin B technique (Fenech, 2000). CBMN test involves the preparation and the staining with May-Grunwald/Giemsa of slides and the reading of 1000 binucleated cells for slides by microscopy. Therefore, some problems have been highlighted, including the number of cells analyzed, the high subjectivity of the reading at the microscope and the long analysis times. The OECD guideline no. 487 however, indicates that the MN test can be conducted by using other techniques such as flow cytometry, laser scanning cytometry or image analysis. For this reason, in this work, a new protocol for the automation of the micronucleus test by FCM, that seems to be the most promising platform to solve some problems that afflict the validated technique, was proposed. This protocol has also led to publication of the following work: **"**Flow cytometry *vs* optical microscopy in the evaluation of the genotoxic potential of xenobiotic compounds" (Lenzi M., Cocchi V., Hrelia P. 2018 Cytometry B).
For this purpose, the two methods have been compared. PBL were treated with six known mutagens and then genotoxicity was analysed by carrying out in parallel, the micronucleus test according to the new method proposed on the one hand (Lenzi *et al.,* 2018), and the classical method on the other (Fenech, 2000). The obtained results, with the FCM technique compared with those obtained with the validated method, demonstrated that the increased micronuclei percentage observed is perfectly comparable (Figure 33).

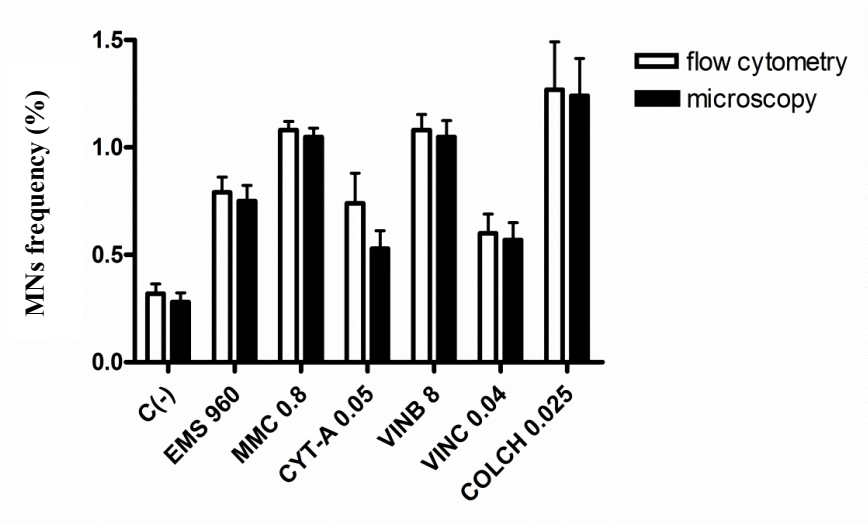


Figure 33: Flow cytometry vs Microscopy

Comparison of percentage of micronuclei in PBL treated for 3h with mutagens indicated and detected by FCM and optical microscopy

Furthermore, the reproducibility of the proposed method using fluorophores commonly used in FCM was demonstrated analysing the samples also with the flow cytometer Epics Elite Beckman Coulter and BRITE HS BioRad as well as the Guava EasyCite 5HT (Figure 34 A, B).

A B

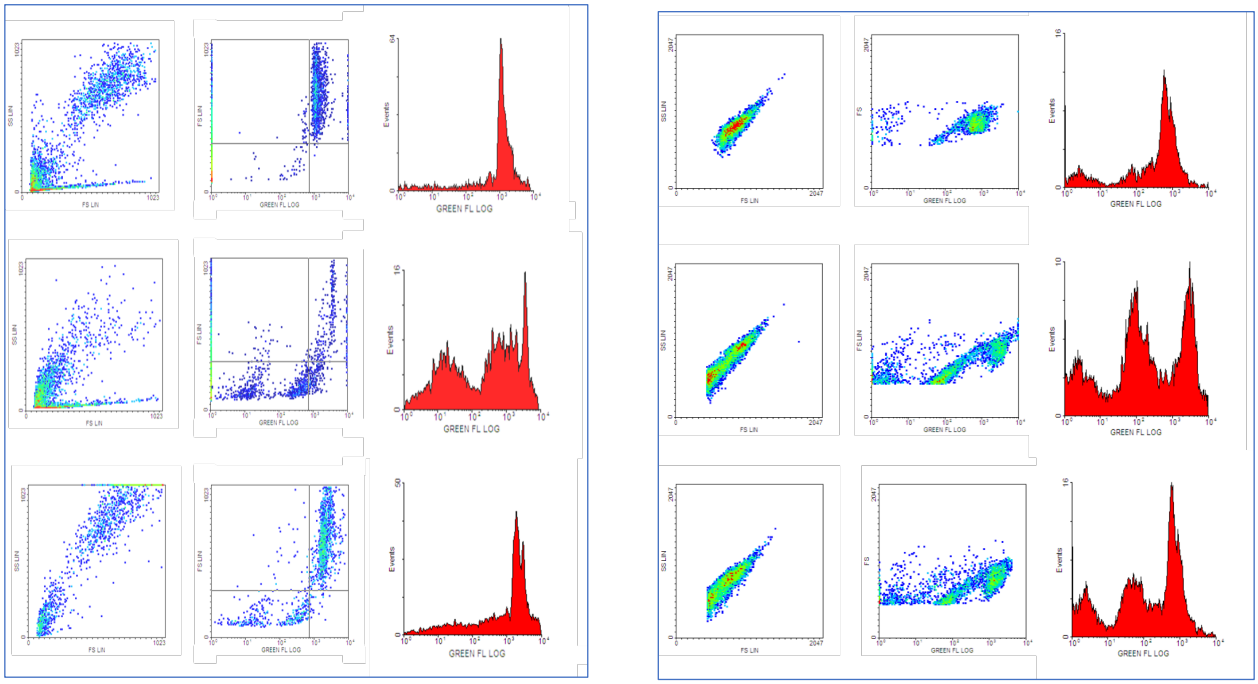


Figure 34: Reproducibility of the proposed method by FCM

Samples analyzed with the flow cytometer Epics Elite Beckman Coulter (A) and BRITE HS BioRad (B)

For these reasons, the study of mutagenesis and antumategenesis, reported below, were performed using the protocol developed for the flow cytometry (Lenzi *et al,* 2018).

Mutagenesis of 6-MITC in TK6 cells/Analysis of micronucleus by FCM

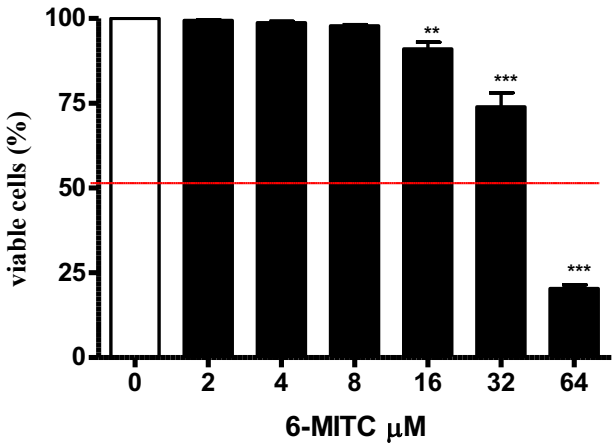
Short term treatment

In the first phase of the mutagenesis analysis, it was evaluated the 6-MITC-induced cytotoxicity and cytostasis after 3h treatment followed by 23h of recovery in complete medium at different concentrations (2, 4, 8, 16, 32, 64 µM). In fact, the OECD guideline no. 487 recommend proceeding to assess the genotoxicity of a xenobiotic, only if the treated population show a viability and a cell proliferation not less than 55±5% with respect to control cultures.

