UNIVERSITY OF PALERMO
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PhD in Cell Biology (Cell and developmental biology)

NUTRIGENOMICS EFFECTS OF PHYTOCHEMICAL INDICAXANTHIN IN *in vitro* CELL SYSTEMS

PhD Student:

Dr. Flores Naselli

PhD School Coordinator:

Prof. Gabriella Sconzo

Tutor:

Dr. Fabio Caradonna, PhD

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I dedicate this thesis to God, my husband and my families

for their constant support and unconditional love.

I love you all dearly.

ABSTRACT

Nutrition plays a key role in many aspects of health, indeed epidemiological studies provide evidence that diet can play essential roles in reducing the risk of chronic diseases. Accumulating evidence indicates that dietary compounds (phytochemicals) from fruit and vegetables can modulate multiple cancer-inflammation pathways which are relevant for chemoprevention and are commonly deregulated by epigenetic mechanisms in cancer cells, including drug detoxification, cell cycle regulation, apoptosis induction. Epigenetic refers to heritable alterations in gene expression that do not involve modification of the underlying genetic DNA sequence. Although epigenetic changes are heritable in somatic cells, these modifications are also potentially reversible, which makes them attractive and promising avenues for tailoring cancer preventive and therapeutic strategies.

The aim of this study was to investigate the antiproliferative potential of a Cactus pear pigment extract (Indicaxanthin) to assess the phenotypic effects on colorectal cancer cell lines and to investigate the biological function and/or epigenetic mechanisms for Indicaxanthin's activity.

The antiproliferative effects of Indicaxanthin (Ind), were investigated on a number of human cancer cell lines including hepatocarcinoma cells (HepG2, Ha22T, HUH 7), breast cancer cells (MCF7), cervix epithelial carcinoma (HeLa) and colorectal carcinoma cells (Caco2, LOVO1, DLD1, HT29, HCT116). Ind caused a clear dose-dependent decrease in the proliferation of Caco2, with minor effect on the other cell lines. Flow cytometric analysis showed a pro-apoptotic effect of the pigment at 48h in Caco2 cells. Incubation of proliferating Caco2 cells with Ind (10µl to 100µl) remarkably reduced the global DNA 5-methyl cytosine methylation and caused a stable demethylation of the tumor suppressor *p16*INK4a gene promoter, with reactivation of the silenced mRNA expression and accumulation of p16INK4a protein. A decrease of the hyperphosphorylated retinoblastoma protein in favour of its hypophospohrylated status was observed, with unaltered level of the cycline-dependent kinase CDK4. Analysis of cell distribution in the cell cycle phases after Ind treatment showed arrest of Caco2 cells in the S- G2/M- phase.

The effect of Ind on LINE-1 methylation levels in the other colorectal cancer cell lines was therefore explored as well as the effect of Ind on promoter methylation status of other genes implicated in colon carcinogenesis. Ind is able to alter the methylation status on global and specific gene level. To rationalize the mechanism of DNA methylation changes induced

by Ind, the effect of the phytochemical on the activity and the level of DNA methyltransferase (DNMT) was evaluated. Ind induced a dose-dependent inhibition of DNMT activity on Caco2 cells, while did not affect DNMT1 and DNMT3b level. However a significant increment of DNMT3a was evident in Caco2 cell line. Aberrant expression of DNMTs and their isoforms has been found in many types of cancer and their contribution to aberrant DNA methylation has been proposed. Following this hypothesis, the effect of Ind on the expression of DNMT splice variant, was investigated. The expression of DNMT increased appreciably only in some cell lines with respect to only some DNMT genes after Ind treatment. DNA demethylation may take place as an active mechanism by the activity of specific enzymes. In order to assess whether Ind is able to induce an alteration in DNA demethylase expression, which may explain the effects of Ind on DNA methylation in the tested cell lines, the effect of Ind on the DNA demethylase expression was investigated. Ind induced an increased expression of some enzymes with demethylating activity (TET2, MBD4) relatively only in some cell lines.

In this work it appears that the phytochemical Indicaxanthin induces different effects (both epigenetic and biochemical) on colorectal cancer cell lines tested (reduction of proliferation, induction of apoptosis, induction of cell cycle arrest, change in DNA methylation, alteration of gene expression). The effects brought by Ind, are often variable depending on the cell line used, such phenomenon can be attributed to the cell lines themselves. Furthermore from in silico molecular imaging tests, in this study it was supposed that epigenetic effects of Ind are not mediated by a direct alteration of DNMT expression, rather by an influence of Ind on their activities. Then, Ind binding the DNMT and altering their methyltransferase function could cause an imbalance of global DNA methylation.

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1. INTRODUCTION

Modernization and its resulting lifestyle trends have ushered in a new era of chronic illness; one in which an unprecedented number of people are estimated to contract cancer and other inflammatory diseases. Observational studies have suggested that lifestyle factors such as tobacco, obesity, alcohol, and a sedentary lifestyle are major contributors to cancer risk. Moreover it is well documented that individuals adhering to a vegetables/fruits-depleted diet and a relatively sedentary lifestyle are more likely to develop obesity, contract chronic conditions such as diabetes, cardiovascular disease, and cancer (GLOBOCAN Colorectal Cancer Data 2008). The importance of dietary habits in achieving and maintaining a healthy status is widely acknowledged. Indeed epidemiological studies provide evidence that diet can play essential roles in reducing the risk of chronic diseases (cardiovascular disorders, type-2 diabetes, various inflammation-based health problems, and cancer) (Willett 1994; Lipkin et al., 1999).

Plants are recognized to have a wide molecular basis of preventive mechanisms. Many contain compounds that act as direct or indirect antioxidants, participate in detoxification and elimination of waste, activate regulatory T-cell responses, and modulate cell signalling to induce apoptosis in damaged cells while promoting differentiation in others (Sokolosky and Wargovich 2012). Studies on a wide spectrum of plant secondary metabolites extractable as natural products from fruits, vegetables, teas, spices, and traditional medicinal herbs have identified various bioactive plant compounds (phytochemicals) that regulate multiple cancer-inflammation pathways. In addition they are cost effective, exhibit low toxicity, and are readily available. In this context dietary components have appeared of critical importance and the eventual roles of dietary phytochemicals in tumour chemoprevention have attracted considerable scientific interest in the latest two decades (Kelloff et al., 2000). Indeed the global demand for more affordable therapeutics and concerns about side effects of commonly

used drugs has renewed interest in phytochemicals and traditional medicines which allow chronic use (Harvey 2008; Li & Vederas, 2009; Singh 2007).

1.1. Cancer disease and dietary chemoprevention

Cancer cells are distinguished by several distinct characteristics, such as self-sufficiency in growth signal, resistance to growth inhibition, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2011). In several familial cancer syndromes, genome instability develops due to inherited mutations in the ‘‘DNA caretaker’’ genes essential for DNA repair or the DNA damage response (Kinzler and Vogelstein 1997). Instead, in sporadic tumorigenesis it has been proposed that in early stages activated oncogenes induce replication stress through deregulation of cell cycle progression, causing chromosomal instability (Gorgoulis et al., 2005). While oncogene activation can induce replication stress, it also activates DNA damage response checkpoints and causes cellular senescence, forming a barrier to cancer progression (Bartkova et al., 2006). Without inactivating mutations in DNA damage response genes or cell cycle checkpoints, transformation does not occur. In any case the deregulation of multiple genes and their accumulation lead to the acquisition of these properties driving tumorigenesis. Rationally designed drugs that target a single gene product are unlikely to be of use in preventing or treating cancer. Moreover, targeted drugs can cause serious and even life-threatening side effects or therapy resistance (Hanahan and Weinberg, 2011). Often, cancers have a long latency period, 20 years or more. By the time they are clinically detectable, the system has degenerated into disorganized chaos which point it may be beyond repair (Sporn, 2011). Therefore, there is an urgent need for safe and effective chemo-preventive multifunctional drugs that act on entire networks in the body, rather than single targets (Deocaris et al., 2008).

Colorectal cancer (CRC) is one of the most common forms of malignancy and the second leading cause of cancer-related death in the Western world (Center et al., 2009). Most of the knowledge about colon cancer progression is derived from the study of the inherited form, familial adenomatous polyposis (FAP), an autosomal dominant CRC syndrome caused by the Adenomatous Polyposis Coli (APC) gene mutation which account for about 10 % of cases (Galiatsatos and Foulkes 2006). Mutant APC promotes WNT signaling pathway activation, resulting in transcription of several genes involved in cell proliferation, differentiation, migration and apoptosis (Fearnhead et al., 2001). This highlights the importance of this pathway to carcinogenesis, which is exemplified by the fact that it is over-active in > 90% of CRC.

Increasing evidence recently claimed that colorectal tumors consist of a heterogeneous population of cells hierarchically organized, with colorectal cancer stem cells (CCSCs) at the top of this pyramid model. The concept that this subset of cells may arise from normal stem cells, as a result of genetic and /or epigenetic mutation (Barker et al., 2009) is appealing for several reasons, (such as the possibility of reprogramming them or inducing differentiation by therapy). Healthy stem cells (normally reside at the base of the intestinal crypts) and CCSCs share many properties, including the self-renewal and multi-lineage differentiation capacity. CCSCs also possess altered DNA repair machinery and high expression levels of anti-apoptotic genes, which may explain the failure of current anti-cancer treatments (Todaro et al., 2007).

The intra-tumor cellular organization is influenced by several stimuli coming from the contiguous microenvironment that also provides protection by sheltering CCSCs from diverse genotoxic insults, contributing to their enhanced therapy resistance (Sun and Nelson 2012)

Under physiological conditions, colon homeostasis is highly regulated, and it is the result of a perfect balance between stem cells, differentiated cells and the microenvironment.

Sometimes, however, this balance is disrupted laying the foundations for the emergence and progression of a tumor. Although the mechanisms that promote and sustain colon carcinogenesis are not yet known, progression from adenomas to colon cancer is a multistep process, involving mutations in several genes. There is evidence that a complex interaction between environmental carcinogens and genetic alterations facilitates the selective growth of transformed cells, thus leading to the development of colonic dysplasia and cancer (Fearon et al., 1990).

Colonoscopy screening, which is aimed at identifying and removing pre-cancerous lesions (*i.e.* polyps), represents the gold standard of the preventive strategies for CRC, even though it is an invasive procedure that reduces the compliance and participation of CRC high-risk subjects in the screening programs.

In light of the characteristics of this heterogeneous type of cancer, and the difficulty of finding an effective therapy, a key role in the "cure" of colon cancer is represented by prevention.

It is interesting to note that people exhibiting the lowest rates of colorectal cancer are also more likely to follow a pastoral way of life reflecting reliance upon the natural world. The reduced risks of cancer and other chronic diseases enjoyed by these individuals are attributable in some ways to genetic disposition, but also correlate largely with environmental factors arising from their retention of preventive dietary and lifestyle practices (GLOBOCAN Colorectal Cancer Data, 2008).Therefore food is now also a conditioning environment that may determine stress adaptive responses, influence metabolism, immune homeostasis and the physiology of the body (Tennant et al., 2010). In this respect cancer chemoprevention with natural phytochemical compounds is an emerging strategy to prevent, impede, delay, or cure cancer. Essentially the concept of cancer chemoprevention by dietary phytochemicals is to arrest or reverse the progression of premalignant cells towards full malignancy using

physiological mechanisms, such as the attenuation cancer-inflammation pathways. Indeed according to the chemical behaviour in either solution or biological environment, anti-carcinogenic effects of phytochemicals such as flavonoids and non-flavonoid phenols have long been ascribed to their redox properties, finally resulting in antioxidant and anti-inflammatory activities (Eastwood 1999).

Chronic inflammation associated with infections or autoimmune disease may precede tumor development and can contribute to it through induction of oncogenic mutations and genomic instability. Prolonged exposure to environmental irritants may also result in low-grade chronic inflammation that promotes tumor progression through the mechanisms mentioned above.

1.2. A particular phytochemical: Indicaxanthin

The coloration of flowers and fruits is due to the accumulation of pigments such as flavonoids (including anthocyanins), carotenoids, and betalains (Tanaka et al., 2008). Anthocyanins and carotenoids are widely distributed in angiosperms, whereas betalains are water-soluble nitrogenous pigments found only in some plants in the order Caryophyllales and in some higher fungi (Stafford et al., 1994), with beetroot (*B. vulgaris*), swiss chard (*B. vulgaris* var. *cicla* L.) and cactus pear (*Opuntia ficus-indica*) fruits as the main dietary sources.

Betalains, which include two classes of compounds: the red-violet betacyanins and the yellow betaxanthins, are biosynthesized from the amino acid tyrosine by several enzymatic and spontaneous chemical steps (Fig. 1) (Strack et al., 2003). The first step is the conversion of tyrosine to dihydroxyphenylalanine (L-DOPA). L-DOPA is converted to betalamic acid (BA), which is an important precursor of betalain biosynthesis. BA conjugates with an amino

acid or amine to form yellow betaxanthins. BA may also condense with cyclo-DOPA to form betacyanins.

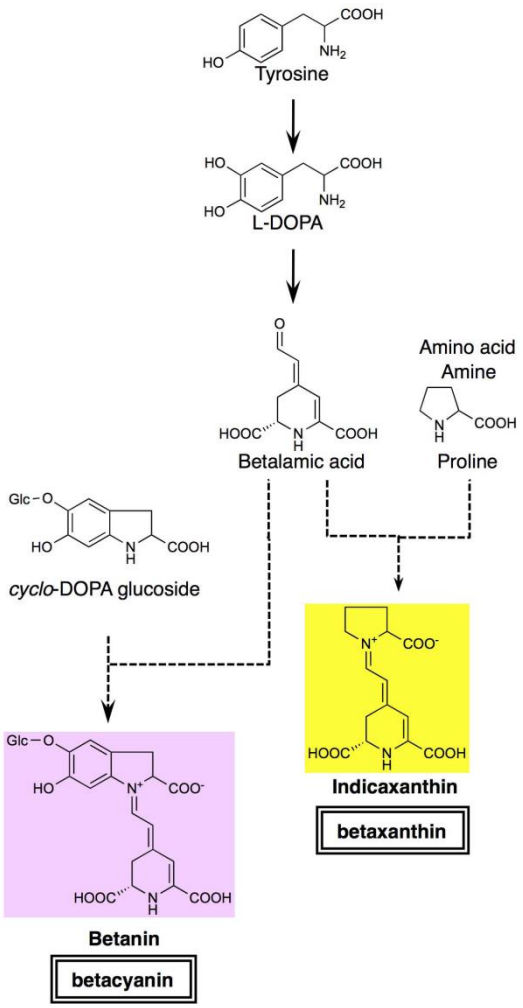


Fig. 1- Betalain biosynthetic pathway. Dotted arrows show spontaneous reactions. Betalamic acid may condense with cyclo-DOPA to form batacyanin or BA may condense with proline to form Indicaxanthin

The antioxidant and antinflammatory properties of betalains has been demonstrated in a wide range of assays (Kanner et al., 2001) and it was reported that enrichment of human low-density lipoproteins by betalains effectively increased resistance to oxidation (Tesoriere et al., 2003).

In addition, betalain-rich extracts from various sources have also been explored for their antitumoral potential in both animal models and in cancerous cell lines. The inhibition of skin

and liver tumor formation has been demonstrated in mice following oral administration of beetroot extract (Kapadia et al., 2003), and a protective effect of red beetroot juice against the tumorigenic effect of gamma radiation has also been shown in these animals (Lu et al., 2009). Other authors have reported that cactus pear extracts effectively inhibited cell growth in several immortalized and cancer cell cultures, suppressed ovarian tumor growth, and modulated the expression of tumor-related genes in nude mice (Zou et al., 2005).

With regards to purified betalains, betanin, the main betacyanin in the betalain foods, has been shown to inhibit the growth of breast, colon, stomach, and lung cancer cells (Reddy et al., 2005), induce apoptosis in human K562 chronic myeloid leukemia cell lines (Sreekanth et al., 2007) and has appeared a weak epigenetic regulator in MCF-7 breast cancer cells (Paluszczak et al., 2010). Activity of betaxanthins on transformed cells has not been reported so far.

Cactus (*Opuntia ficus-indica*) has been used for many years as a common vegetable and as a medicine by the Native Americans and Mexicans (Knishinsky 1996). In Chinese medicine, cactus fruit is considered a weak poison and used as medicine for the treatment of inflammation and pain (Wang 1988). In recent years the properties and bioactivities of Indicaxanthin (Ind), a betaxanthin highly concentrated in the edible fruits of cactus (Kanner et al., 2001), have been researched. This molecule can behave as a radical scavenger and antioxidant (Butera et al., 2002), possesses physico-chemical characteristics allowing its interaction with and location in membranes (Turco Liveri et al., 2009) and it may affect redox-sensitive signaling pathways inside the cells by preventing alteration of the cell oxidative balance and dysregulation of intracellular Ca^{2+} homeostasis in human monocyte/macrophage THP-1 cells (Tesoriere et al., 2013). On a nutritional perspective, Ind has been shown to be stable under digestive conditions. It infact undergoes only a 20% loss during the gastric-like digestion, with no further loss in the postintestinal digest. Ind is not

metabolized by the enterocytes and because of its stability and solubility in the postintestinal digest it is highly bioavailable in its native form (Tesoriere et al., 2004). In addition it has been shown that it has the potential to act at the level of cells and tissues (Tesoriere et al., 2006).

1.3. Biological functions targeted by phytochemical chemoprevention

Chemoprevention comprises multiple intervention strategies, using either pharmacological or dietary agents to prevent, arrest or reverse the carcinogenesis process at various stages. Conventional classification of chemo-preventive agents as either blocking or suppressing agents based on the underlying mechanisms by which they exert their protective effects at a specific stage of multistep carcinogenesis. Blocking agents can hinder initiation either by inhibiting carcinogen formation from procarcinogens or by preventing the electrophilic and carcinogenic species from interacting with critical cellular target molecules such as DNA, RNA, and proteins (Surh 2003). Suppressing agents, in turn, inhibit initiated cells either in the promotion or progression stages.

One relevant aspect of carcinogenesis is recognized to be the involvement of the inflammatory response, which may be prevented by hindering oxidative stress conditions through antioxidants phytochemicals. Reactive oxygen/nitrogen species (ROS/RNS) are formed as a consequence of normal and abnormal metabolic reactions; ROS can damage proteins, DNA and RNA, as well as oxidize fatty acids in cell membranes thus increasing the risk of mutations. Naturally occurring phenolic acids and analogs (e.g., caffeic and gallic acids) are known to display a wide variety of biological functions which are mainly related to modulation of carcinogenesis (Gomes et al., 2003). One of the chemoprevention mechanisms of phytochemicals such as phenolic compounds is associated with their scavenging properties

of deleterious reactive species (e.g., superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide, and peroxynitrite) (D'Alessandro et al., 2003).

One of the hallmarks of cancer cells is their ability to evade growth-suppressing signals. Various genes affecting cell cycle progression have been identified as tumor suppressor genes, such as p53 and pRB (Hanahan and Weinberg 2011). Progression through the cell cycle is regulated through activation and inactivation of cyclin-dependent kinase (Cdks) that form sequential complexes with cyclins A-D-E during the different phases (G1, S, G2, and M) of the cell cycle. During G1 phase, Cdk2–cyclin E and Cdk4/6–cyclin D1 complexes promote entry into S-phase by phosphorylation of pRB, thereby releasing the transcription factor E2F (Pan and Ho 2008). The activity of Cdks is controlled by binding of Cdk inhibitors (CKIs) to Cdk–cyclin complexes. CKIs p21, p27, and p57 preferentially interact with Cdk2– and Cdk4–cyclin complexes, whereas CKIs p15 ^{INK4B} and p16^{INK4A} are more specific for Cdk4– and Cdk6–cyclin complexes and block their interaction with cyclin D inhibiting entry into S phase (Fig. 2) (Pan and Ho 2008).

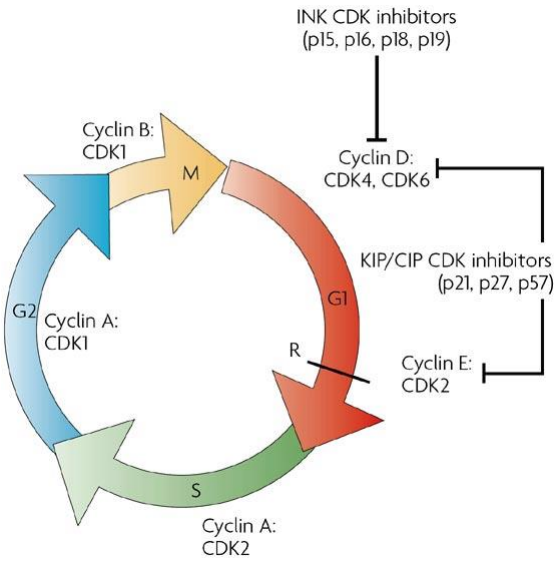


Fig. 2 - The complex regulatory and signaling pathways that regulate cell-cycle progression are highly conserved in eukaryotes. Two cell-cycle checkpoints control the order and timing of cell-cycle transitions (G1–S and G2–M) and ensure that critical events such as DNA replication and chromosome segregation are completed correctly before allowing the cell to progress further through the cycle.

Cell cycle deregulation and overexpression of growth promotion kinases (i.e. cyclin D1 and CDKs) are accepted to be associated with carcinogenesis. Recent studies have shown that numerous phytochemicals can inhibit various tumour cell lines at different cell phases: G1, S, S/G2, and G2 (Gusman et al., 2001). Nevertheless, the effects on cell cycle arrest can be either direct or indirect. For instance, epigallocatechin-3-gallate (EGCG) (phytochemical from green tea) has been shown to directly inhibit CDKs (Liang et al., 1999) or indirectly by inducing the expression of p21 and p27 genes and inhibiting the expression of cyclin D1 and Rb phosphorylation (Semczuk et al., 2004).

Therefore, cell cycle arrest can represent a chemopreventive mechanism by subsequent induction of apoptosis. Tissue homeostasis is balanced by cell proliferation and cell death. Evading apoptosis (programmed cell death) has been recognized as one of the hallmarks of cancer cells (Hanahan and Weinberg 2000). Apoptosis can be triggered when cells sense abnormalities such as DNA damage, imbalance in signaling by aberrant activation of oncogenes, lack of survival factors, or hypoxia (Hanahan and Weinberg 2000). Apoptosis is regulated by several proteins, including p53, and the Bcl-2 and caspase families (Fig. 3). Activation of these cell death proteins by phytochemicals, may be beneficial if it occurs in pre-neoplastic or tumor cells, but it may result in toxicity when taking place in normal cells. Nevertheless, some phenolic compounds such as curcumin (pigment from *Curcuma longa*), EGCG, resveratrol (flavonoid from grape skins), seem to induce apoptosis only in immortalized malignant cells (Jiang et al., 1996, Kuo et al., 2002, Yang et al., 1998). Further molecular mechanisms underlying induction of these pathways resulting from chemo-preventive action by phytochemicals, are still being researched.

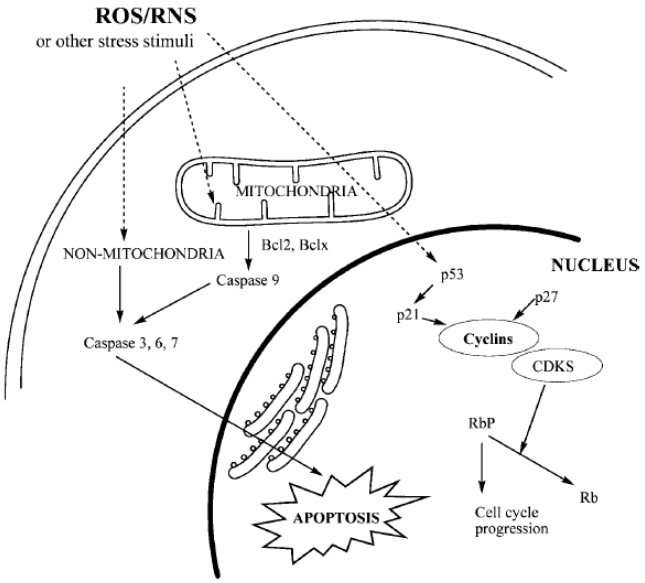


Fig. 3 - Reactive oxygen/nitrogen species (ROS/RNS) and other stress stimuli can interfere with the cell cycle regulation (increasing expression of cell cycle regulatory genes such as p53, cyclins, and CDKs), activation of caspase cascades and induction of apoptosis. These pathways are also described as possible targets for chemoprevention.

1.4. Epigenetics and Cancer

Genome-wide association studies have identified hundreds of genetic mutations associated with complex human diseases like cancer. Despite the success of genome-wide association studies in identifying loci associated with cancer, a substantial proportion of the causality remains unexplained. Only a minority of cancers are caused by germline mutations, whereas the vast majorities (90%) are linked to somatic mutations and environmental factors (Anand et al., 2008). It is now well known that cancer initiation and progression are driven by the concurrent changes in the expression of multiple genes that occur via both genetic and epigenetic alterations (Szyf 2005).

Epigenetics pertains to heritable alterations in gene expression that do not involve modification of the underlying genetic DNA sequence. In particular the term “epigenetics” refers to modifications in gene expression caused by heritable changes in DNA methylation

and chromatin structure. Major epigenetic mechanisms of gene regulation include DNA methylation, modifications of the chromatin structure by histone tail acetylation and methylation, and small non-coding RNAs that affect gene expression by targeted degradation of mRNAs or inhibition of their translation (Fig. 4) (Choudhuri 2011). Although catalyzed by different enzymes and controlled by different protein complexes, all the elements of the epigenome influence each other at the level of the chromatin structure (Strahl and Allis, 2000; Bergmann and Lane 2003).

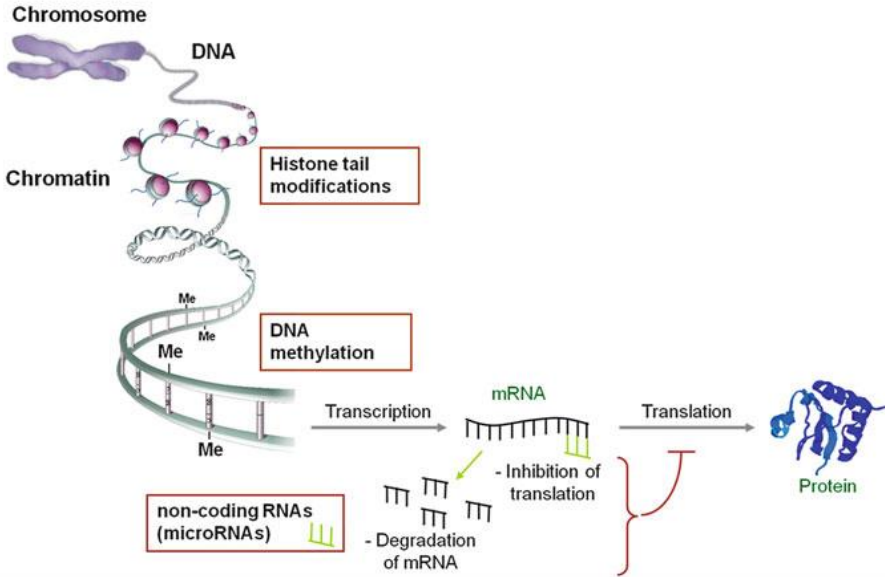


Fig. 4 - Overview of epigenetic mechanisms including DNA methylation, histone tail modifications and non-coding (micro) RNAs, targeting DNA, N-terminal histone tails and mRNA

Epigenetic mechanisms are essential to control normal cellular functions and they play an important role during development. Distinct patterns of DNA methylation regulate tissue specific gene expression and are involved in X-chromosome inactivation and genomic imprinting (Berdasco and Esteller 2010). Interestingly, epigenetic profiles can be modified to adapt to changes in the environment (e.g., nutrition, chemical exposure, smoking, radiation) (Suter and Tillery 2009) underlining a mechanistic link between environmental risk factors and the development of disease. Consequently, alterations in DNA methylation and histone

marks may eventually contribute to the development of age-related and lifestyle-related diseases (Jones and Baylin 2007). Indeed epigenetic deregulation has been associated with a variety of human diseases, including cancer, neurological disorders, and autoimmune diseases (Jaenisch et al., 2003).

Epigenetic alterations have been identified as promising new targets for cancer prevention strategies as they occur early during carcinogenesis and represent potentially initiating events for cancer development. But the most important feature of epigenetic changes that make them an important target for cancer chemoprevention is that they are potentially reversible. This has led to various research efforts aimed at identifying dietary phytochemicals (nutri-epigenomics) as environment factors, which can reverse epimutations and/or prevent cancer progression.

Accumulating evidence shows that natural compounds can alter epigenetic patterns by directly interacting with enzymes responsible for adding or removing epigenetic marks or indirectly regulating the expression of genes that encode proteins implicated in the epigenetic machinery (Stefanska et al., 2012).

1.5. Chromatin states and Epigenomic landscape

Chromatin consists of DNA, histones and non-histone proteins, and plays a role in compacting DNA, preventing DNA damage, controlling gene expression and DNA replication (Kouzarides 2007). In general, DNA is wrapped around nucleosomes, which are arranged as regularly spaced beads (146 bp DNA/nucleosome) along the DNA. Typically, nucleosomes consist of a histone octamer of histones (H)2A/B, H3 and H4. The DNA bridging two adjacent nucleosomes is normally bound by the linker histone H1 and is termed linker DNA. The positively charged amino group of amino acid residues on histones core

interacts with the negatively charged phosphate groups of DNA, which results in the compaction of chromatin and the formation of transcriptionally inactive heterochromatin. While the core histones are bound relatively tightly to DNA, chromatin is largely maintained by the dynamic association with its architectural proteins. It is well established that post-translational modifications of the histone tails regulate gene expression by determining chromatin structure. These modifications are dynamic and controlled by a variety of enzymes (Histone-(de)acetylases (HDAC/HAT), Histone-(de)methylases (HMT/HDMT), ubiquitin ligases, small ubiquitin-related modifier (SUMO) ligases) that add and remove specific groups and together establish specific chromatin states involved in transcription (Ernst & Kellis 2010). Specific sets of histone modifications are associated with genes that are actively transcribed or are repressed, a phenomenon defined as the "histone code" (Chi et al., 2010). In analogy to allosteric control of enzymes, "histone code" may determine specific gene activity by spatial organization of a gene locus, by altering the higher order structure of chromatin or by generating a binding platform for effector proteins (Nolis et al., 2009). The dynamic time-dependent combinations of histone modifications or three dimensional locus configuration, as nucleosome positioning, further increase the complexity of information contained in chromatin (Van Steensel 2011).

Although still controversial, non-coding RNAs (ncRNA) have been included as a component of the epigenome. The term non-coding RNA is commonly employed for RNA that does not encode a protein. ncRNAs, and more specifically micro-RNAs (miRNAs), are involved in heritable changes in gene expression. ncRNAs may regulate gene expression by controlling translation of mRNA into proteins either by imperfect base-pairing to the mRNA 3’-untranslated regions to repress protein synthesis, or by affecting mRNA stability (Brait and Sidransky 2011).

Each miRNA is expected to control several hundred genes, in fact, miRNAs are involved in the regulation of key biological processes, including development, differentiation, apoptosis, and proliferation, and are known to be altered in a variety of chronic degenerative diseases including cancer (Calin and Croce 2006). Furthermore, there is good evidence that noncoding RNAs also regulate chromatin architecture (Gupta et al., 2010). More interestingly, they are implicated in reciprocal interconnections between all the components of the epigenome. Aberrant miRNA regulation especially that associated with cancer can be influenced by DNA methylation and histone covalent modifications (Iorio et al., 2010). miRNA may also affect the epigenome by targeting enzymes of the epigenetic machinery (Zhou et al., 2010).

1.6. DNA Methylation

DNA methylation is a covalent modification of DNA that in mammalian cells takes place mainly at the fifth position of the cytosine pyrimidine ring located predominantly within CpG sequences (Gruenbaum et al., 1981). CG-rich regions, called CpG islands (CGI), are mostly unmethylated in normal cells and located in regulatory regions of housekeeping genes, tissue-specific genes and tumour suppressors (Hermann et al., 2004). CGI are also present in promoters of some oncogenes, where their methylated state is involved in the formation of an inactive chromatin structure leading to transcriptional silencing (Szyf et al., 2004). Recent data indicate that methylation can also occur in cytosines within dinucleotide sequences other than CpG, although the role of non-CpG methylation remains to be elucidated (Lister et al., 2009).