Figure 35 shows that the viability remains for all concentration abundantly higher than the threshold required by the OECD at all concentrations tested, as evidenced by the red line, except for the 6-MITC 64µM (Figure 35A).

At the same time, using RPD, the cytostasis measured in treated cultures and compared with that obtained in control cultures was checked in order to respect the threshold established by OECD and to define the concentrations (red values) to be used in the subsequent experiments (Figure 35 B).

A



B

Treatment	RPD %
6-MITC 0 µM	100
6-MITC 2 µM	97.2±0.4
6-MITC 4 µM	98.5±0.2
6-MITC 8 µM	90.4±0.6
6-MITC 16 µM	82.2 ±0.7
6-MITC 32 µM	44.7 ±0.2
6-MITC 64 µM	8.6 ±0.3

Figure 35: Effect of 6-MITC on cytotocicity and cytostasis inTK6 cells

Percentage of viable (A) and RPD (B) in TK6 cells treated with 6-MITC for 3h followed by 23h of recovery in complete medium. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

**** p<0.001 vs control*

Subsequently, the induction of apoptosis was evaluated as alternative cell death mechanism and to avoid the possible confounding effect of apoptotic bodies whit MNs. In this case it is important that the percentage of apoptotic cells measured in treated cultures remains comparable to that present in the control cultures.

In particular, with respect to the control cultures, a similar trend was detected a 2µM, 4µM and 8 µM (where doubling is not achieved), while a 2.2 and 3.4 time increase was detected at 16µM and 32µM respectively (Figure 36).

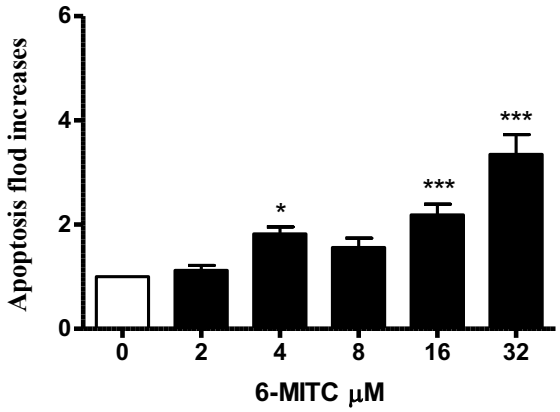


Figure 36: Effect of 6-MITC on apoptosis of TK6 cells

Apoptosis fold increase in TK6 cells treated with 6-MITC for 3h followed by 23h of recovery in complete medium. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

**p<0.05 vs control; *** p<0.001 vs control*

Therefore, on the basis of the obtained results, 2 and 4μM concentrations were selected to be used to assess the potential genotoxicity induced by 6-MITC.

For this purpose, the MNs frequency was measured in control and treated cultures and compared with MCC 400 ng/mL and VINB 25 ng/mL, known mutagens, used as a positive control. As shown in Figure 37 the MNs frequency increase in cell cultures treated with 6-MITC was not statistically significant compared to the control cultures, while a 2 and 5.4 time increase was detected for the mutagens MMC 400 ng/mL and VINB 25 ng/mL respectively (Figure 37 A, B).

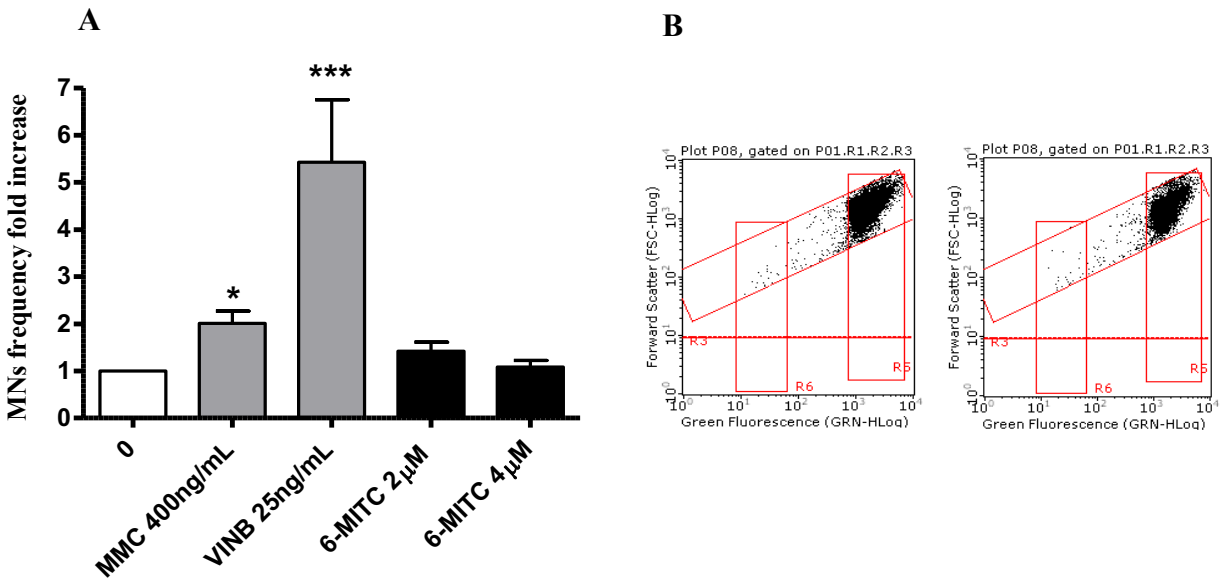


Figure 37: Effect of 6-MITC on mutagenesis in TK6 cells

*MNs frequency fold increase (A) and dot plot related to the cytofluorimetric analysis of micronuclei in the negative control (left) and 6-MITC 4 µM (right) (B) in TK6 cells after 3 hours of treatment followed by 23h of recovery in complete medium. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test. *p<0.05 vs control ***P<0.01 vs control*

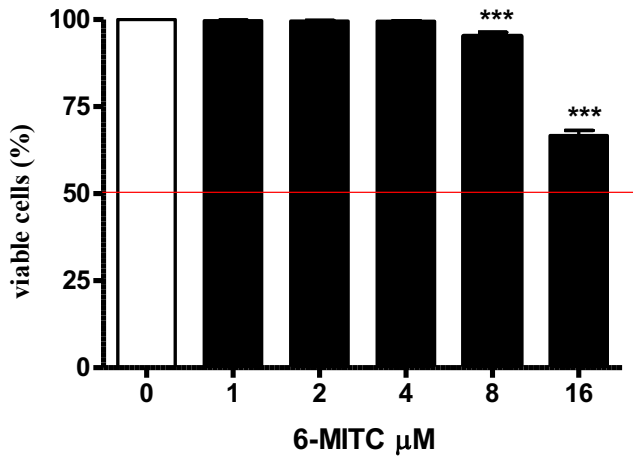
Long term treatment

In order to exclude the genotoxicity of a substance, the OECD guideline no. 487 suggests to perform a short term treatment and a long term treatment. For this reason, TK6 cells were treated with different concentrations of 6-MITC (0,1,2,4,8,16µM) for 26h.

As previously described for the short time treatment also in this case, in a first phase, were selected non-cytotoxic and non-cytostatic concentrations.

Figure 38 shows that the viability was remained abundantly higher than the threshold (evidenced by the red line) required by the OECD guideline no. 487 for all concentration tested (Figure 38A). The calculated RPD values proved to be consistent at all concentrations except the highest concentration tested where a cytostasi of 89.6 % (cell proliferation of 10.4 %) was observed (Figure 38B). For this reason, the 16 µM concentration was excluded from the apoptosis test.

A



B

Treatment	RPD %
6-MITC 0 µM	100
6-MITC 1 µM	95.5±0.2
6-MITC 2 µM	95.6±0.4
6-MITC 4 µM	80.9±0.7
6-MITC 8 µM	55.2±0.9
6-MITC 16 µM	10.4±0.5

Figure 38: Effect of 6-MITC on cytotocicity and cytostasis inTK6 cells

Percentage of viable (A) and RPD (B) in TK6 cells treated with 6-MITC for 26h. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

**** $p<0.001$ vs control*

Annexin V-PE/7-AAD double staining allowed to calculate the apoptotic fold increase. As shown in Figure 39 a similar apoptotic effect was observed at all concentrations tested, except for the 6-MITC 8µM where a population doubling was detected (2.3-fold increase compared to the control negative) (Figure 39).

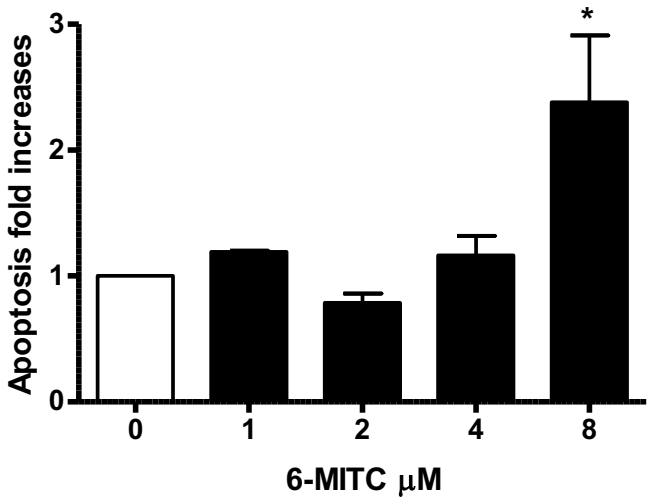


Figure 39: Effect of 6-MITC on apoptosis of TK6 cells

Apoptosis fold increase in TK6 cells treated with 6-MITC for 26h. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

**p<0.05 vs control*

Therefore, on the basis of the obtained results, 1 and 2μM concentrations were selected to be used to assess the potential genotoxicity induced by 6-MITC.

As shown in Figure 40 the MNs frequency increase in cell cultures treated with 6-MITC was non-statistically significant compared to the control cultures, while a 3.8 and 4.7 time increase was detected for the mutagens MMC 200 ng/ml and VINB 6.25 ng/ml respectively (Figure 40A, B).

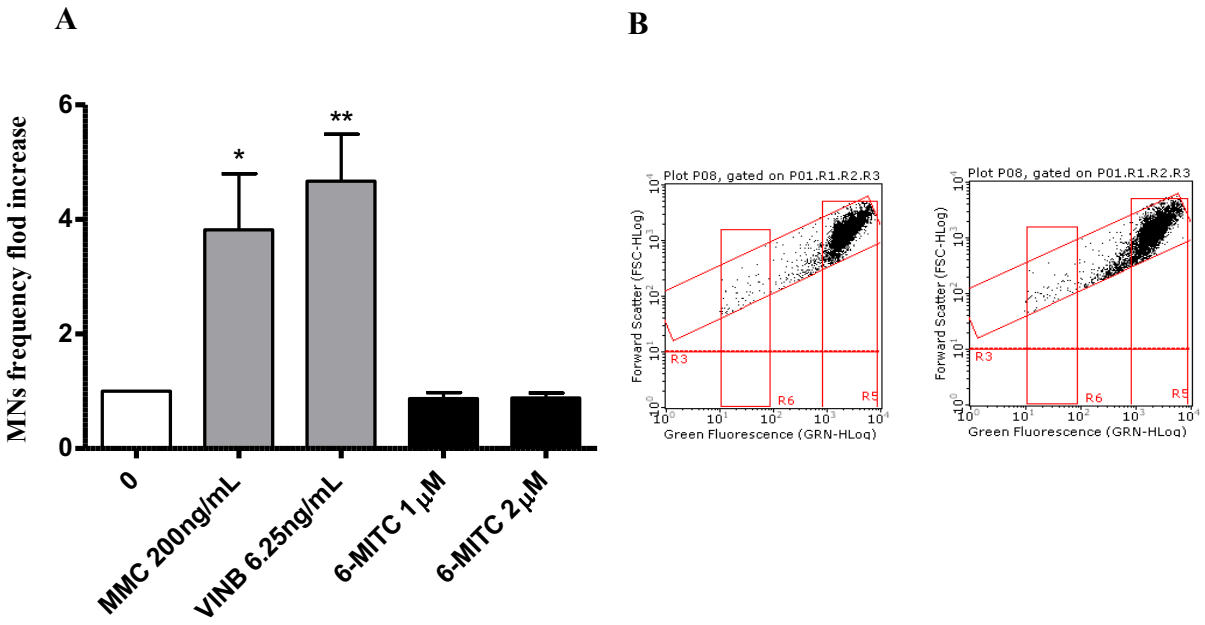


Figure 40: Effect of 6-MITC on mutagenesis in TK6 cells

MNs frequency fold increase (A) and dot plot related to the cytofluorimetric analysis of micronuclei in the negative control (left) and 6-MITC 2 µM (right) (B) in TK6 cells after 26h treatment. Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnetpost-test.

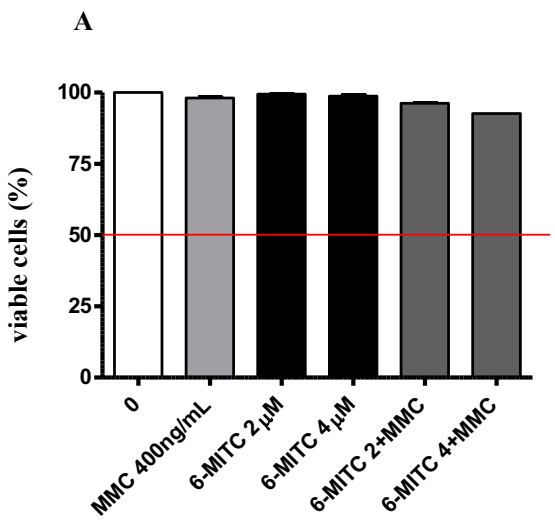
**p<0.05 vs control; **P<0.01 vs control*

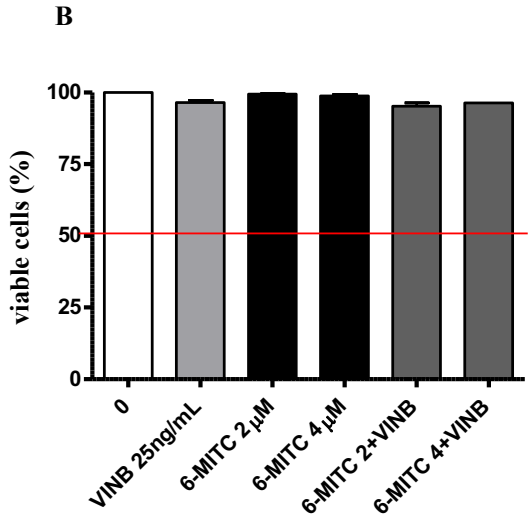
Antimutagenesis of 6-MITC inTK6 cells/Analysis of micronucleus by FCM

Short term treatment

Demonstrated the non-mutagenicity of isothiocyanate both short- and long-term treatment, the study continued evaluating the possible antimutagenic activity of 6-MITC against the previously mutagens analyzed.