DNA methylation in normal cells is implicated in oncogene repression, the control of expression of genes crucial for cell proliferation, differentiation and normal development as

well as in parental imprinting, X chromosome inactivation, and preservation of chromosomal integrity by the silencing of transposons and repetitive elements (Szyf et al., 2004). Several mechanisms were proposed for DNA methylation-mediated transcriptional silencing. Firstly, some transcription factors, such as CREB (cAMP response element binding protein), E2F (elongation 2 factor), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-2 (activator protein-2), are unable to recognize specific sequences when they are methylated or methylation occurs in their proximity (Paluszczak and Baer-Dubowska, 2005). Secondly, the binding of transcription factors to regulatory elements within promoters and enhancers can be hindered by methyl- CpG-binding domain proteins (MBDs) that bind with high affinity to methylated DNA and cover recognition elements (Reik and Dean, 2001). The third mechanism is associated with the MBD-mediated recruitment of HDACs and HDMT that set up a compacted inactive chromatin state around the gene (Das and Singal, 2004).

The highly controlled pattern of DNA methylation is disrupted during ageing, development of chronic diseases and carcinogenesis.

Numerous data show that a hallmark of cancer is global DNA hypomethylation and hypermethylation of specific regions, mainly within promoters of tumour suppressor genes. The increase in promoter DNA methylation was reported as a common mechanism of tumour suppressor gene silencing observed in many types of cancer (Baylin et al., 2001; Szyf et al., 2004) and reversal of the aberrant DNA hypermethylation reactivated gene transcription (Stefanska et al., 2010). DNA hypermethylation at gene promoter regions is responsible for silencing more than 600 cancer-related genes and this number is still rising (Mulero et al., 2008). Besides effects on tumour suppressor genes, DNA methylation changes have also been detected in oncogenes as well as genes involved in the cell-cycle regulation, DNA repair, angiogenesis, metastasis and apoptosis (Herceg 2007). This show how changes in DNA-methylation contribute to the 6 hallmarks of a cancer cell i.e. limitless replicative potential,

self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis and tissue invasion and metastasis.

1.6.1. DNA Methyltransferase (DNMTs)

DNA methylation in mammals is an enzymatic process which is primarily mediated by the three known active DNA cytosine methyltransferase (DNMT1, 3A, 3B), which catalyze the transfer of a methyl group from the ubiquitous methyl donor, S-adenosyl-L-methionine (SAM), to the fifth position of a cytosine pyrimidine ring (Gruenbaum et al., 1981).

1.6.1.1. DNMT1

DNMT1 is the best known and studied member of the DNMT family. It is primarily a maintenance methyltransferase and plays an important role in cell division and development. During cell division, methylation patterns in the parental strand of DNA are maintained in the daughter strand by the action of DNMT1 which catalyses the transfer of a methyl group to the cytosine residues, restoring the symmetrically methylated CpG dinucleotide pair.

The human *DNMT1* gene is located at human chromosome 19p13.2 and encodes a 183 kDa protein. DNMT1 comprises a large N-terminal domain with regulatory functions and a smaller 500 amino acid C-terminal catalytic domain (Yen et al., 1992). The N-terminal regulatory domain harbors different motifs including different start codons, a nuclear localization signal (NLS), a PCNA (proliferating cell nuclear antigen) interacting domain (Chuang et al., 1997), a replication foci targeting region (RFTS) involved in targeting the enzyme to replication foci during S phase (Leonhardt et al., 1992), and a CXXC domain (a cysteine-rich Zn2_-binding motif) implicated in binding DNA sequences containing CpG dinucleotides (Bestor et al., 1992). These specific domains allow DNMT1 to directly interact

with various transcriptional regulators such as DNA methyltransferase 1 associated protein 1 **(**DMAP1) and histone deacetylases (HDACs), thereby influencing gene regulation through epigenetic signaling (Rountree et al., 2000). The C-terminal domain of DNMT1 contains all the conserved motifs characteristic of cytosine-C5-methyltransferases and shares a set of 10 conserved amino acid motifs (Cheng 1995) (Fig. 5). The catalytic process involves a conserved mechanism that has been best studied in the bacterial cytosine-C5 methyltransferase (MTase). Briefly, this mechanism involves MTase binding to the DNA, eversion of the target nucleotide so that it projects out of the double helix ("base flipping") into the catalytic pocket of the enzyme, covalent attack of a conserved cysteine nucleophile on cytosine C6, transfer of the methyl group from S-adenosylmethionine to the activated cytosine C5, and the various release steps. Moreover, base flipping plays an important role in the enzymatic reaction by providing high accessibility of the target base to the enzyme, thereby allowing for intricate chemical reactions to occur and for accurate recognition of the flipped base (Zhang et al., 2006).

DNMT1 is ubiquitously expressed in proliferating cells, localizes to replication foci, and interacts with the proliferating cell nuclear antigen (PCNA) (Leonhardt et al., 1992).

Homozygous knockout of *DNMT1* is lethal to the embryo in mammals, suggesting a crucial role for DNMT1 in embryonic development. However, studies on DNMT1-overexpression in embryonic stem cells also resulted in lethality to the embryo suggesting accurate expression of DNMT1 is a key factor in maintaining embryonic development (Biniszkiewicz et al., 2002).

As expected for a maintenance methyltransferase, DNMT1 has a 30- to 40-fold preference for hemimethylated sites (Jeltsch 2006). Further investigations proved that DNMT1 activity is required for *de novo* methylation at non-CpG cytosines (Grandjean et al.,

2007). However, increased DNMT1 expression occurs in the process of malignant genesis leading to the silencing of expression of tumor suppressor genes (Robert et al., 2003).

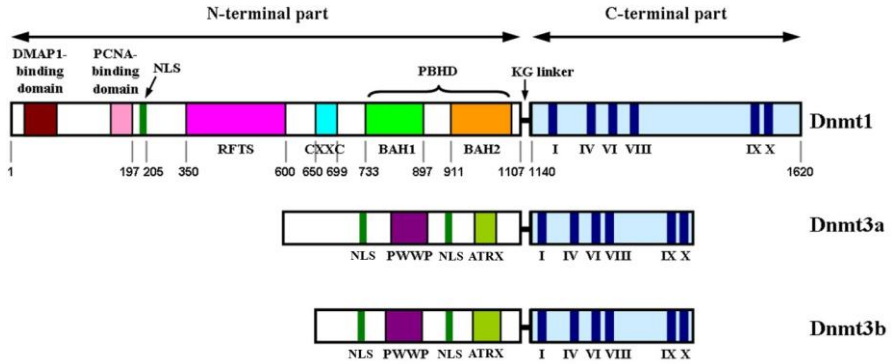


Fig. 5 - Schematic representation of DNMT structure, they carry a similar C-terminal part, but a different N-terminal domain that determines the interaction with diferent proteins.

1.6.1.2. DNMT3 Family

The DNMT3 family includes two major members, DNMT3A and DNMT3B, which play an important role in mediating *de novo* methylation processes. Targeted disruption of both DNMT3A and DNMT3B in mouse embryonic stem cells blocked *de novo* methylation, but had no effect on maintenance of an imprinted methylation pattern (Okano et al., 1999). The DNMT3 enzymes have an equal preference for hemi and unmethylated DNA substrates in vitro (Aoki et al., 2001). The distinct biochemical properties and biological functions exhibited by the de novo and maintenance methyltransferases are partly due to the structural differences of these enzymes. Both the DNMT1 and DNMT3 families of methyltransferases contain the highly conserved C-5 methyltransferase motifs at their C-terminal domain, but they show a variable region at their N-terminal regions (Chen et al., 2004).

The DNMT3A and DNMT3B proteins are very similar in structural organization. Their N-terminal regulatory domains contain a variable region (280 amino acids in DNMT3A and

220 amino acids in DNMT3B) followed by two conserved regions, a PWWP domain and a cysteine-rich domain that shares homology with a region in ATRX (Okano et al., 1998). The ATRX homology domains of DNMT3A and DNMT3B have been shown to interact with HDACs and repress transcription in reporter assays (Fuks et al., 2001). The PWWP domain is a moderately conserved region of 100 to 150 amino acids. There is evidence that the PWWP domains of DNMT3A and DNMT3B are involved in functional specialization of these enzymes (Chen et al., 2004).

Both DNMT3A and DNMT3B exhibit specialized roles. These phenomena may be due to the different distribution of these two enzymes, whereby DNMT3A is ubiquitously expressed but DNMT3B localizes its activity to the pericentromeric repeats carrying high CG content. In fact DNMT3B appears to be critical for the methylation of a particular compartment of the genome, for instance, DNMT3B mutations are linked with a syndrome called ICF (immunodeficiency, centromeric instability, facial abnormalities), a rare recessive autosomal disorder characterized by hypomethylation at pericentromeric satellite regions (Okano et al., 1999). DNMT3A cannot replace DNMT3B in this function, possibly because of its distribution mechanism, which is less efficient in methylating highly CG rich DNA (Shirohzu et al., 2002).

Over-expression of *DNMT3B* has been shown in various human tumors, while the expression level of *DNMT3A* is only modestly increased in certain types of tumors (Robertson et al., 1999), indicating that DNMT3B plays a more important role in tumorigenesis than DNMT3A.

DNMT3B contains 24 exons spanning 47kb of genomic DNA, but unlike DNMT1 and DNMT3A, DNMT3B is the only DNMT that is expressed as alternatively spliced variants or by alternative promoter usage (Chen et al., 2002). Several of the DNMT3B variants are missing key regions of their C-terminal catalytic domain, rendering them catalitycally inactive

(DNMT3B3; DNMT3B4; DNMT3B5). Two alternative 5' exons are used (DNMT3B1; DNMT3B2), but the same full-length DNMT3B protein is expected from both transcripts (Okano et al., 1999). ΔDNMT3B, consists of at least seven transcriptional variants by alternative pre-mRNA splicing lacking 200 amino acids at the regulatory N-terminal domain of DNMT3B. The predicted proteins from ΔDNMT3B5, ΔDNMT3B6, and ΔDNMT3B7 contain no enzymatic domain of the methyltransferase due to premature translational termination. (Wang et al., 2006) (Fig. 6)

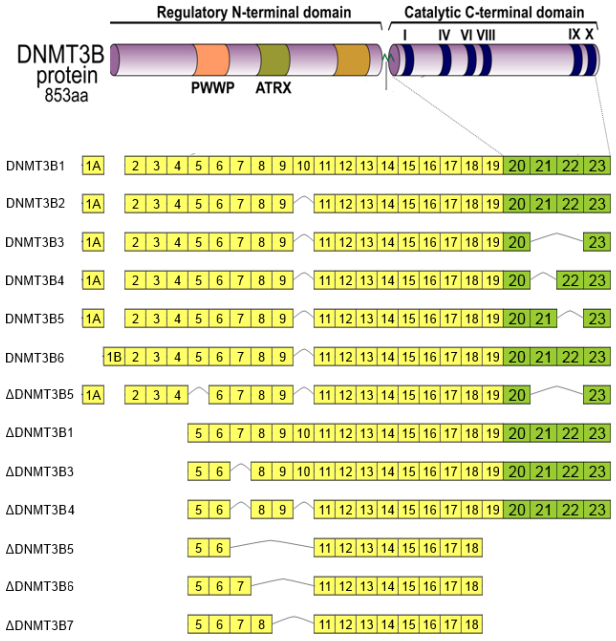


Fig. 6 - Schematic representation of DNMT3B splice variant structure. Some of them have alteration in catalytic domain. The ΔDNMT variants lack the first exons in the N-terminal domain.

Splicing patterns and spatio-temporal patterns of isoform expression appear to be conserved between humans and mice, suggesting that these isoforms carry biological significance (Okano et al., 1998). Despite this conservation, their functions in normal and disease states remain largely unclear. Increased expression of *DNMT3B* has been frequently observed in human cancer cell lines and primary tumors (Okano et al., 1998). Some of the

variants may compete with each other, perhaps resulting in DNA hypomethylation (Saito et al., 2002). This possibility suggests a complex biological role of the DNMT3B variants. Though the expression of inactive DNMT3B variants is associated with changes in DNA methylation, little is known about the mechanisms that underlie these changes at the molecular level.

1.6.2. DNA Demethylation

DNA methylation has a profound impact on genome stability, transcription and development. Although enzymes that catalyse DNA methylation have been well characterized, those that are involved in methyl group removal have remained elusive until recently.

DNA methylation is relatively stable compared with most histone modifications. DNA methylation has been considered as a non-reversible reaction for decades. It was assumed that demethylation can only occur in a passive way by blocking the activity of DNMTs in dividing cells. Nevertheless, loss of DNA methylation, or DNA demethylation, has been observed in different biological contexts and this alteration can take place actively or passively (Ooi and Bestor 2008)

Active DNA demethylation refers to an enzymatic process that removes or modifies the methyl group from 5-methylcytosine (5mC). By contrast, passive DNA demethylation refers to loss of 5mC during successive rounds of replication in the absence of functional DNA methylation maintenance machinery. Although passive DNA demethylation is generally understood and accepted, the evidence for active DNA demethylation and how it occurs has been controversial (Wu and Zhang 2010).

One if the most studied biological contexts in which active DNA demethylation takes place is mammalian embryogenesis (Surani et al., 2010). In fact establishing and editing genomic methylation patterns seems to be particularly relevant during development and cellular differentiation. After fertilization the paternal genome, but not the maternal, goes rapidly through a complex remodeling of DNA methylation patterns suggesting an active 5mC editing process (Jenkins et al., 2012; Mayer et al., 2000)

Besides global loss of DNA methylation in zygotes, DNA demethylation has also been observed at specific loci in rapid response to environmental stimuli or in post-mitotic cells, supporting the relevance of active demethylation in various biological settings in the absence of cellular replication (Kangaspeska et al., 2008).

The discovery of a family enzymes (TET) that can modify 5mC through oxidation sheds light on DNA demethylation mechanisms, introducing 5-hydroxymethylcytosine (5hmC) as a key intermediate in active demethylation pathways (Tahiliani et al., 2009). Even more strikingly recent reports providing insights into 5hmC genomic distribution suggest it is potentially highly relevant in gene regulation (Ndlovu et al., 2011)

The TET enzyme family not only catalyze oxidation of 5mC to 5hmC, it is demonstrated that they are capable of iterative oxidation, yielding 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al., 2011). Notably, 5hmC, 5fC and 5caC are chemically distinct modifications of C that could be specifically recognized by different DNA-binding proteins to regenerate unmodified C, although 5hmC is significantly more prevalent than 5fC and 5caC (Song et al., 2012). Thus 5hmC has taken on a new central role in epigenetics. The base sits at an important branch point, with at least three potential outcomes. 5hmC could have an independent epigenetic role related to the base's interaction with chromatin-associated proteins, either through direct recruitment by 5hmC or through disruption of 5mC-specific interactions. However other potential pathways for demethylation following 5mC

oxidation to 5hmC have been proposed and they could have a role in remodeling DNA methylation patterns (Kohli and Zhang et al., 2013).

Kohli and Zhang revisited the definitions of active and passive demethylation describing the latter as the replication-dependent dilution of 5mC only. Instead active modification (AM) of 5mC to generate 5hmC involving TET is best viewed as active demethylation. This base (5hmC) can be further processed through either passive dilution (PD) to regenerate unmodified C through DNA replication, or active restoration (AR) through further enzymatic modification (Kohli and Zhang 2013) (Fig. 7).

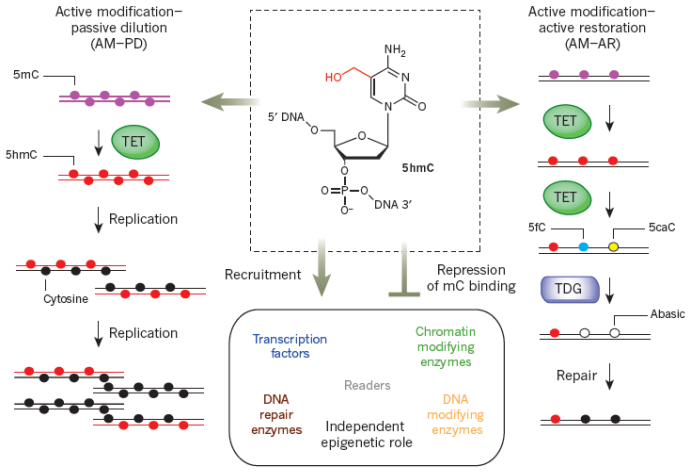


Fig. 7 - Schematic representation of the pathways that drive DNA demethylation. Active modification and Passive Dilution involves the oxidation of 5mC to 5hmC and subsequent cycles of replication- induced base loss. Alternatively the activity of repair enzymes provides the modification of 5hmC regenerating the C in an active restoration (AR).

A pathway for active restoration of C could involve DNA repair enzymes, which remove an entire modified base with its subsequent repair to replace the residue with unmodified C.