A co-treatment of 3h followed by 23h of recovery in complete medium was performed and, also in this phase, cytotoxicity, cytostasis and apoptosis were checked. As show in Figure 41 cell viability, cell replication were found abundantly above the threshold established by the OECD no. 487 (Figure 41 A, B, C, D).





C

Treatment	RPD %
6-MITC 0 µM	100
6-MITC 2 µM	97.2±0.4
6-MITC 4 µM	98.5±0.2
MMC 400 ng/ml	72.8±0.9
6-MITC 2 + MMC	67.1±0.4
6-MITC 4 + MMC	60.9±0.6

D

Treatment	RPD%
6-MITC 0 µM	100
6-MITC 2 µM	97.2±0.4
6-MITC 4 µM	98.5±0.2
VINB 25 ng/ml	74.4±0.5
6-MITC 2 + VINB	66.3±0.7
6-MITC 4 + VINB	93.8±0.6

Figure 41: Effect of 6-MITC on cytotoxicity and cytostasis in TK6 cells

Percentage of viable in TK6 cells treated with 6-MITC for 3h followed by 23h of recovery in complete medium with MMC (A) and VINB (B). Value of RPD in TK6 cells treated with 6-MITC for 3h followed by 23h of recovery in complete medium with MMC (C) and VINB (D). Data represents the mean ± SEM of five independent experiments.

Apoptosis does not reach a doubling at all concentrations tested except for co-treatments where an increase of 3.5 and 2.4 was observed in the 6-MITC+MMC associations (Figure 42A) and an increase of 3.5 and 2.4 for associations with VINB (Figure 42B).

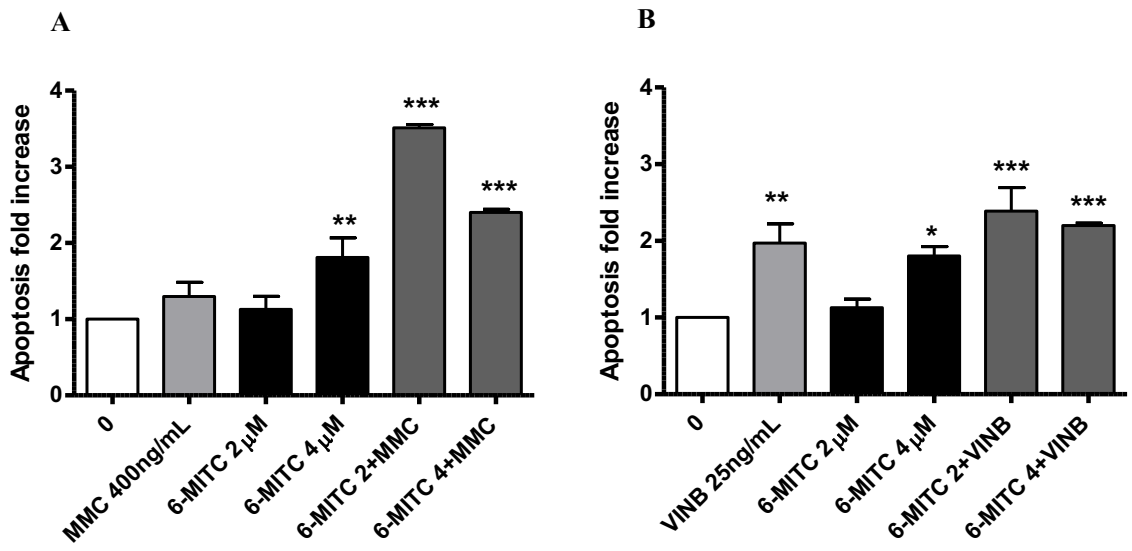


Figure 42: Effect of 6-MITC on apoptosis in TK6 cells

*Apoptosis fold increase in TK6 cells treated with 6-MITC for 3h followed by 23h of recovery in complete medium with MMC (A) and VINB (B). Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test. *p<0.05 vs control; ** p<0.01 vs control; *** p<0.001 vs control*

Overall, the obtained results were allowed to proceed with the MN test. The MNs frequency increase in the cultures treated with 6-MITC 2µM in association with MMC was comparable than cultures treated with the only mutagen while, the co-treatment MMC and 6-MITC 4µM was showed a statistically significant increase in MNs frequency compared to that measured in cultures treated with MMC alone (4.1 times *vs* 2.0 times in MMC) (Figure 43 A, C). On the contrary, in the case of aneuploidogen VINB, a decrease in MNs frequency increase was observed for both associations tested with respect to treatment with the mutagen alone, however, a statistically significant decrease was observed only at the highest concentration tested (5.4 times *vs* 4.2 times) (Figure 43 B, D).