The involvement of base excision repair (BER) pathway in demethylation identified a particular thymine DNA glycosylase (TDG) that has a role as a DNA repair enzyme that can

remove a normal base, T, from a genomic T·G mismatch (Cortázar et al., 2007). Therefore deamination of 5mC or 5hmC by DNA cytosine deaminases (AID) lead to T·G mismatches that are substrates of TDG for which repair could regenerate unmodified C (Cortellino et al., 2011). These deamination-mediated pathways for demethylation could also involve the DNA damage response protein GADD45 or even MBD4 as a glycosylase for excision of T·G mismatches (Barreto et al., 2007; Niehrs and Schafer 2012) (Fig. 8).

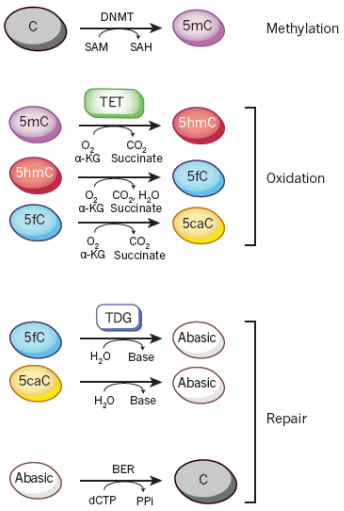


Fig. 8 - The individual reactions in the pathway for dynamic modifications of C are shown with all reactants depicted. The BER pathway involves excision of the abasic site, replacement of the nucleotide using unmodified deoxycytidine triphosphate (dCTP) by a DNA polymerase and ligation to repair the nick.

Interestingly a recent study showed that DNMT enzymes, in addition to converting C to 5mC on DNA, can also demethylate 5mC on DNA under specific conditions in vitro; in particular in the presence of Ca^{2+} ion and in the absence of reducing reagents. In other words, the covalent addition of the methyl group to the 5-position of cytosine on DNA, as catalyzed by DNMTs, is reversible (Chen et al., 2013). Furthermore Mizuno et al showed the increase, instead of reduction, of the levels of DNMT1, DNMT3A, and DNMT3B in cancer cells that could not be easily correlated with the typical global hypomethylation of cancer genomes (Mizuno et al., 2001; Feinberg et al., 2004). The finding of the Ca^{2+} dependent oxidation

state-facilitated DNA demethylase activities of the three enzymes, in contrast to their DNA methyltransferase activities, provides a plausible basis for the above correlation.

Oxidative modifications of 5mC and related repair mechanisms have expanded the possibilities by which the genome can retain great flexibility while maintaining the integrity of its coding information.

1.7. Interplay between chemo-preventive and epigenetic mechanisms and the effects of natural compounds

Since epigenetic changes are reversible, developing drugs that control epigenetic regulation now attracts substantial research investment, including the development of functional foods or supplements as nutrition based epigenetic modulators for cancer chemoprevention (Arasaradnam et al., 2008; Parasramka et al., 2012)

Over the last few years, evidence has accumulated that natural products and dietary constituents with chemo-preventive potential have an impact on DNA methylation (Fig. 9), histone modifications (Fig. 10), and miRNA expression. (Stefanska et al., 2012; Druesne-Pecollo and Latino-Martel 2011; Li et al., 2010).

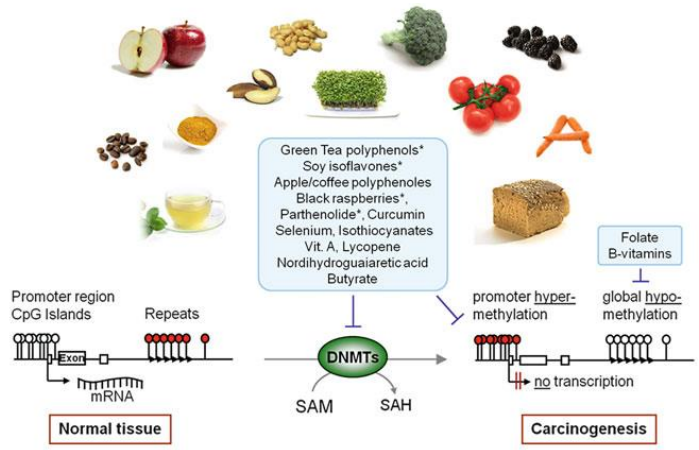


Fig. 9 - Overview of DNA methylation changes during carcinogenesis and cancer chemopreventive agents inhibiting the activity of DNMTs, thereby preventing aberrant (promoter) hypermethylation or genome wide hypomethylation. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) using S-adenosylmethionine (SAM) as a substrate. Empty circles: unmethylated CpG dinucleotide; red circles: methylated CpG site.

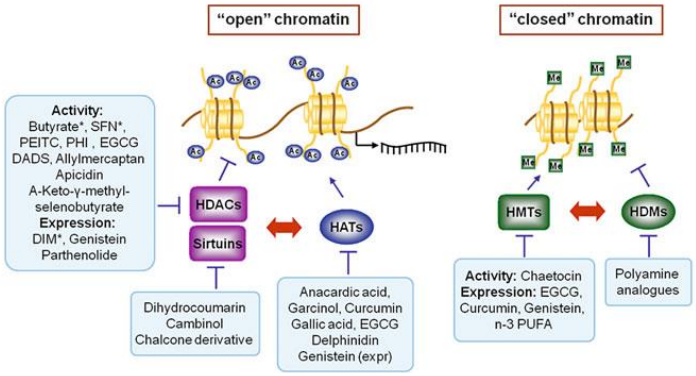


Fig. 10 - Simplified overview of histone modifying enzymes with a focus on histone deacetylases (HDACs), histone acetyltransferases (HATs), histone methyl transferases (HTMs), and histone demethylases (HDM), and their influence on chromatin structure. Sirtuins represent a NAD+- dependent subclass of HDACs (class III).

As indicated in Fig. 9, folate and B-vitamins have a potential impact on DNA hypomethylation. They affect the "one-carbon metabolism" which provides methyl groups for methylation reactions. Folate is an important factor for the maintenance of DNA biosynthesis and DNA repair, and folate deficiency leads to global DNA hypomethylation, genomic

instability, and chromosomal damage. As an essential micronutrient, folate needs to be taken up from dietary sources, such as citrus fruits, dark green vegetables, whole grains, and dried beans. Epidemiological studies have indicated that low folate levels are associated with an increased risk for colorectal, breast, ovary, pancreas, brain, lung, and cervix cancer (Lamprecht and Lipkin 2003; Duthie 2011). Consequently, the relationship between folate status, DNA methylation, and cancer risk has been analysed in numerous rodent carcinogenesis models and in human intervention studies. Overall, the results are inconclusive and depend on various parameters, for example dose and timing of the intervention, the severity of folate deficiency, and health status (Kim 2005). Excessive intake of synthetic folic acid (from high-dose supplements or fortified foods) may even increase human cancer risk by accelerating growth of precancerous lesions (Duthie 2011). Therefore folate supplementation cannot be generally recommended, and deficiencies should be prevented by dietary intake.

In this respect medical benefits of dietary compounds as epigenetic modulators, especially with regard to their chronic use as nutraceutical agents in cancer chemoprevention (individual targeted diet or diet supplements) deserve to be further investigated to better understand their epigenetic role during embryogenesis, early life, aging, as well as during carcinogenesis.

1.8. Altered epigenetic mechanism and phytochemical effects

In the last decade, the anti-cancer activity of multiple bioactive food components with a diverse range of molecular targets has been proven by intensive research. Accumulating evidence indicates that dietary compounds can alter pathways which are relevant for chemoprevention and are commonly deregulated by epigenetic mechanisms in cancer cells, including drug detoxification, cell cycle regulation, apoptosis induction, DNA repair, tumor-associated inflammation, cell signaling that promotes cell growth, and cell differentiation.

There is now a lot of evidence for phytochemicals that may induce cell cycle arrest by epigenetic alterations that lead to downstream re-expression and reactivation of key cell cycle regulators.

Remarkably, DNA methylation is involved in the regulation of CKI expression, as exemplified by $p16^{INK4A}$ and $p21^{CIP1/WAF1}$. Moreover in epidermoid carcinoma cells, EGCG decreased global methylation and inhibited DNMT activity as well as expression of DNMT1, DNMT3A, and DNMT3B, which led to the re-expression of p16 mRNA and protein (Nandakumar et al., 2011).

The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) negatively regulates the phosphatidylinositol 3-kinase (PI3K)-AKT pathway that transmits anti-apoptotic survival signals and regulates cell proliferation, growth and motility (Vivanco Sawyers 2002). Downstream signaling is indirectly mediated via transcription factors such as NF-kB (Hollander et al., 2011). Epigenetic inactivations of PTEN by promoter methylation or miRNA silencing are common in multiple tumor types. Silencing through epigenetic mechanisms frequently occurs in breast, prostate, thyroid, and lung cancer, glioma, and melanoma.

Studies report that vitamin D3 treatment is able to reduce methylation and to enhance concomitantly expression of PTEN. This was associated with down-regulation of DNMT1 and upregulation of p21 after incubation with vitamin D3 and resveratrol (Stefanska et al., 2012).

Furthermore, cancer, like multiple other conditions such as diabetes, allergy, rheumatoid arthritis and cardiovascular disease, has been linked to chronic inflammation and metabolic stress. Studies have shown that epigenetics constitute the crossroads of cancer, inflammation and metabolic stress (Mantovani et al., 2008). Signalling pathways responsive to chronic

inflammation activate downstream mediators such as transcription factors (NF-kB) that drive changes in the expression of target genes. However, binding of the transcription factors to DNA depends on the chromatin structure, which is regulated by the epigenetic machinery. Thus, inflammatory gene expression is dependent on epigenetic mechanisms. Dietary phytochemicals that modulate epigenetic components, such as EGCG and gallic acid, can thereby suppress the expression of inflammatory genes and induce phenotypic alteration in inflammation-related disorders (Choi et al., 2009).

Normal cells do not proliferate without mitogenic stimulatory signals. Consequently, "self-sufficiency in growth signals" was defined as one of the hallmarks of cancer cells (Hanahan, Weinberg 2000). WNT signalling is one of the most important pathways that regulate proliferation as well as embryonic development, differentiation and cell migration. Endogenous WNT antagonists that inhibit WNT signaling through direct binding to WNT are frequently disrupted by DNA methylation in various cancers. These include secreted frizzled-related proteins (sFRPs) and WNT inhibitory factor 1 (WIF-1), as well as WNT5A (Klaus Birchmeier 2008). Several recent studies indicate that the chemopreventive agents EGCG, genistein, and black raspberries reactivate silenced WNT inhibitory genes by promoter demethylation, leading to a reduction of proliferation and apoptosis in lung and colorectal cancer cells (Wang et al., 2011; Gao et al., 2009; Wang et al., 2010).

Thus numerous data have shown that alterations in epigenetic modifications produce various effects on the phenotype. Natural compounds that directly affect DNA methylation can induce changes in other components of the epigenome due to a trilateral relationship that exists between DNA methylation, histone covalent modifications and non-coding RNAs. This increases the epigenetic modulatory potential of dietary compounds, which attracts more scientific interest, given their advantages in costs, their availability and their non-toxicity that allows a long period of use.

Therefore, natural chemicals can be efficacious in both cancer prevention and cancer therapy and are becoming potential targets of drug development. The main challenges in the investigation of phytochemicals as epigenetic agents are new strategies for increasing their bioavailability, as most of them show poor bioavailability in vivo. Study on a genome-wide level may help to better understand the importance of epigenetic mechanisms in gene regulation in cancer chemoprevention as well as determining the role of natural compounds alone or in combination with existing drugs in improving cancer treatment.

2. AIM OF STUDY

The aim of this study was to investigate the antiproliferative potential of a Cactus pear pigment extract (Indicaxanthin) and outline its features. In particular, to assess the phenotypic effects on tumour cell lines and to investigate the biological function and/or epigenetic mechanisms for Indicaxanthin's activity in order to determine the potential chemo-preventive or therapeutic properties of this phytochemical towards cancer.

3. MATERIALS AND METHODS

The study was carried out at the Department STEBIFEC University of Palermo (Italy), both in the cytogenetics and cell biology laboratory (Tutor: Dr Fabio Caradonna) and in the Biochemistry laboratory (Prof. Maria Antonia Livrea). Part of this research was also carried out at the Institute of Food Research (Norwich, UK) in Dr Nigel Belshaw Lab.

3.1. Indicaxanthin extraction and purification

Indicaxanthin (Ind) was isolated from cactus pear (*Opuntia ficus-indica*) fruits (yellow cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25 (Butera et al., 2002). Fractions containing the pigment were submitted to cryo-dessiccation (Freezone 4.5, Labconco, Kansas City, Mo, USA) and purified according to Stintzing et al (Stintzing et al., 2002). Briefly, the dessiccated material was re-suspended in 1% acetic acid in water and submitted to semi-preparative HPLC using a Varian Pursuit C18 column (250 x 10 mm i.d.; 5mm; Varian, Palo Alto, CA), eluted with a 20 min linear gradient elution from solvent (1%acetic acid in water) to 20% solvent B(1% acetic acid in acetonitrile) with a flow-rate of 3ml/min. The eluent was monitored spectrophotometrically at 482 nm. The eluted fractions containing indicaxanthin were collected. Samples after cryo-dessiccation were re-suspended in 5 mM phosphate buffered saline (PBS), pH 7.4, at a suitable concentration and used immediately or stored at -80°C. The concentration of the samples was evaluated spectrophotometrically in a DU-640 Beckman spectrophotometer by using a molar coefficient at 482 nm of 42,800 (Piattelli et al., 1964). Indicaxanthin was filtered through a Millex HV 0.2 μm filter (Millipore, Billerica, MA) immediately before the use.

3.2. Cell lines and treatment

All tumour cell lines used for the experiments, obtained from the American Type Culture Collection (Rockville, MD), were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin and were maintained at 37 °C in 5% CO_2, and 95% humidity. The medium was changed 2-3 times per week.

Cell lines analysed were: Human Liver cell line (Chang Liver); Human malignant liver cell lines (HepG2, Ha22T, HUH7); Breast Cancer cells (MCF7); Cervix Cancer cells (HeLa); Human epithelial colorectal adenocarcinoma cells (Caco2).

Caco2 cells are of particular interest for two reasons. Firstly, they are a model of the intestinal ephitelium which may be exposed to high concentrations of Ind from the diet. Furthermore Caco2 cells are a model system for the study of cell differentiation of human enterocytes of intestinal origin. Caco2 cells, in fact, are able to differentiate spontaneously into polarized cells with morphological and biochemical characteristics of normal intestinal enterocytes. The differentiation process begins when the cells reach confluence, which means when the first cell-cell contacts are established, and it is considered complete after about 3 weeks in standard culture condition (Pinto et al., 1983). Therefore, these cells are of particular interest not only for the study of various biological processes such as differentiation, proliferation, but also because they are a tool for the study of how the intestinal cells transport nutrient and metabolize substances of pharmacological interest.

In addition to these cell lines in the last part of this work spent abroad at the Lab of Dr. Nigel Belshaw (Institute of Food Research, Norwich, UK), were also studied other tumour

cell lines derived from colorectal cancer (HCT116, LOVO1, DLD1, HT29). This is to assess whether Ind had a widespread effect on cell derived from the same tissue of origin.

3.3. MTT Assay

Cell sensitivity to Ind was evaluated by MTT assay. This colorimetric assay allows the assessment of cell proliferation that is directly proportional to the metabolic activity of the cell. MTT is a water-soluble tetrazolium salt that is metabolized by living cells and converted into formazan by the action of a dehydrogenase enzyme. The formazan is violet and is typically viewed as insoluble material at the bottom of the culture plate. Formazan is solubilized in DMSO to form a purple solution, the intensity of which is proportional to cell density.

In a typical experiment exponentially growing tumour cells were seeded into 96-wells culture plates (Corning Costar Inc, Corning, NY) at a density of 2.0 x 10^4 cells/cm^2 and incubated for 24 h prior to treatment, with Ind for 24 h or 48 h. In some experiments Caco2 cells were grown 3 weeks after confluence, to allow differentiation, and then treated with either 50 μM or 100 μM Ind. Following treatment, the medium was removed and the cells were washed with PBS. Serum-free medium containing 5 mg/mL MTT was added and the cells were incubated for 2 h at 37°C. Then, the medium was discarded, and the formazan blue formed was dissolved in DMSO (200μl). The absorbance at 560 nm of MTT-formazan was measured in a microplate reader (GloMaxÂ®-Multi Microplate Reader (Promega Corporation, Madison, WI, USA) and the values were expressed as percentage of control (untreated cells).