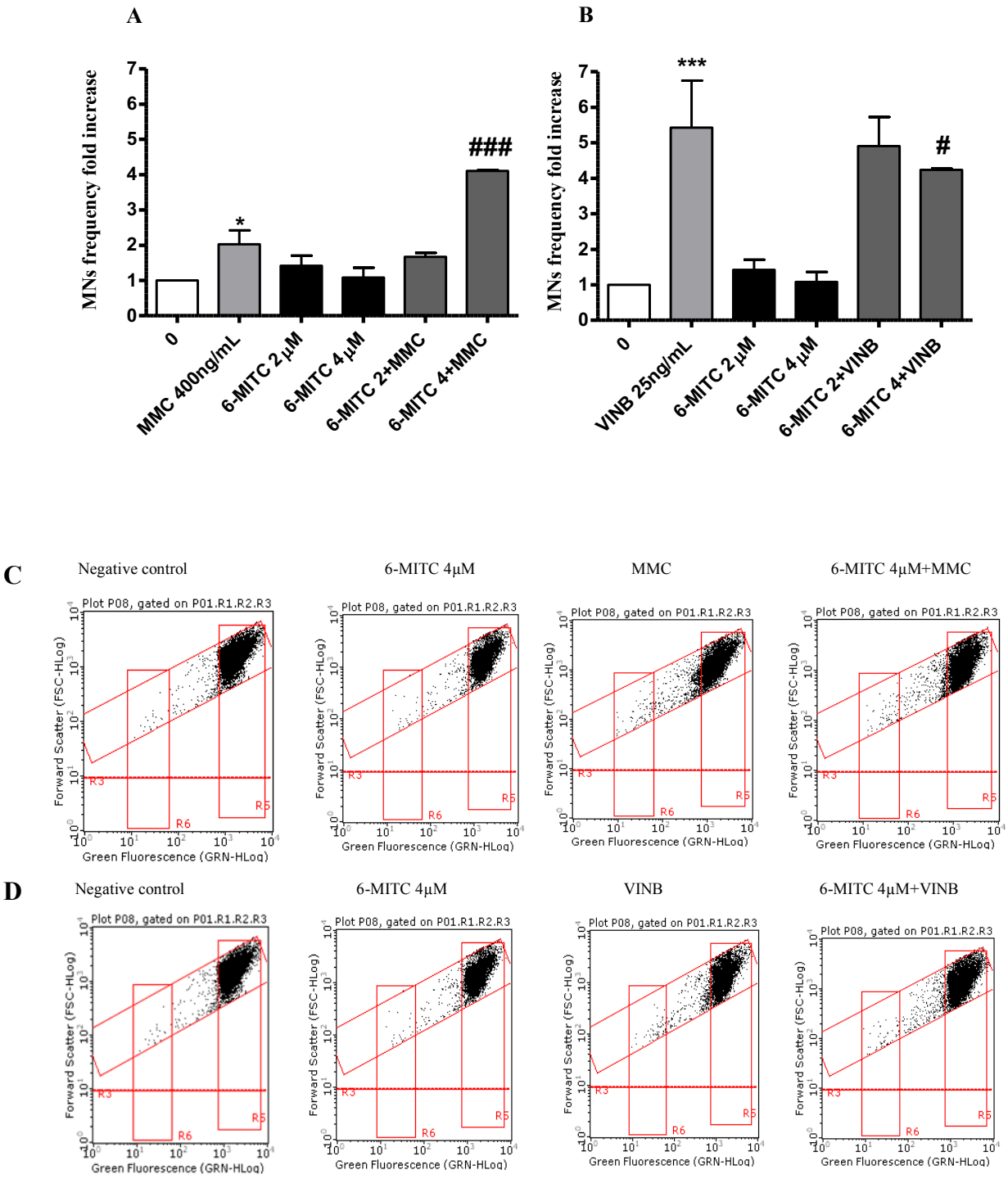


Figure 43: Effect of 6-MITC on mutagenesis in TK6 cells

MNs frequency fold increase in TK6 cells treated for 3h followed by 23h of recovery in complete medium with MMC (A) and VINB (B). Dot plot related to the cytofluorimetric analysis of micronuclei in MMC (C) and VINB (D) Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnetpost-test.

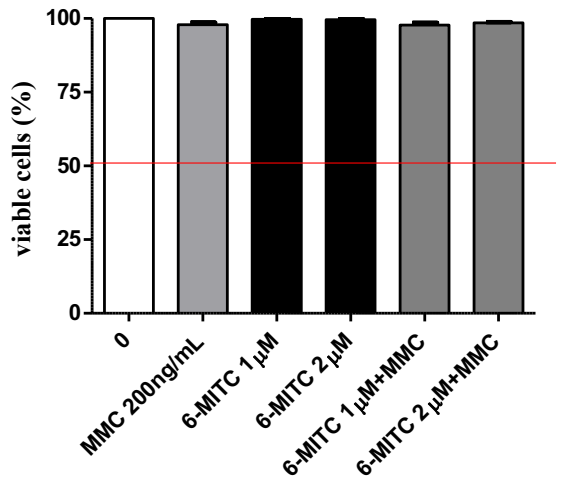
***p<0.01 vs control; ***P<0.001 vs control*

p< 0.001 vs MMC; #p<0.05 vs VINB

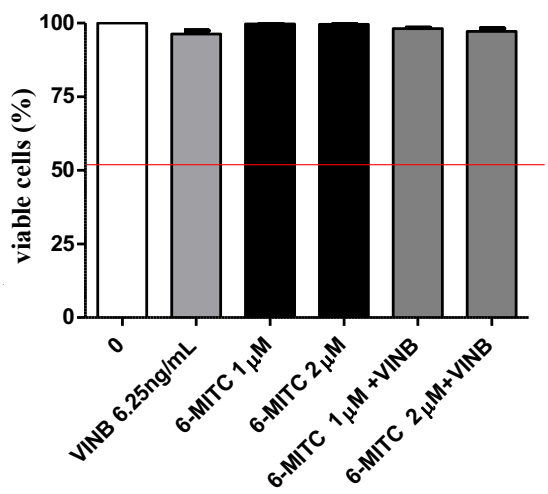
Long term treatment

The study was concluded by evaluating the antimutagenic activity of 6-MITC also at 26h. Similarly, to the short-term treatment, cytotoxicity and cytostasis values respected the established threshold at all the conditions analyzed (Figure 44A, B, C, D).

A



B



C

Treatment	RPD %
6-MITC 0 µM	100
6-MITC 1 µM	95.5±0.2
6-MITC 2 µM	95.6±0.4
MMC 200ng/mL	65.2±0.7
MMC+6-MITC 1	66.3±0.3
MMC+6-MITC 2	57.5±0.9

D

Treatment	RPD %
6-MITC 0 µM	100
6-MITC 1 µM	95.5±0.2
6-MITC 2 µM	95.6±0.4
VINB 6.25 ng/mL	78.8±0.3
VINB+6-MITC 1	77.0±0.2
VINB+6-MITC 2	77.1±0.4

Figure 44: Effect of 6-MITC on cytotocicity and cytostasi inTK6 cells

Percentage of viable in TK6 cells treated with 6-MITC for 26h with MMC (A) and VINB (B). Each bar represents the mean ± SEM of five independent experiments. Value of RPD in TK6 cells treated with 6-MITC for 26h with MMC (C) and VINB (D). Data represents the mean ± SEM of five independent experiments Data were analysed using repeated ANOVA followed by Dunnet post-test.

Moreover, as shown in Figure 45 apoptosis, at all concentrations tested, does not reach a doubling except for VINB alone and co-treatment 6-MITC 1 µM+VINB where an increase of 2.2 and 2.4 respectively was observed (Figure 45A, B).

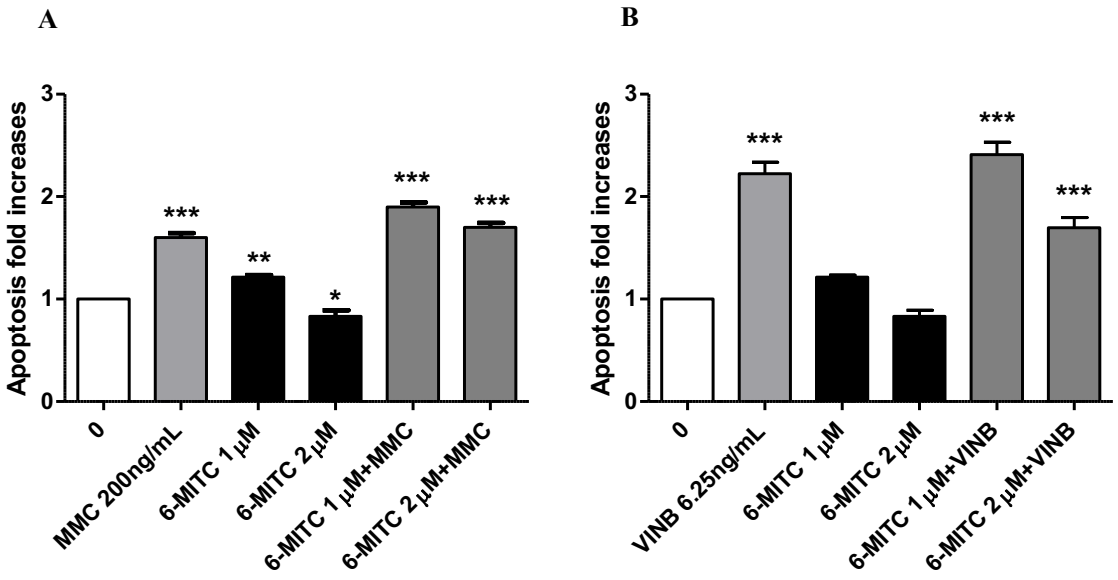


Figure 45: Effect of 6-MITC on apoptosis in TK6 cells

Apoptosis fold increase in TK6 cells treated with 6-MITC for 26h with MMC (A) and VINB (B). Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnet post-test.