3.4. Annexin-V apoptosis assay in Caco2 cells

The externalization of phosphatidylserine to the cell surface was detected by flow cytometry by double staining with Annexin V/propidium iodide (PI), using the Annexin V apoptosis detection kit FITC, according to the manufacturer's instructions (Cat. No. 88-8005, eBiosciences Inc., San Diego, CA, USA). Cells were seeded in triplicate at a density of 2.0 x 10^4 cells/cm^2. After an overnight incubation, cells were washed with fresh medium and incubated with 25 μM to 100 μM Ind in DMEM. After 48 h, cells were harvested by trypsinization and adjusted to 1.0 x 10^5 cells/0.1 mL with combining buffer (50 mM TRIS, pH 7.4, 100 mM NaCl, 1% BSA). After addition of 5 μL Annexin V provided by the kit and 5 μL of PI (20 μg/ml), cells were incubated at room temperature, in the dark, for 15 min. After incubation the volume was adjusted to 0.5 mL PBS and samples of at least $1.0x10^4$ cells were subjected to fluorescence-activated cell sorting (FACS) analysis by Epics XL™ flow cytometer using Expo32 software (Beckman Coulter, Fullerton, CA), using an appropriate bi-dimensional gating method.

3.5. Measurement of mitochondrial trans-membrane potential

Mitochondrial trans-membrane potential ($\Delta\psi_m$) was assayed by flow cytofluorometry, using the cationic lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3), Molecular Probes Inc., Eugene, OR, USA) which accumulates in the mitochondrial matrix. Changes in mitochondrial membrane potential are indicated by a reduction in the DiOC6-induced fluorescence intensity. After treatment, $5x10^5$ cells were incubated in 0.5 mL PBS containing 40 nmol/L DiOC6(3) for 15 min at 37 °C. After centrifugation, cells were washed with PBS and resuspended in 500 μL PBS. The fluorescent intensities were analysed in at least 1.0 x 10^4 cells for each sample.

3.6. Measurement of Intracellular Reactive Oxygen Species (ROS) level in Caco2 cells.

ROS level was determined by measuring fluorescence changes which resulted from intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFDA). DCFDA, at 10 μM final concentration, was added into the cell medium 30 min before the end of treatment. After trypsinization the cells were collected by a 5 min centrifugation at 2000 rpm at 4 °C, washed, resuspended in PBS and immediately subjected to FACS analysis. At least 1.0 x 10^4 cells were analysed for each sample.

3.7. Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction (MeS-AP-PCR)

After treatment of Caco2 cells with Ind or 5aza-2'deoxycytidine (Aza), DNA extraction was performed using a PureLink Genomic DNA Kit (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's instructions. The concentration and purity of the samples were assessed using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The methylated CpG islands were detected by MeSAP-PCR, carried out according to Liang et al (Liang et al., 2002) with some modifications (Caradonna et al., 2007). Briefly, DNA from Caco2 cells (8μg) was digested in a total volume of 10 μl for at least 16 h, at 37°C, with 10 U of AfaI restriction endonuclease (single digested DNA, SDD). Then, one half of SDD digested was further treated with HpaII, a methylation-sensitive restriction endonuclease unable to cut DNA if methylated cytosine is present in its recognition site (CC*GG) at 37°C for 16 h (double digested DNA, DDD). SDD and DDD were heat inactivated for 20 min at 65°C and separately amplified by Arbitrarily-Primed PCR using two subsequent amplification cycles, AP1-M and AP2-M, in a T1 Plus Thermocycler (Biometria,

Gottingen, Germany). In the AP1-M (low stringency cycle), a permissive annealing temperature, a high salt and primers concentration were set to allow arbitrary primer annealing to the best matches in the template with the highest preference for all the genomic CpG sites since they are provided with a 3' tail complementary to these sites. The reproducibility of the reactions was ensured as all experiments were conducted using the same rates of buffers reaction.

PCR was performed in a total volume of 25 µl under the following conditions:

Genomic DNA	250 ng
21-mer arbitrary primer (5'- AAC TGA AGC AGT GGC CTC GCG-3')	10 µM
dNTPs	200 µM
Taq DNA polymerase (Roche Diagnostics, Milano, Italy) in 10 mM Tris-HCl, pH 8.3, containing 1.5 mM $MgCl_2$ and 50 mM KCl	0.8 U

Cycle profile was:

A Cycle	94°C	5 min
4 Cycles at low stringency conditions	94°C	30 sec
	40°C	60 sec
	72°C	90 sec

AP2-M (high stringency cycle), runs immediately after AP1-M. Reaction mixture consisting of 75µl of:

Tris-HCl, pH 8.3	10mM
$MgCl_2$	1.5 mM
KCl	50 mM
dNTPs	200 µM
Taq DNA polymerase	2.5U

was added to 25 µl of AP1-M mix, immediately before AP2-M cycle.

Cycle profile was:

A Cycle	94°C	60 sec
29 Cycles at high stringency conditions	60°C	60 sec
	72°C	2 min
	72°C	90 sec

Amplified DNA was resolved by non-denaturating 6% acrylamide-bisacrylamide (29:1 ratio) gel electrophoresis. DNA fingerprinting was obtained by Sybr safe staining, and submitted to densitometric scanning and analysed by SigmaGel image analysis software (Jandel Scientific, San Rafael, CA, USA).

3.8. Methylation-Sensitive Restriction Endonucleases Multiplex-Polymerase Chain Reaction (MSRE-PCR).

In order to analyse the methylation status of the CpG island of *p16*^{INK4a} gene promoter region, MSRE-PCR was performed (Longo et al., 2013). In detail, genomic DNA (0.25 µg) from Caco2 cells was digested with excess of CfoI a Methylation-Sensitive Restriction

Endonuclease (MSRE) (5U/µg DNA), for at least 16 h at 37°C, according to the manufacturer's instructions (Invitrogen) Then, samples of either genomic or MSRE-digested DNA were amplified by Multiplex-PCR (M-PCR) in the presence of primers flanking the CpG island of *p16*^{INK4a} gene promoter. M-PCR of MSRE-digested DNA will give a product only when MSRE has not cut the DNA, leaving the template intact and suitable for amplification.

Reaction mixture of M-PCR reaction contained:

Genomic DNA	250 ng
dNTP	0.2 mM
***p16*^{INK4a} sense (5'-ACTCCCTCCCCATTTTCCTATCT-3')**	0.2µM
***p16*^{INK4a} antisense (5'-CCGCGATACAACCTTCCTAACT-3')**	0.2µM
$MgCl_2$	1.2 mM
Taq polymerase in 1X buffer provided by manufacturer (Invitrogen)	2.5 U

Amplification was carried out in a total volume of 100 µl using a T1 Plus Thermocycler.

Cycle profile was:

A cycle	94°C	5 min
30 Cycles of	94°C	2 min
	55°C	2 min
	72°C	2 min
final extension	72°C for	10 min

Co-amplification of IL-4 internal region (998 bp product) was concurrently carried out as a control.

IL4-2A sense	5’-CCCCAAGTGACTGACAATCTGG-3’
IL4-2B antisense	5’-GTGAGAGTATTTGGTTTTTCAGAAAT-3’

These primers result in a PCR product using either genomic or MSRE-digested DNA as template, since the sequence to be amplified has no recognition site for the methylation-sensitive endonuclease used.

The M-PCR products were resolved by non-denaturing 6% acrylamide-bisacrylamide (29:1, ratio) gel electrophoresis, stained with Sybr Safe by Imaging System. (ChemiDoc XRS, Bio-Rad, Milano, Italy).

3.9. Real-Time Polymerase Chain Reaction (RT-PCR) of $p16^{INK4a}$

Total RNA was extracted from Caco2 cells using Trizol according to the manufacturer's protocols (Invitrogen); the RNA was eluted in diethyl pyrocarbonate (DEPC) treated water (0.01% DEPC) and stored at -80°C until RT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (NanoDrop 1000 Spectrophotometer). First-strand cDNA was synthesized from total RNA (250 ng) using a SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. mRNA expression levels were determined using first strand cDNA as the template by quantitative real-time PCR (qPCR), with Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) in a 7300 Real Time PCR system (Applied Biosystem, Philadelphia, PA, USA).

The sequences of the *p16*^{INK4a} primers used were:

***p16* Forward**	5'-GTGGACCTGGCTGAGGAG-3'
***p16* Reverse**	5'-CTTTCAA TCGGGGATGTCTG-3'

β-Actin was used as an internal control with the following primers:

Actin Forward	5′-GCCCACATAGGAATCCTTCTGAC-3′
Actin Reverse	5′-AGGCACCAGGGCGTGAT-3′

PCR data obtained by the instrument software were automatically analysed by the Relative Quantification Study Software (Applied Biosystem) and expressed as target/reference ratio.

3.10.SDS-Page and western blotting analysis

After treatment, Caco2 cells were rinsed twice with ice-cold PBS and harvested by scraping with ice-cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1.5 µg ml soya bean trypsin inhibitor, 7 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.1 mM benzamidine and 0.5 mM DTT) and incubated for 15 min on ice. The lysates were centrifuged at 13,000 g for 5 min and supernatants (cytosolic fraction) were aliquoted and stored at -80 °C for up to two weeks. Protein concentration was determined using the Bradford protein assay reagent (Bio-RAD, Milan, Italy) and 30 µg protein was separated on 8-12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane at 400 mA for 1h. The nitrocellulose membrane was incubated overnight at 4 °C with blocking solution (5% skimmed milk), followed by incubation with anti-p16^{INK4a} monoclonal antibody (clone F-12, Cat No sc-1661, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CDK4 monoclonal antibody (clone C-22, Cat No sc-260 Santa Cruz Biotechnology), anti-

retinoblastoma protein (RB, clone G3-245, Cat No 554136, BD Biosciences, San Jose, CA, USA) or IgG-anti DNMT1 (AbCam) for 1 h at room temperature. Blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti-IgG antibody for 1 h at room temperature. Blots were again washed five times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). Immunoreactions were also performed using anti-β-tubulin (clone H-235, Cat no sc-9104, Santa Cruz Biotechnologies) antibody as a loading control. Immunoblots were submitted to densitometric scanning and analysed by Sigma Gel Image Analysis software.

3.11. Cell cycle analysis

Cell cycle stage was analysed by flow cytometry using a Propidium iodide (PI) stain. Propidium iodide is an intercalating agent and a fluorescent molecule that is widely used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis. PI is used to quantitatively assess DNA content, it binds to DNA by intercalating between the nitrogenous bases with a stoichiometry of one dye per 4–5 base pairs of DNA, this allows to distinguish the different phases of the cell cycle on the basis of a cell's DNA content during the various stages of the cell cycle.

Once the dye is bound to nucleic acid, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted ~30–40 nm to the red and the fluorescence emission maximum is shifted ~15 nm to the blue. When PI is bound to nucleic acid, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm.

For cell cycle synchronization, cells were cultured in serum-free medium for 24 h. After addition of Ind, cells were re-stimulated with 10% FBS and incubated for the indicated times.

Cells were harvested by trypsinization and washed with PBS. Aliquots of 5.0 x 10^5 cells were incubated in the dark in 0.5 mL PBS containing 20 μg/ml PI, 0,1% Triton X and 200 μg/ml RNase for 30 minutes at room temperature. Samples were, then immediately subjected to FACS analysis. At least 1.0 x 10^4 cells were analysed for each sample.

3.12. Genomic DNA and RNA isolation

Genomic DNA from all cell lines analysed (DLD1, HCT116, HT29, Lovo1, Caco2) was extracted following a phenol-choloroform protocol. The media was removed from cultured cells and DNA extraction buffer (500μl) (0.2 M TrisHCl ph 8.8, 0.25 M NaCl, 25mM EDTA, 0.5% (w/v) SDS) added. After adding Proteinase K (10 mg/ml), solutions were incubated at 50 °C for 1h. A solution of phenol/chloroform/isoamyl alcohol (500μl) (Sigma), was added mixed and centrifuged at 1300g for 5 mins. The upper aqueous layer was collected and added to chloroform (500μl), mixed and centrifuged. The upper aqueous layer was transferred to a new eppendorf and 400 μl isopropanol added mixed and centrifuged for 30 min. The pellet was washed with ice-cold 70% ethanol, and left to air dry. DNA was resuspended in TE buffer.

RNA was extracted from all cell lines using the RNeasy mini Kit (Qiagen) following the manufacturer's instructions.

The NanoDrop spectrophotometer (Labtech International,UK) was used to quantify DNA/RNA concentration and purity by absorbance measurement at 260 and 280 nm.

3.13.Bisulphite conversion and DNA recovery

All cell lines analysed (DLD1, HCT116, HT29, LOVO1, Caco2) were seeded in p6-well plates to perform three different biological replicates. Genomic DNA was extracted from all cell lines after 48h treatment with 10µM, 50 µM and 100 µM indicaxanthin as well as from no treated cell lines as controls.

Genomic DNA, up to 2µg , in a 17µl volume was added to NaOH to a final concentration of 0.3 M in a 20 µl volume, and the sample was incubated at 37°C for 15 min.

Sodium bisulphite solutions were prepared on the day of use by adding 1.9 g sodium metabisulphite (Sigma) to 3ml of 0.46 M NaOH and heating at 50°C to dissolve the bisulphite. After addition of hydroquinone (0.7 ml of a 0.75 M solution), 0.4 ml of this solution was mixed with each DNA sample and incubated at 50° C for 4.5 h.

Following bisulphite conversion, the DNA was recovered and purified using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's specifications. To desulphonate the sample, 3µl of 10M NaOH was added and incubated for 15 min at 37°C, followed by neutralization with 70µl of 10 M Ammonium Acetate. The bisulphite-converted DNA was precipitated with 525 µl Ethanol at -20°C overnight. The bisulphite-converted DNA was pelleted by centrifugation at full speed at 4°C for 30 min, washed with 70% ethanol and air dried. DNA was resuspended in Qiaquick elution buffer and stored at -20°C.

3.14. Quantitative methylation analysis of LINE-1.

There are half a million long interspersed nucleotide elements (LINE-1 elements) in the human genome (Kazazian 2002) that are normally heavily methylated, and it is estimated that more than one-third of DNA methylation occurs in repetitive elements (Bestor 1998). Thus, analysing the methylation of repetitive elements can serve as an alternative marker for global genomic DNA methylation. There are several methods of detecting total 5-methylcytosine content in the genome, but most of them have the disadvantage of being labor intensive and/or requiring large amounts of good quality DNA.

Global methylation assay by LINE-1 was performed as described by Iacopetta et al (Iacopetta et al., 2007). It involves two Real-Time PCRs. One, the unmethylated reaction (U), uses primers to TpG containing sites to quantify the number of unmethylated LINE-1 elements, whilst the other, the methylated reaction (M) uses primers containing CpG sites to quantify the number of methylated alleles.

Primers used were:

Unmethylated LINE-1 forward primer	TGTGTGTGAGTTGAAGTAGGGT
Unmethylated LINE-1 reverse primer	ACCCAATTTTCCAAATACAACCATCA
Methylated LINE-1 forward primer	CGCGAGTCGAAGTAGGGC
Methylated LINE-1 reverse primer	ACCCGATTTTCCAAATACGACCG

Methylated and unmethylated LINE-1 sequences were quantitatively evaluated using bisulphite treated DNA as template.

Real-time PCR was conducted using the ABI-PRISM 7700 Sequence Detection System (Applied Biosystems, Foster, CA, USA) in the following amplification conditions:

Immomix (Bioline's hotstart Taq master mix)	2.5ul
BSA (10mg/ml)	0.5ul
MgCl2	0.05µl
Sybr Green 1:100 diluition	0.125µl
Rox	0.1µl
Forward Primer (U/M)	2pmol
Reverse Primer (U/M)	2pmol
Ultrapure water	To 3.5µl

A plasmid containing both unmethylated and methylated LINE-1 amplicon was created prior to measurements for use as a constant reference. DNA from this plasmid was used as a standard for the measurement of both unmethylated and methylated LINE-1, and serial dilutions were used to obtain accurate and reproducible results.

Bis-Mod DNA from DLD1, HCT116, HT29, LOVO1 and Caco2, (each DNA performed in three different biological replicates) treated with 10µl, 50µM and 100µM Ind, as well as control no treated cells, was added (1.5µl) to every well plate in triplicate.

Real-time reactions for unmethylated and methylated LINE-1 sequences and the standard, were performed simultaneously in triplicate in one 384 well plate.

PCR conditions were:

1 Cycle	95°C	30 sec
40 Cycles	95°C	30 sec
	60°C	30 sec
	72°C	30 sec

The percentage of methylated LINE-1 was calculated using the equation:

$$\frac{100 \times methylated\ copies}{unmethylated\ copies + methylated\ copies}$$

3.15. Gene specific methylation analysis by Combined Bisulphite Restriction Analysis (COBRA) assay

The COBRA assay has been used as a quantitative technique for methylation analysis (Xiong and Laird 1997). The assay measures the methylation status of 1 CpG within the CpG Island of interest; in fact the methylation status of any CpG within a CpG island tends to be proportional to the methylation status of all the CpG's within that island (Xiong and Laird 1997). After bisulphite modification and PCR amplification, the PCR product is digested with a restriction enzyme (whose recognition sequence is affected by bisulphite modification) and quantitated using gel electrophoresis and densitometry.

PCR was always carried out in 10 µl reaction mixture:

Hotstar Taq polymerase mix	2.5µl
Forward Primer	1pmol
Reverse Primer	1pmol
$MgCl_2$ (50mM)	0.05µl

PCR conditions were:

1 Cycle	95°C	15 min
35 Cycles	95°C	30 sec
	T =optimum annealing temperature for the 2 primers	30 sec
	72°C	1 min

p16 Forward	GGTTTTTTTTAGAGGATTTGAGGGATA
p16 Reverse	CTACAAACCCTCTACCCACCTAA
DNMT3B Forward	GGGTGGAAGGAAGGGAGGAA
DNMT3B Reverse	CCTAACCCCCTACCCTCCCAA
ESR1 Forward	GGGATGGTTTTATTGTATTAGATTTAAGGG
ESR1 Reverse	CTATTAAATAAAAAAAAACCCCCCAAAC
NOD2 Forward	TGATGTAGTTGTTGGGAGGATA
NOD2 Reverse	TGATGTAGTTGTTGGGAGGGGATA
GATA4 Forward	GAGTTTGGATTTTGTTTGTT
GATA4 Reverse	GTGATGTTTTAGGGGTTT
WNT Forward	GTGGGGGAGGGTGTTTTAGG
WNT Reverse	CAATCTAACTTTAACAACCCTAAAAAC
WNT5A Forward	ATTAGGTTTTGTTTTTGTTGT
WNT5A Reverse	CACTAAACACCTACCTTCATAA
SFRP1 Forward	GTTTTTTAAGGGGTGTTGAGT
SFRP1 Reverse	CAAACTTCCAAAAACCTCC
HPP1 Forward	TGTGTGTGAGTTGAAGTAGGGT
HPP1 Reverse	ACCCAATTTTCCAAATACAACCATCA

After amplification, the PCR products were precipitated by adding 3M sodium acetate pH5.5 (1/10 th volume) and 2.5 vols cold absolute ethanol.

For enzymatic digestion, in a final volume of 10 µl containing specific enzymes for each recognition site analysed, in appropriate buffer was added to each DNA and incubated at specific temperature for 2h. The digested DNA was separated on 5% PAGE gels and stained with SYBR green solution. Bands were analysed using Totallab software.

Different restriction enzymes used are summarized in the following table

p16	Sau3AI 37°C
DNMT3B	BstUI 60° C
ESR1	DpnI 37°C
NOD2	TaqI 65°C
GATA4	RsaI 37°C
WNT	TaqI 65°C
WNT5A	DpnI 37°C
SFRP1	EcoRI 37°C
HPP1	TaqI 65°C

% Methylation was calculated using the formula:

$$100 \times \frac{\text{digested fragments}}{\text{undigested fragments} + \text{digested fragments}}$$

3.16.Activity study of the DNA Methyl- Transferase (DNMT)

DNMT activity was analysed by EpiQuik ™ DNMT Activity / Inhibition Assay Kit Ultra. This is a colorimetric assay that allows the study of DNMT activity / inhibition in nuclear extracts. A universal DNA substrate permanently conjugated to the bottom of a 96 well plate should be methylated by nuclear extract samples. The methylated substrate is, then, recognized by an antibody anti -5- methylcytosine. The amount of the methylated DNA, which is proportional to the enzyme activity, is subsequently measured by a spectrophotometer plate reader at 450nm. The DNMT enzymes activity will be proportional to the intensity of measured optical density. Inhibition inducted by Ind was calculated following the formula:

$$DNMT\ Inhibition\ \% = 1 - \frac{Inhibitor\ Sample\ OD - Blank\ OD}{No\ Inhibitor\ Sample\ OD - Blank\ OD} \times 100$$

In this regard, the nuclear proteins of control CaCo2 (10µg) cells were extracted through Cell Extraction Buffer (Invitrogen) and subsequently, the inhibitor (Ind) was added at different concentrations directly to the appropriate wells containing the nuclear protein extracts, according to the manufacturer's specifications.

3.17.Molecular modelling of Ind and DNMT interactions

The crystallographic structure of human DNMT1 in complex with DNA double helix and adenosyine-homocysteine bound was extracted from Protein Data Bank (3PTA). The missing residues (1480-1483) were modelled using Prime (Prime v2.1 2009). The model was further refined using the Protein Preparation Wizard implemented in Maestro (Maestro 9.0 2009). H-bond networks were optimized and orientation/tautomeric states of Gln, Asn, and His residues were flipped. At the end, a geometry optimization was performed to a maximum RMSD of 0.3 Å with OPLS2005 force field.

The refined model was used to perform a mixed Molecular Docking/Dynamics protocol, called Induced Fit Docking (IFD) (Sherman et al., 2006), with Indicaxanthin. In an interative manner, this approach combines ligand-docking techniques with those used to model receptor conformational changes. The Glide docking software package (Glide v.5.5 2009) was used for ligand flexibility, while the refinement module in the Prime program was used to account for receptor flexibility: the degrees of freedom of side chains were mainly sampled, while minor backbone movements were allowed through minimization. The strategy used was to first dock ligands into a rigid receptor using a softened energy function such that steric clashes do not prevent at least one pose from assuming a conformation close to the correct one (the "ligand sampling step"). The degrees of freedom of the receptor were then sampled, and global

ligand/receptor energy minimization was performed for many ligand poses; this attempted to identify low free-energy conformations of the whole complex (the “protein sampling step”). After that, a second ligand docking step was performed on the refined protein structures, using a hard potential function to sample the ligand’s conformational space within the refined protein environment (the “ligand resampling step”). Finally, a composite score function was applied to rank the complexes; this accounted for the receptor/ligand interaction energy as well as strain and solvation energies (the “scoring step”). The composite score, which was used to perform the final ranking of the compounds, was derived as follows:

$$\text{IFScore} = \text{Glide Score} + 0.05\ \text{Prime Energy}$$

The validity of the whole process was previously tested (Almerico et al., 2012; Almerico et al., 2009). The best scored ligand/protein complex was submitted to a nanosecond-scale (2ns) molecular dynamics (MD) simulation using Desmond [16]. The system was solvated with a cubic box of water molecules (SPC water model), and was first relaxed using the Desmond relaxation model. The completed equilibration run was followed by a production run performed with NPT conditions using the Berendsen thermostat (Berendsen 1984) (300K and 1.103 bar).

3.18.DNMT and Demethylase Real-Time Polymerase Chain Reaction (RT-PCR)

RNA from different cell lines (Caco2, LOVO1, DLD1, HCT116, HT29) treated with 0 or 100µM Ind or with 10µM 5-Azacytidine was extracted using Qiagen RNeasy mini - kit according to the manufacturer's instructions. A reverse transcription was performed from 500 ng RNA to obtain cDNA using qSCRIPT cDNA SuperMix (Quanta Biosciences), through a single cycle:

1 Cycle	25 ° C	5 min
	42 ° C	30 min
	85 °C	5 min

The cDNA was used as template for the subsequent quantitative PCR (Real-Time PCR) using Platinum SYBR Green qPCR SuperMix - UDG with Rox (Invitrogen, Life Technologies, Paisley, UK). The ribosomal subunit 18s gene expression, whose expression is constant, was used as reference gene. The latter was analyzed using a TaqMan probe hybridized to gene target sequence. The probe contains two dyes: FAM (6-carboxy-fluorescein) covalently linked to the 5' end and TAMRA (6-carboxy-tetramethyl-rhodamine) as a fluorescent quencher covalently linked to the 3 ' end. During the amplification, however, the probe is destroyed by hydrolysis of 5'- exonuclease 3 ' activity associated with the polymerase. During amplification polymerase induces a detachment of the reporter that, no longer sustained quencher inhibition, and emits fluorescence. Thus fluorescence intensity emitted during PCR cycles is directly proportional to the amount of amplified cDNA.

3.19.Statistical analysis

Calculations and graphs were obtained by INSTAT-3 statistical software (GraphPad software Inc, San Diego, CA, USA). Results are given as mean±SD. Three independent observations were carried out for each experiment replicated three times. Comparison between individual group means was performed by unpaired Student's t-test. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Bonferroni's test. In all cases, significance was accepted if the null hypothesis was rejected at $P<0.05$ level.

In addition Pearson correlation was used to investigate relationships of CGI methylation within different genes and to identify differences between gene methylation and expression.

4. RESULTS

4.1. Indicaxanthin stability and its anti-proliferative effects in tumor cell lines

Previous studies by other investigators have demonstrated that purified betalains, betanin in particular (red pigment of cactus pear), inhibit the growth of breast, colon, stomach and lung cancer (Reddy et al., 2005) cells. However the activity of betaxanthins on transformed cells has not been reported.

Here the antiproliferative activity of Ind on different cell lines was investigated, in order to evaluate any cell-type specificity.

A preliminary experiment to assess the stability of Ind in cell culture conditions showed that Ind was unstable at incubation conditions; with a 40% decrease in Ind concentration after 48h (Fig. 11)

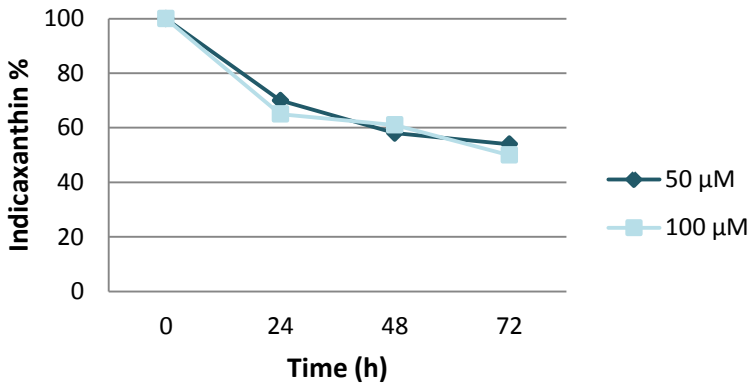
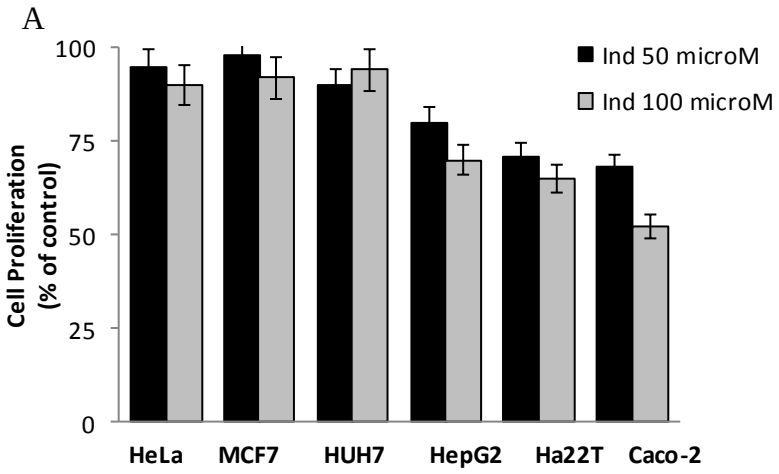


Fig. 11 - Spectophotometric analysis of indicaxanthin concentration after 24h, 48h, 72h of incubation at 37°C in 5% CO_2, and 95% humidity.

The effect of Ind on cell proliferation was investigated by treating cells during the exponential growth phase, as determined by a Trypan blue assay.

The anti-proliferative activity of Ind at 50 μM and 100 μM was assayed in a number of human cancer cell lines (Fig. 12). A 48 h treatment provided evidence that whereas proliferation of HeLa, HUH-7 and MCF-7 cells was not affected by the phytochemical, the growth of HepG2, Ha22T and Caco2 cells was inhibited by 30%, 35% and 48%, respectively, with Ind at 100 μM. Subsequent investigation, including mechanism of cytotoxicity and epigenetic activity of Ind, was then carried out on Caco2 cells, because of their intestinal origin, where Ind may reach a high concentration at physiological conditions.



B

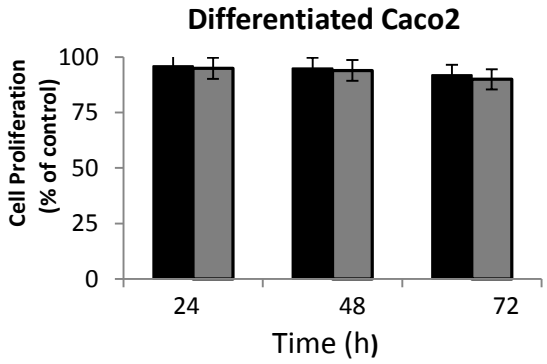


Fig. 12 – (A) Cell Proliferation of HeLa MCF7, HUH7, HepG2, Ha22T and Caco2 after 48h treatment with Ind 50uM and 100uM, compared to the control (B) Cell Proliferation of differentiated Caco2 cells treated with Ind 50uM and 100uM, for 24, 48, 72h.

The response of differentiated Caco2 cells to Ind (50µM, 100µM) treatment was also investigated, in order to evaluate potential differences compared with undifferentiated Caco2 sensitivity. As Fig. 12 B shows Ind doesn't affect differentiated Caco2 proliferation at any time or concentration used.

Since there was a highly significant impact of Ind on the colorectal cell line Caco2 and the intestine may be exposed to high concentrations of Ind from diet, the effect of Ind on the proliferation of other colorectal cancer cell lines (DLD1, HCT116, HT29 and LOVO1) was investigated.

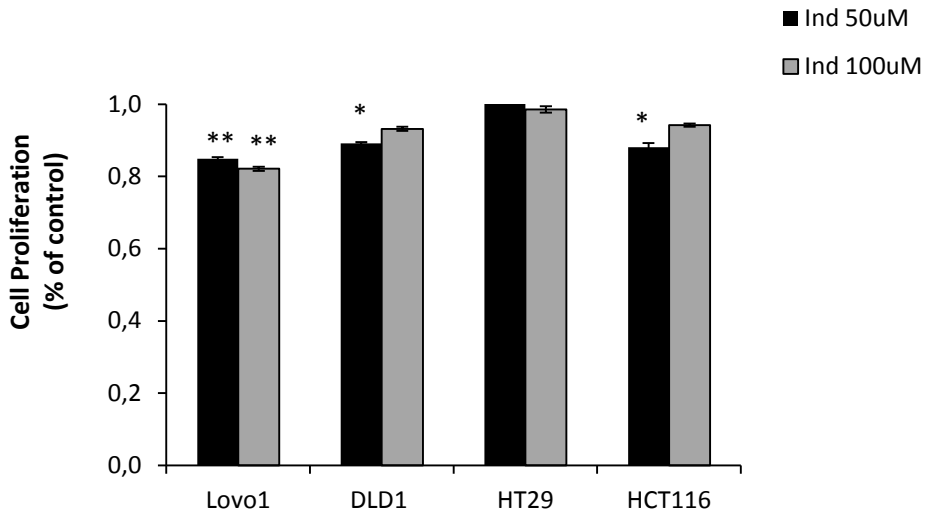


Fig. 13- Cell proliferation of LOVO1, DLD1, HT29, HCT116 after 48h treatment with Ind 50uM and 100uM, compared to the control. (*)pValue<0,05; (**)pValue<0,002

Fig. 13 shows cell proliferation after 48h treatment with Ind at 50 and 100 µM compared to control untrated cells. Whereas HT29 proliferation was not affected by Ind treatment, the other cell lines were weakly but significantly influenced by Ind.

4.2. Pro-apoptotic effects of Ind in Caco2 cell line

Treatment of proliferating Caco2 cells with Ind for 48 h resulted in a dose-dependent cell growth inhibition. The calculated IC_{50}, i.e. the Ind concentration that caused a 50% decrease of cell proliferation, was 115±15 µM (n=9) (Fig. 14). No differences in trypan blue uptake were observed when either untreated cells or Caco2 cells treated with Ind were compared, ruling out events leading to cell rupture. More importantly, no variation of cell viability was observed when Caco2 cells grown 20 days post-confluence, were treated with 25 µM to 250 µM Ind, indicating absence of toxicity even for differentiated cells (Fig. 14).