**p<0.05 vs control; ** p<0.01 vs control *** p<0.001 vs control*

Therefore, checked cytotoxicity, cytostasis and apoptosis, the study ended by evaluating the 6-MITC antimutagen activity, after 26h treatment. The MN test confirmed the results obtained at the short term treatment. In fact, also in this case, the association with the MMC led to a statistically significant increase in MNs frequency at the highest concentration tested, compared to treatment with the clastogen alone (3.8 times *vs* 5.5 times) whereas, the association with the VINB reduced in a statistically significant manner the MNs frequency respect to the treatment with aneuploidogen alone at both concentration tested (2.3 times and 3.3 times *vs* 4.7 times) (Figure 46A,B, C, D).

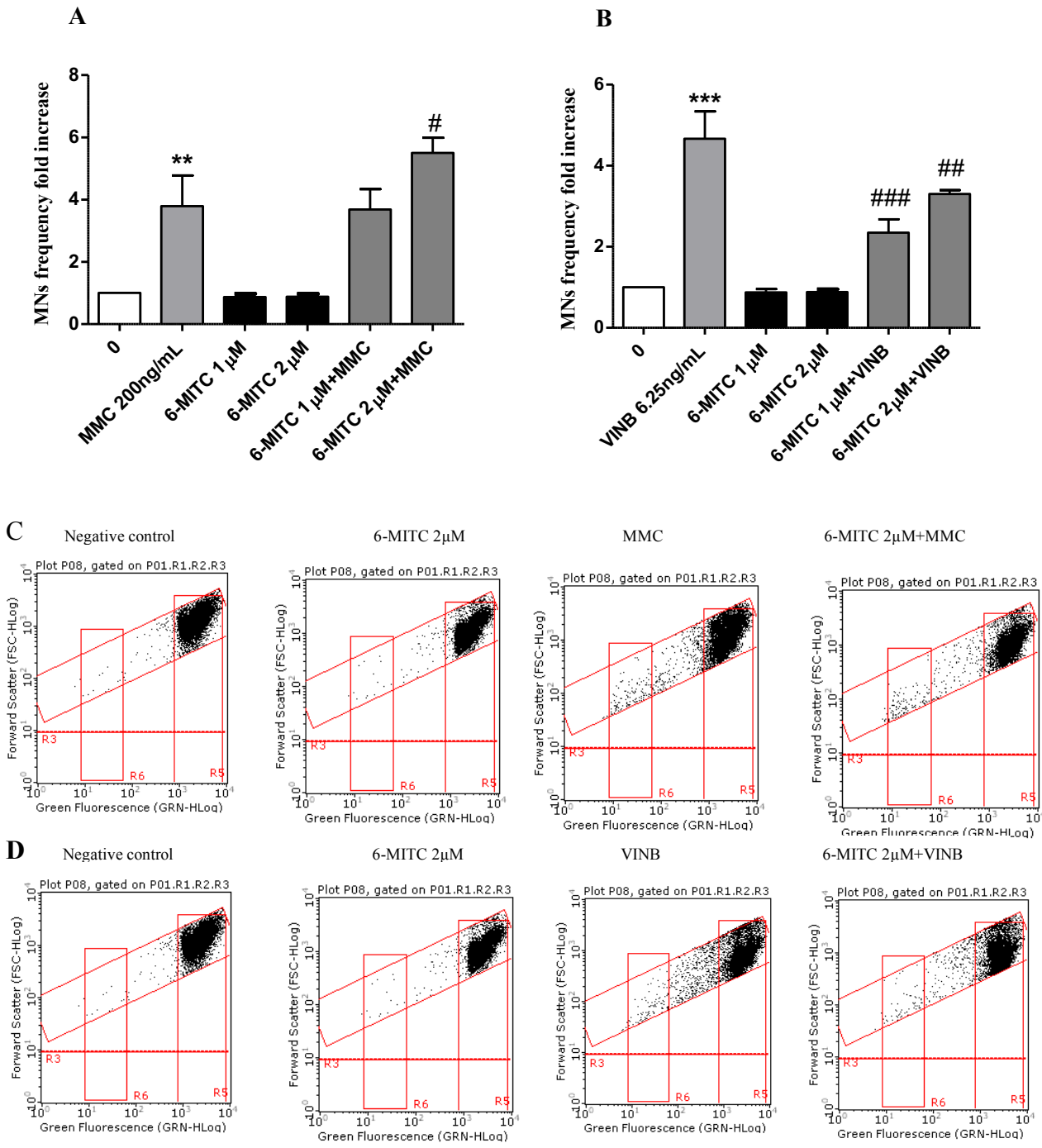


Figure 46: Effect of 6-MITC on mutagenesis in TK6 cells

MNs frequency fold increase in TK6 cells treated for 26h with MMC (A) and VINB (B). Dot plot related to the cytofluorimetric analysis of micronuclei in MMC (C) and VINB (D) Each bar represents the mean ± SEM of five independent experiments. Data were analysed using repeated ANOVA followed by Dunnetpost-test.

***p<0.01 vs control; ***P<0.001 vs control*

#p< 0.05 vs MMC; ##p<0.01 vs VINB; ###p<0.001 vs VINB

CHAPTER 5
DISCUSSION

In recent years there has been a growing interest in the chemopreventive potential of several naturally occurring substances; laboratory research and epidemiological studies have shown that the risk of developing various types of cancer may be reduced by the use of compounds that act with multiple mechanisms (Maru *et al*., 2016; Lenzi *et al*., 2017).

In this context, a major role is exerted by ITCs, a broad group of highly reactive compounds characterised by a common sulphur-containing functional group (-N = C = S) and a variable alkyl or allyl portion. In particular, 6-MITC has recently aroused the interest of researchers due to the proven anti-inflammatory, antioxidant and neuroprotective properties that have led to hypothesise its use as a chemopreventive agent (Nagai *et al*., 2009; Hsuan *et al*., 2106; Trio *et al.*, 2016; Lenzi *et al*., 2017).

In this study we show that 6-MITC, the main isothiocyanate found in the rhizome of *Wasabia japonica* exerts strong anti-leukemia effects through the modulation of different critical targets. In order to do so, we evaluated the cytotoxic, cytostatic and cytodifferentiating effects of 6-MITC on cancer cells and checked its selectivity of action by monitoring the same effects on healthy cells.

The analysis of the specific mechanism of cell death (apoptosis and/or necrosis) demonstrated the ability of 6-MITC to induce apoptosis in a concentration- and time-dependent manner in both cell lines tested. In fact, at the highest concentration, tested for the longest treatment time, the fraction of apoptotic cells in treated cultures increased 7 times in Jurkat cells and 8 times in HL-60 cells, compared to control cultures. Moreover, statistically significant induction of apoptosis occurred in Jurkat cells at 2μM and in HL-60 cells at 4μM. These concentrations are respectively 8 and 4 times lower than those required to induce apoptosis in PBL. The results suggest that this ITC has an important chemopreventive potential, by acting selectively on transformed cells and inducing low toxicity in non-transformed cells. A further confirmation came by the observation of an IC_{50} value in PBL more than 10 times higher than in Jurkat cells and more than 5 times higher than in HL-60 cells. It is therefore possible to define a range of concentrations in which 6-MITC acts selectively.

Depending on their activation the apoptotic pathways are generally classified as intrinsic, mitochondrial, and extrinsic, death receptor. In the extrinsic pathway the activation of death receptor results in the cleavage of pro-caspase-8 and activation of caspase-8 (Fimognari *et al*., 2011).

An analysis of molecular pathways highlighted the interesting capacity of 6-MITC to trigger apoptosis through the involvement of the extrinsic pathway. This contrasts with many widely studied phytochemicals such as *Hemidesmus Indicus*, or other ITCs, such as SFN, phenethyl isothiocyanate

and benzyl isothiocyanate, which all generally modulate the mitochondrial pathway (Fimognari *et al*., 2011; Sehrawat *et al*., 2016; Sehrawat *et al*., 20117).

In fact, after 72h of treatment at 8µM, the increase in activated caspase-8 apoptotic cells in both cell lines perfectly matched with the previously reported increase in the Annexin V-PE positive/7-AAD negative cells. These data suggest that the pro-apoptotic effect of 6-MITC was caused exclusively by the involvement of the extrinsic pathway, since the apoptosis did not seem to correlate with a loss of mitochondrial transmembrane potential. This hypothesis is further corroborated by the comparable number of cells observed in the treated and control cultures with a depolarised mitochondrial membrane potential. BAX and BCL-2 are two proteins located on the mitochondrial membrane. More specifically, BAX exerts pro-apoptotic activity while BCL-2 is an anti-apoptotic protein that inhibits apoptosis and is overexpressed in cancer (Autret *et al*., 2009). Most cancer targeted therapies are based on stimulating the expression of BAX protein and/or suppressing BCL-2 protein. Conversely, in this study the BAX and cytochrome c levels remained unaltered in both the control and treated cultures, while BCL-2 expression was upregulated in the treated cultures, resulting in a reduced BAX/BCL-2 ratio. This effect could represent a possible attempt at resistance of the cancer cells through a compensatory mechanism in response to the apoptosis induced by the ITC, which is, however, able to induce it by triggering the extrinsic pathway. BCL-2 is certainly a component of the apoptotic machinery; however, it can regulate autophagy via interaction with autophagy proteins. In fact, normally BCL-2 is bound to Beclin 1 preventing interaction with PIK3C3 and consequently inhibiting autophagy. Under autophagy inducing cell stress, instead, BCL-2 dissociates from Beclin 1 and activates the autophagic process (Booth *et al*., 2014; Parzych and Klionsky, 2014). Our study reported that 6-MITC induced autophagy in Jurkat and HL-60 cell lines with a mechanism that probably depends on H_2O_2 generation, which explains the high levels of BCL-2 observed.

Another interesting result in relation to the autophagic process is the sharp rise in the percentage of autophagic vacuoles that is observed when the 6-MITC is associated with CQ. The combination of 6-MITC with autophagy inhibitor might represent a potential strategy to enhance the anti-leukemia efficacy of 6-MITC. In fact, several studies show that simultaneous stimulation and autophagic blockage increase apoptosis levels, thus stimulating cell death of tumor cells (Li *et al*., 2016; Xia et *al.,* 2016).

6-MITC showed antiproliferative effects in both cell lines, as the distribution of cells in the different phases of the cell cycle proves. In particular, it successfully limits Jurkat cell replication by slowing down the cell cycle, causing a statistically significant decrease in the percentage of S phase cells after 24h of treatment at both tested concentrations. The reduction was still observable after 48h only at the highest tested concentration, and no longer visible after 72h. These results also suggest a cross-

talk between apoptosis and cell cycle regulation. In fact, the different treatment times allowed us to detect a statistically significant percentage of apoptotic cells at 24h, which continues to increase after 48h and 72h, while the decrease in S phase cells, statistically significant at 24h, gradually disappears as time progresses. Therefore, either the apoptotic cells observed were directly induced or those who exited from the cell cycle were slowing down.

6-MITC has also been shown to be able to induce a strong inhibitory effect on HL-60 cell proliferation, resulting in a blockage of the cell cycle progression in the G_1 phase, statistically significant after 48h of treatment and constant after 72h. At the same time, a statistically significant decrease in the S phase was observed. In human cells, cell cycle progression is controlled at three checkpoints, G_1, S andG_2/M, by a series of cyclins and cyclin-dependent kinase (CDK) complexes. In particular, the progression from G_0/G_1 phase to S phase is regulated by D-type cyclins and E-type cyclins. D-type cyclins act precociously in the progression through the G_1 phase while E-type cyclins become upregulated later during the transition (Lee *et al*., 2017). In the current study, 6-MITC increased the expression of cyclin D3 but did not modulate cyclin E2 levels. A possible explanation could be that 6-MITC blocks HL-60 cell-cycle acting precociously on the G_1/S transition and that the potential critical factor for G_1 arrest is cyclin D3.

Recently Wang et al. (Wang *et al*., 2016) demonstrated that the overexpression of cyclins sometimes leads to apoptosis. Therefore, we hypothesise that cyclin D3 overexpression might be involved in 6-MITC-indiced apoptosis as well.

Moreover, the different treatment times in this case allowed us to hypothesise that the cytotoxic and cytostatic effects showed by 6-MITC on HL-60 cells were connected. In fact, 6-MITC stimulates apoptosis either as a direct action or indirectly through the blockage of the cell cycle. A further confirmation of its selectivity of action, is the fact that 6-MITC does not exercise any kind of activity on healthy cell proliferation.

In light of these demonstrated activities, p53 expression levels were analysed in Jurkat cells; p53, in fact, is a tumour suppressor gene that plays an important role in the cell cycle and apoptosis. In non-transformed cells, p53 levels are normally low and rise as a result of DNA damage or other injuries. Its increase causes a slowdown/blocking of the cell cycle and/or apoptosis induction. However, most tumour cells are p53-mutated or p53-null and thus proliferate indiscriminately and beyond the normal mechanisms of cell survival regulation (Yu, 2006).

An investigation of this aspect is particularly interesting because ITC may be able to modulate the expression of p53mon p53-mutated cells (Jurkat) while causing its effect without any involvement, as demonstrated in HL-60 cells that are p53-null. Data analysis showed no change in p53 levels at

any treatment time, supporting the hypothesis that ITC induces cytostatic and cytotoxic effects with an independent p53 mechanism.