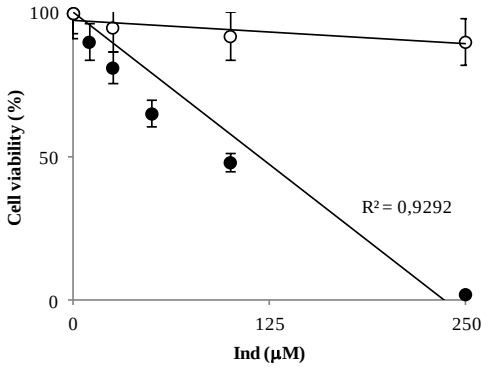


Fig. 14 - Effect of Ind on viability of undifferentiated (closed symbol) or differentiated (open symbol) Caco2 cells. Cells were incubated for 48 h with the phytochemical and cell viability was measured by MTT test as reported in Methods. Values are the mean ± SD of three triplicate experiments.

Induction of apoptosis by Ind was investigated to evaluate the mechanism of the pigment-induced effect on cell proliferation.

Phosphatidylserine externalization on cell surface is an early marker of programmed cell death. AnnexinV is a protein that binds phosphatidylserine exposed on cell membranes surface, while the PI is a DNA intercalating molecule, permeable only through damaged plasma membrane cells, thus, it represents an index of necrosis. Therefore using both compounds allows the discrimination of early or late apoptotic cells from necrotic cells.

Flow cytometry analysis of annexin V-FITC and PI stained Caco2 cells, after a 48 h treatment with 25 to 100 µM Ind showed cells in early apoptosis, with a dose-dependent relationship between Ind concentration and the percentage of Annexin V-FITC positive cells (Fig. 15).

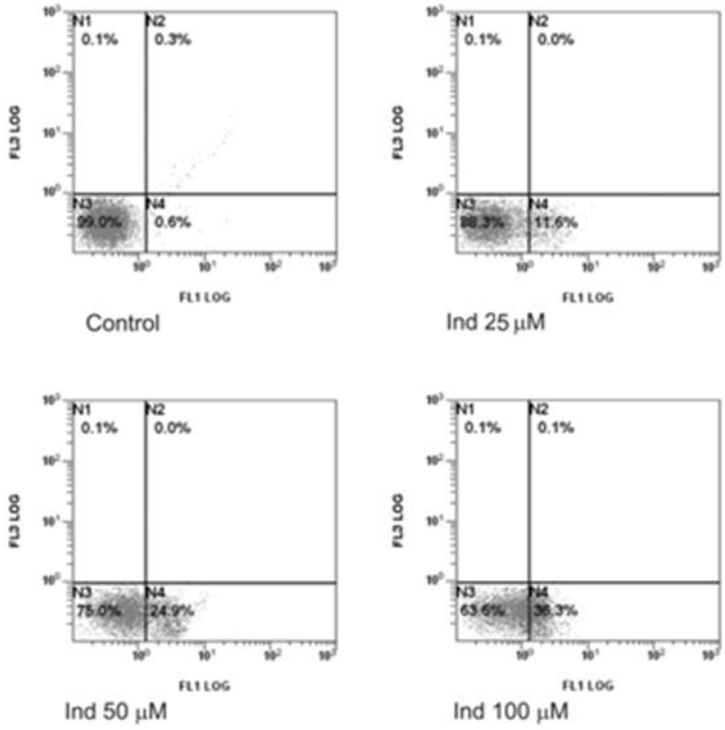


Fig. 15 - Apoptotic effect of Ind in Caco2 cells. % of Annexin V/PI double stained cells. Caco2 cells were incubated for 48 h either in the absence (control) or in the presence of Ind. Phosphatidylserine externalization was measured using flow cytometry as reported in methods. Representative images of three experiments with similar results.

The apoptotic process includes the release of mediator from the inter-membrane mitochondria space (mitochondrial outer membrane permeability MOMP). DiOC6(3), a fluorescent mitochondria-specific and voltage-dependent dye, was used to assess the involvement of mitochondria in the Ind-induced apoptosis.

CaCo2 cells, incubated in the absence or in the presence of the phytochemical, were subjected to flow cytometric analysis after reaction with DiOC6 (3), whose mitochondrial permeability decreases with decreasing organelle trans-membrane potential. Treatment of Caco2 cells with Ind for 48 h, caused a marked dose-dependent decrease of DiOC6(3) uptake, indicating dissipation of $\Delta\psi_m$ (Fig. 16)

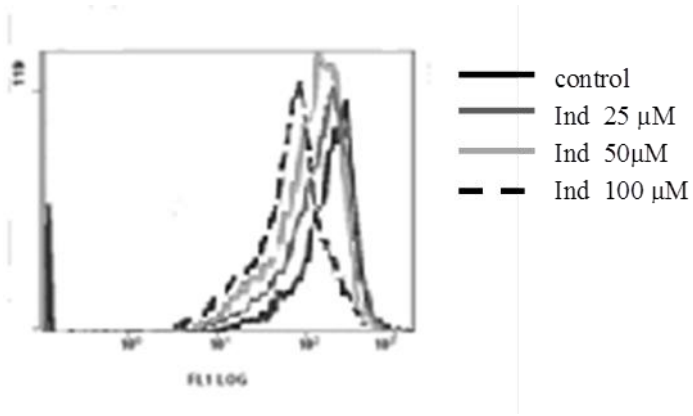


Fig. 16 - Apoptotic effect of indicaxanthin on Caco2 cells. Fluorescence intensity of DiOC6-treated cells as determined by flow cytometry. Caco2 cells were incubated for 48 h either in the absence (control) or in the presence of Ind and mitochondrial dysfunction was measured using flow cytometry as reported in methods. Representative images of three experiments with similar results.

Intracellular reactive oxygen species (ROS) represent important bio-signals involved in many molecular pathways including apoptosis. As Ind is a redox-active compound (Butera et al., 2002), the role of oxidative imbalance in triggering pro-apoptotic death was investigated. Flow cytometric analysis was performed in the absence or presence of the phytochemical after reaction with DCFA, a cell permeable reagent that emits fluorescence after ROS oxidation.

With respect to untreated cells, the level of intracellular ROS, as a measure of the global Caco2 cell redox state, appeared not significantly modified in cells treated with 100 µM Ind within 6 h to 48 h of incubation, indicating that the effects of Ind did not depend on oxidative stress (Fig. 17).

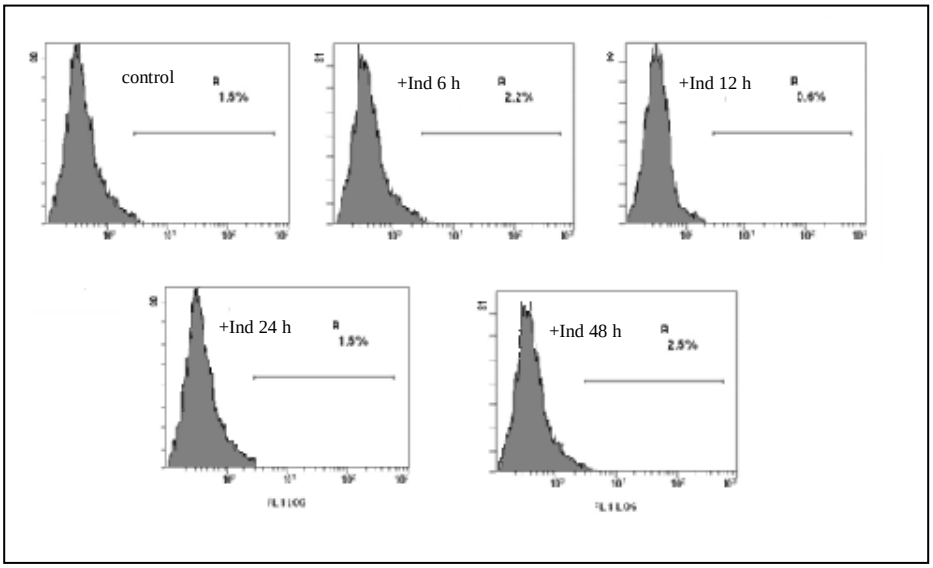


Fig. 17 - Indicaxanthin does not alter ROS levels in Caco2 cells. Caco2 cells were incubated either in the absence (control) or in the presence of 100 μM Ind at different time intervals. Cellular ROS was assayed using flow cytometry (DCFDA staining) as reported in Methods. Representative images of three experiments with similar results.

4.3. Epigenetic effects on genomic methylation status by Ind in caco2 cells

It is known that phytochemicals may exert their protective action on human health through several biological functions; antioxidant activity, modulation of detoxification enzymes, or even regulating gene expression. Changes in genome methylation may be one of the mechanisms through which phytochemicals act as gene expression regulators (Vanden Berghe, 2010). The effect of Ind on DNA methylation patterns in the colorectal cancer cell line was therefore investigated. In particular DNA isolated from Caco2 cells grown for 48 h, either in the absence or in the presence of 10 μM, 50 μM Ind, and 100uM was analysed by MeSAP-PCR, to investigate changes induced by the phytochemical to global methylation of genomic DNA. In accordance with this method, genomic DNA samples are either digested with methylation-insensitive restriction enzyme (SDD), or digested with methylation-

insensitive followed by methylation-sensitive restriction enzymes (DDD). Comparison of the products from multiplex PCR amplification using non-specific primers allows screening for regions of DNA that have altered methylation patterns. Fingerprints of the matched SDD and DDD from either untreated or Ind-treated Caco2 cells are reported in Fig. 18. Inspection of the PCR fragments revealed that treatment with an Ind concentration as low as 10 μM was effective in modifying the global DNA methylation pattern, as shown by the different number, intensity and size of the bands in the matched samples compared with the samples from untreated Caco2 cells. The DNA banding patterns of cells treated with Ind appeared quite comparable with those obtained after treatment with 10 μM 5-azacytidine (Aza), a potent de-methylating agent, suggesting that Ind may induce DNA demethylation

It should be noted that MeSAP-PCR gives no indication of the genomic region affected by methylation / demethylation by an external agent.

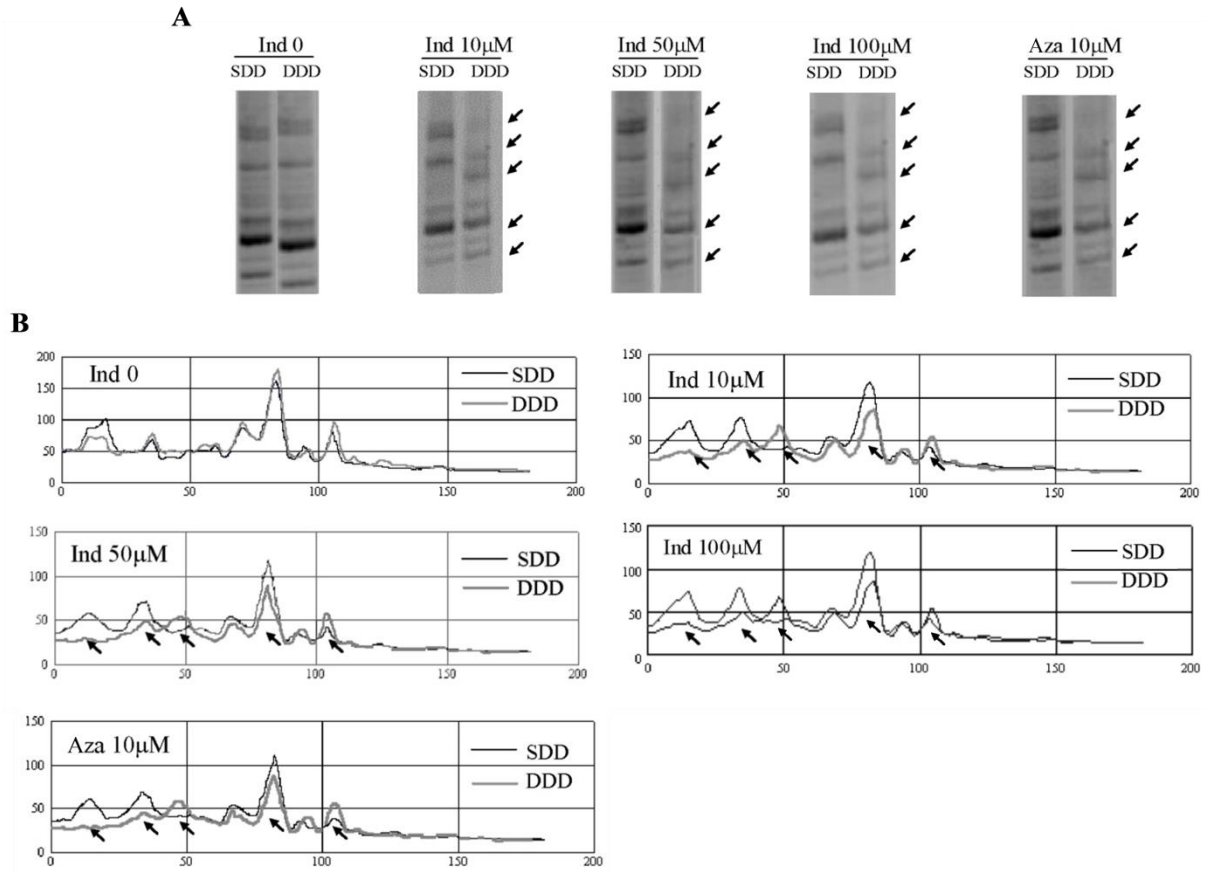


Fig. 18 - Methylation-sensitive fingerprints (A) and relevant densitometry profiles (B) of the matched SDD and DDD pairs from either untreated or Ind-treated Caco2 cells for 48 h. PCR was performed using arbitrary primers after a 16-h restriction enzyme digestion with 10 unit each of AfaI (single digested DNA, SDD) or AfaI+ HpaII (double digested DNA, DDD). Caco2 cells treated for 48 h with 10 μM Aza were taken as positive control. Bands that appeared to be differentially methylated between matched SDD and DDD global DNA are indicated by arrows. Representative images of three experiments with similar results.

4.4. Analysis on methylation status of *p16*^{INK4a} gene promoter region

It is known that abnormal methylation of *p16*^{INK4a} gene promoter region is the most common cause of silencing of this gene in colon cancer cell lines, including Caco2 cells (Herman et al., 1995) because of its role as cell cycle regulator. In fact, the lack of this protein may lead to uncontrolled proliferation in cancer cells The p16 protein, in effect, binds the kinase 4/6 and interferes with their binding to cyclin D, blocking cell cycle progression

(Rocco and Sidransky 2001). Therefore, it may be one of the critical factor in colon epithelial carcinogenesis.

To evaluate the potential influence of Ind on the methylation status of *p16*^{INK4a} gene, MSRE-PCR (Methylation-Sensitive Restriction endonucleases) was performed using the restriction enzyme CfoI. Through multiplex-PCR, the promoter region of *p16*^{INK4a} gene and an inner region of *INTERLEUKIN-4* gene (IL-4), as a control, were simultaneously amplified, using as template DNA extracted from Caco2 cells treated with or without 10µM 50µM and 100µM Ind for 48h.