A large number of malignant cells undergoes mitosis, and these cells are poorly differentiated (Thwe *et al*., 2016). A chemopreventive agent could act as differentiation inducer, stimulating the differentiation of transformed and immature cells into normal and mature cells. By measuring the expression levels of CD-14 and CD-15 (membrane proteins characteristic of macrophages and granulocytes respectively), 6-MITC demonstrated an ability to induce cytodifferentiation of promyelocytic cells into both macrophage and granulocyte phenotypes after the longest treatment time.

Subsequently the research continued evaluating the safety of use of the 6-MITC. It is of fundamental importance to evaluate the toxicological profile of each substance at all levels and, in particular, in terms of analysis of its effects on genetic material, to exclude any genotoxicity and mutagenicity capacity (Combes *et al*., 2007). Of great importance is also the impact that mutations can have on human health, given their involvement in multiple physiological and pathological processes. In particular, if the mutation occurs in the germ line of organisms that reproduce sexually, it will be transmitted to subsequent generations and, as a consequence, there will be potential hereditary anomalies. Moreover, these mutations can contribute to determining pathologies with a complex etiology, such as heart disease, hypertension, diabetes, and represent a possible cause of infertility, teratogenesis or spontaneous abortions. Furthermore, the involvement of a somatic cell could lead to the development of neoplasms, premature aging, damage to the immune system and promote the onset of cardiovascular diseases, such as atherosclerosis, and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Chatterjee and Walker, 2017).

The cytotoxic and mutagenic activity of 6-MITC was therefore initially evaluated both at short times and at long times, in order to exclude the mutagenicity of the compound. The OECD guideline no. 487, in fact, states that for a correct evaluation in order to obtain a negative result it is necessary to test both experimental conditions (short times and long times) (OECD no. 487, 2016). After defining the non-cytotoxic concentration and demonstrating the absence of mutagenicity of the compound under study, the research continued by analysing the antimutagen potential against two known mutagenic agents, with different mechanisms of action: the MMC clastogen and the aneuploidogen VINB.

The ability to inhibit or counteract the mutations at the cellular level, induced by the exposure to a mutagen, has a great therapeutic value, as it represents one of the possible mechanisms through which a chemopreventive can express its activity. Mutagenic and antimutagenic activity was evaluated in terms of increase/decrease of the frequency of MN.

The Micronucleus Test (OECD Guideline for the testing of chemicals: "*In vitro* mammalian cell micronucleus test" no. 487, 2016) is part of a large group of mutagenesis tests which can highlight the mutagenic potential of a xenobiotic. The micronucleus is used as an important biomarker of chromosomal damage, genomic instability and cancer risk. It is an accessory nucleus, morphologically identical to the main one but smaller, easily identifiable by optical microscopy with a technique validated by the European Center for the Validation of Alternative Methods (ECVAM). Although, it has limitations as to the number of analysed cells, exiguous for the purposes of robust analysis, the long analysis times and, moreover, the high subjectivity of the operator. Therefore, in the present thesis a protocol has been used that allows the possible automation in flow cytometry as an alternative platform to optical microscopy (Lenzi *et al*., 2018).
6-MITC has been shown to counteract genotoxicity, in terms of decrease in micronuclei frequency, of aneuploidogen VINB, which acts at the level of cellular mitosis by preventing tubulin polymerization and consequently inhibiting the aggregation of microtubules during the formation of the mitotic spindle. It is though unable to prevent the activity of the clastogen MMC, where the increase in micronuclei frequency in association with 6-MITC is statistically significant, compared to the increase measured in cultures treated with MMC alone. From the results obtained it emerges, therefore, an interesting biological potential, which needs to be confirmed on a greater number of mutagens, in order to define more accurately the mechanism of action of the isothiocyanate.

Overall, the results obtained and summarised in the table shown in Figure 47 demonstrate that 6-MITC successfully modulates many of the molecular and cellular pathways constituting the main chemopreventive mechanisms.

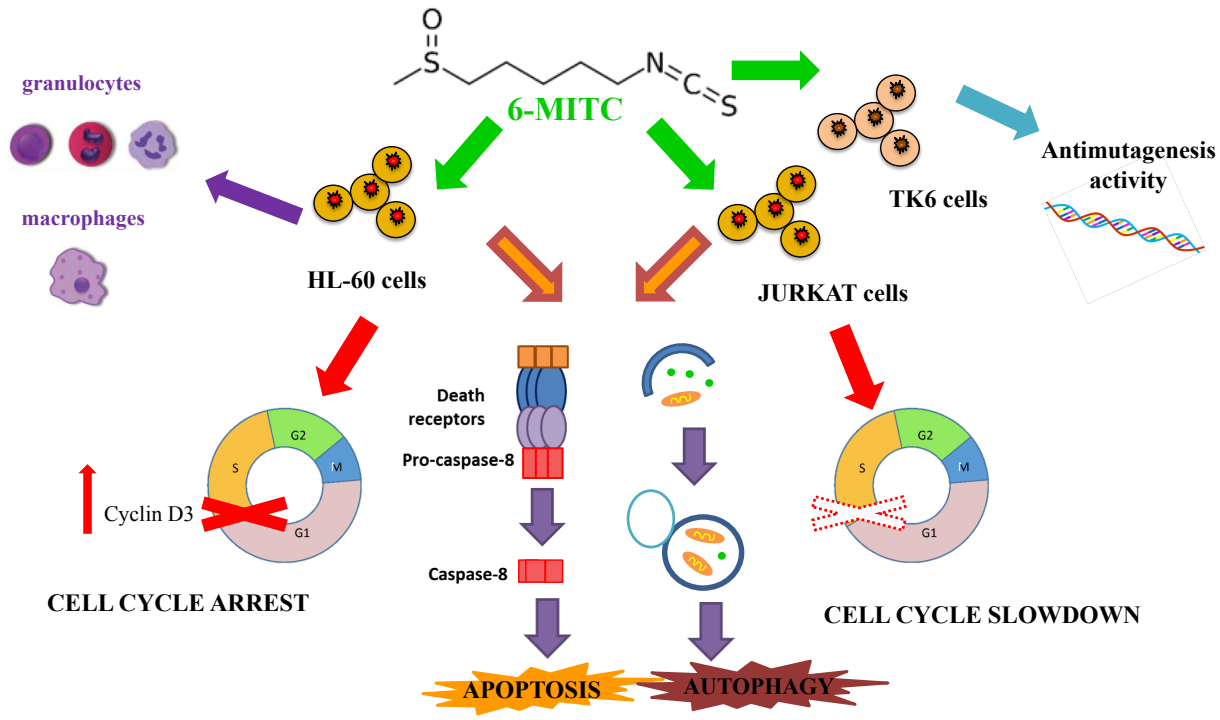


Figure 47: pathways modulated by 6-MITC. *6-MITC induced apoptosis and autophagy on transformed cells. Moreover, apoptosis is triggered by an extrinsic pathway increasing activated caspase-8 level; autophagy is triggered by an increase of H_2O_2 level (orange arrows). 6-MITC can limit tumor growth by slowing down the cell cycle of Jurkat cells and it blocks HL-60 cell cycle by modulating Cyclin D3 expression levels (red arrow). 6-MITC showed the ability to induce cytodifferentiation of promielocytic HL-60 cells into macrophage and granulocytic phenotypes (purple arrow). Finally, 6-MITC is able to counteract the activity of VINB showing antimutagenic activity (blue arrow).*

CHAPTER 6
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