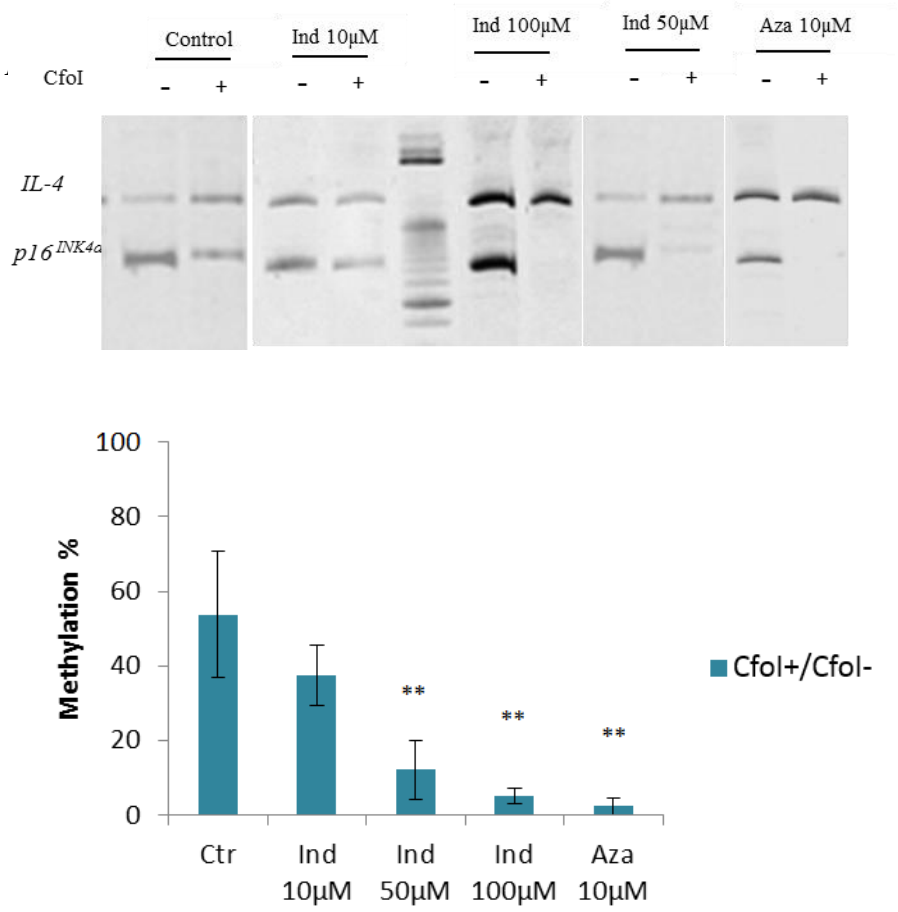


Fig. 19 - Effect of Ind on methylation of p16^{INK4a} *promoter region in Caco2 cells .(A) Matched pairs of multiplex specific PCR products from undigested (-CfoI) or CfoI methylation sensitive restriction enzyme digested DNA(+CfoI) from either untreated or Ind treated Caco2 cells for 48h. IL-4 gene promoter was used as negative control. (B) Data of the densitometric analysis are the mean ±SD. ** significantly different from the untreated cells (control) (p<0.002).*

Treatment of Caco2 cells with 10 μM Ind did not result in an apparent loss of methylation. However demethylation of the $p16^{INK4a}$ promoter region by treatment with 50 and 100 μM Ind is indicated by its reduced PCR amplification, allowing digestion with CfoI. The presence, in the same lane, of the IL4-specific product, used as control, shows the PCR reaction was worked. Negative control consists in not digested amplified DNA (Fig. 19).

The same MSRE experiment carried out on total DNA extracted from Caco2 cells treated with or without 100 μM Ind for at least 5 days without media change, shows that demethylation induced by Ind is stable, since it was shown previously that the Ind concentration decreases rapidly after 48h in culture (Fig. 20). The observed DNA demethylation effect of Ind in Caco2 cells may reflect an effect forwards DNA methyltransferase (DNMTs) enzyme activity.

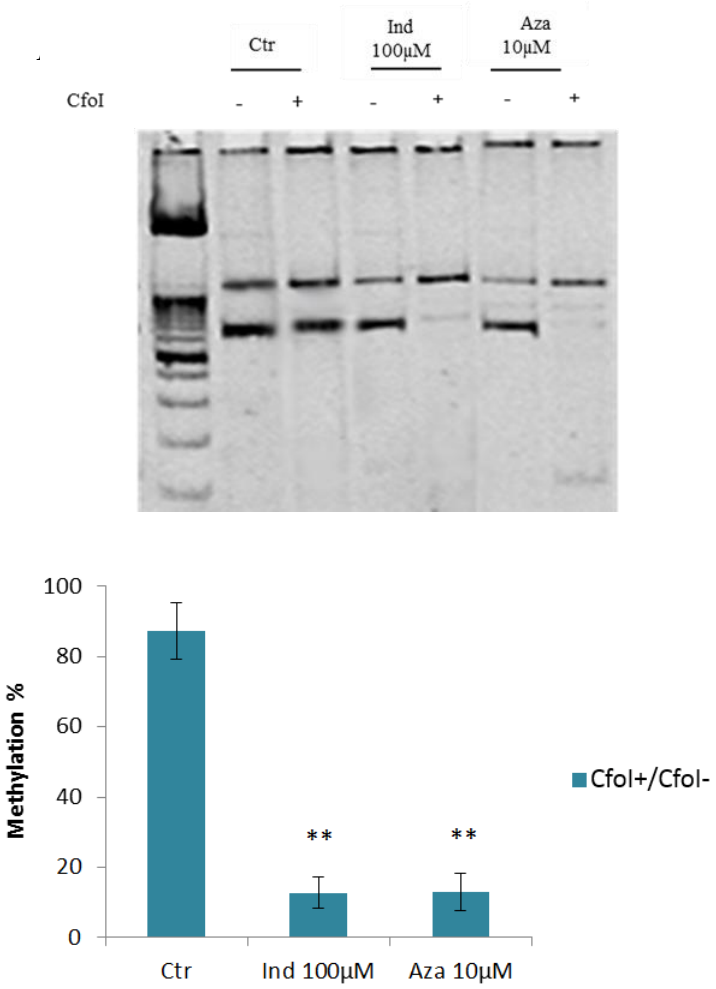


*Fig. 20 - Effect of Ind on methylation of $p16^{INK4a}$ promoter region in Caco2 cells. (A) Matched pairs of multiplex specific PCR products from undigested (-CfoI) or CfoI methylation sensitive restriction enzyme digested DNA(+CfoI) from either untreated or 100µM Ind treated Caco2 cells for 5 days. IL-4 gene promoter was used as negative control. (B) Data of the densitometric analysis are the mean ±SD. ** significantly different from the untreated cells (control) ($p<0.002$).*

4.5. Influence of Ind on the tumor-suppressor *p16*^{INK4a} gene expression in Caco2 cells

In order to validate that demethylation of the $p16^{INK4a}$ promoter gene leads to a reactivation of gene expression a Real Time PCR was carried out. This technique utilises the fluorophore, Sybr Green, which intercalates in a nonspecific manner into the DNA double helix, that is formed during the PCR. Sybr Green when excited emits a green light whose

intensity is proportional to the concentration of amplified DNA. In particular the number of the PCR cycle when sybr green starts to emit fluorescence is proportional to the original amount of cDNA in each sample.

First a reverse transcription of RNA extracted from Caco2 cells treated with or without 10 µM 100 µM Ind and with 10µM Aza, was carried out. Real-time PCR revealed that a 48h treatment of Caco2 cells with 100 µM Ind resulted in a significant reactivation of the mRNA expression (Fig. 21).

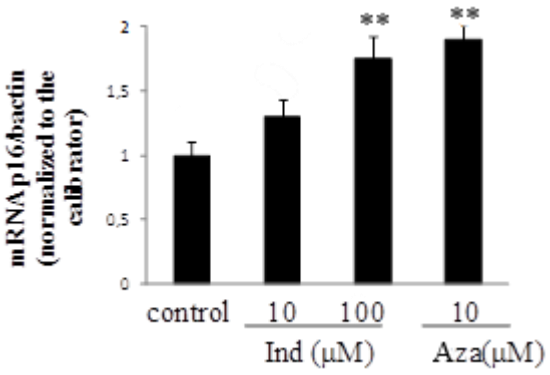


*Fig. 21 - mRNA expression levels in Caco2 cell line performed by real time-PCR with β-actin as an internal control Caco2 cells treated for 48 h with 10 µM Aza were taken as positive control. Values are the mean ±SD of three experiments carried out in triplicate ** significantly different from the untreated cells (control) ($p<0.002$)*

Interestingly a slight $p16^{INK4a}$ mRNA increase was evident even after a treatment with 10 µM Ind under the same conditions indicating some gene reactivation.

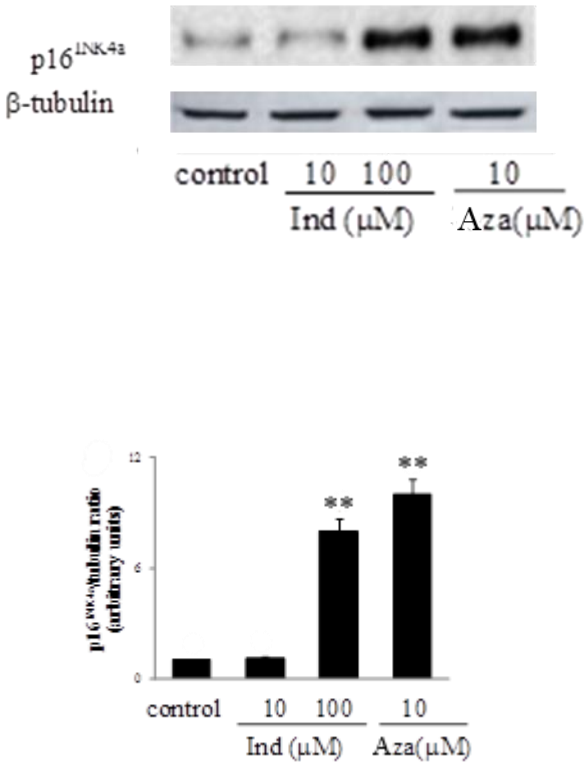


Fig. 22 - p16 protein expression by Western blot analysis and densitometric analysis of immunoblots. Those are representative of three experiments with similar results. Caco2 cells treated for 48 h with 10 μM Aza were taken as positive control. (**) p<0.002

The effect of Ind on p16 protein expression in Caco2 cells was evaluated by Western blot (Fig. 22). The results obtained indicate that, there is increased p16 protein in Caco2 cells treated with 100 μM Ind for 48h, compared to the control, confirming the reactivation gene.

The reversal of *p16*^{INK4a} hypermethylation and re-expression of mRNA and protein was comparable with that obtained after treatment of cells with 5-azacytidine (Aza) (Fig. 19, Fig. 21 and Fig. 22) indicating that Ind al 100μM has a similar demethylating effect to this pharmaceutical agent at this locus. Moreover, this study confirms the literature data that indicates the *p16*^{INK4A} gene is epigenetically silenced in cancer cells of intestinal adenocarcinoma (Rocco. 2001).

4.6. Ind induces a reactivation of p16^{INK4a} protein

Working in concert with other factors, p16^{INK4a} regulates cell cycle progression. Infact the INK4 proteins, such as p16^{INK4a}, destabilize the association of the D-type cyclin with kinase K4 or K6 (CDK4/6) (Sherr, Roberts 1999), thus preventing phosphorylation, and inactivation of the tumour suppressor retinoblastoma protein, RB, a key regulatory step in the pathway controlling proliferation of cancer cells (Knudsen, Knudsen 2008).

To evaluate that, the re-expression of *p16^{INK4a}* leads to the reactivation of functional p16 protein, the protein levels of phosphorylated pRB was analysed.

Western blot analysis showed that treatment of Caco2 cells with 100 μM Ind for 48 h, resulted in a significant decrease of the hyper-phosphorylated RB, with a parallel increase of the hypo-phosphorylated form, indicating that Ind treatment prevented inactivation of RB consistent with a p16^{INK4a} reactivation. The CDK4 levels appeared unchanged with respect to untreated cells (Fig. 23).

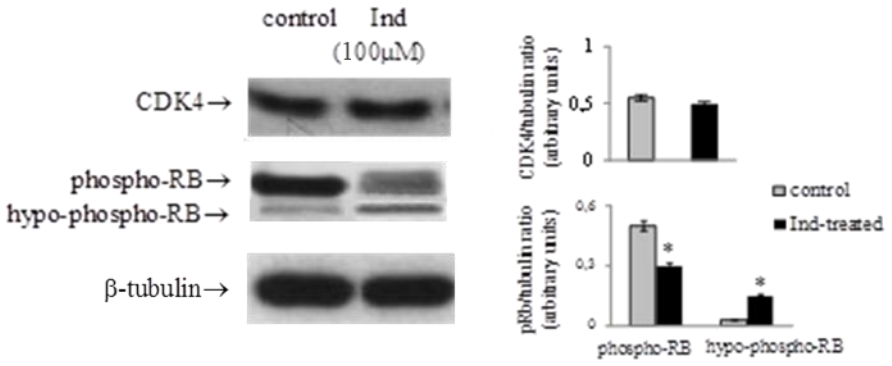


*Fig. 23 - Ind effect on CDK4 and RB levels in Caco2 cells. Caco2 cells were incubated either in absence (control) or in presence of 100 μM Ind for 48 h. CDK4 and RB by immunonoblotting with densitometric analysis of the immunoblots. Representative images of three experiments with similar results. Data of the densitometric analysis are the mean ±SD. *significantly different from the untreated cells (control) ($p<0.05$).*

4.7. Ind influences on cell cycle progression in Caco2 cells

Inhibition of RB phosphorylation is the main mechanism to control cell cycle progression at G1 and S phases (Weinberg RA 1995). In this regard, the phytochemical influence on Caco2 cell cycle progression was studied by flow cytometry using a PI stain that intercalating on nucleic acids gives an idea of cell DNA content during different phases.

The distribution of Caco2 cells in different phases of the cell cycle was analysed after a 48 h treatment with 100 μM Ind and is shown in Fig. 24, compared with untreated cells grown under comparable conditions. DNA content analysis showed that Ind induced a decrease in the percentage of cells in the G0/G1 phase, with a concurrent increase of cells in phase S and G2/M, indicating that despite re-expression of $p16^{INK4a}$ and re-activation of RB the transit of cells from G1 to S phase was not completely prevented.

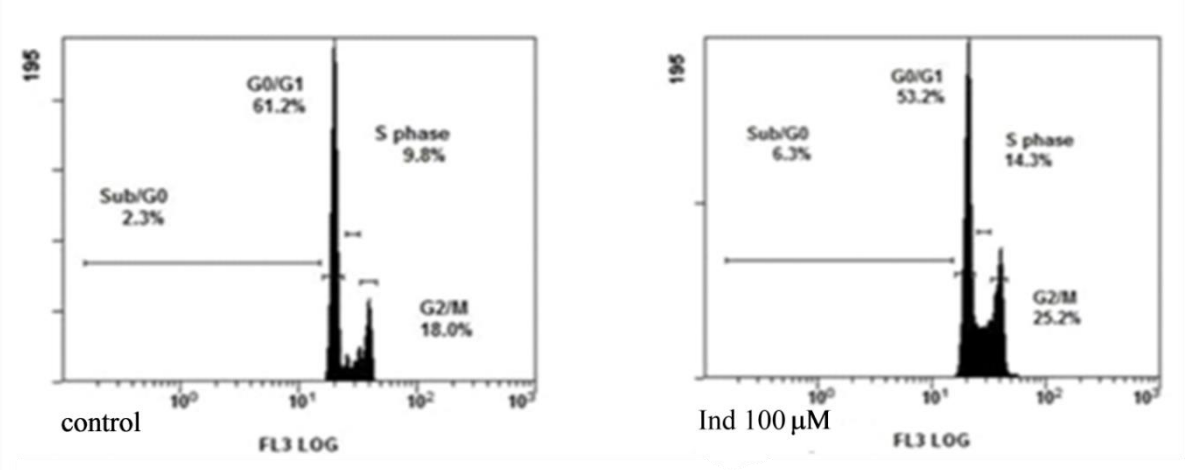


Fig. 24 - Effect of Ind on cell cycle distribution in Caco2 cells. Flow cytometric analysis of propidium iodide-stained cells after treatment with Ind. Caco2 cells were incubated either in the absence (control) or in the presence of 100 µM Ind for 48 h. The percentage of cells in the different phases of the cycle was calculated by Expo32 software. Representative images of three experiments with comparable results.

4.8. Influence of Ind on global DNA methylation in different colorectal cancer cell lines (methylation status of LINE-1)

It is now well established that phytochemicals often function as potent modulators of the mammalian epigenome-regulated gene expression through regulation of DNA methylation, histone acetylation, and histone deacetylation in experimental models (Malireddy et al., 2012).

This studies indicate that Ind is able to affect the epigenome in Caco2 cells. In this context the epigenetic effect of Ind on DNA methylation in other colorectal cancer cell lines was investigated.

Long interspersed nuclear element-1 (LINE-1), a transposon in the human genome, has been considered as a good representative for measuring global DNA methylation (Kazazian and Goodier, 2002).

The effect of Ind on LINE-1 methylation levels was therefore explored. DNA from the cell lines (Caco2, LOVO1, DLD1, HT29, HCT116) treated with 10, 50 and 100µM Ind for 48h was extracted and subsequently treated with sodium bisulphite. The latter is known to deaminate cytosine to thymine, while 5-methylcytosine resists this bisulphite action. Thus performing two subsequent real-time PCR, with specific primers amplifying methylated sequences or unmethylated sequences, it is possible to quantify the amount of total methylation as well as any change in total methylation.

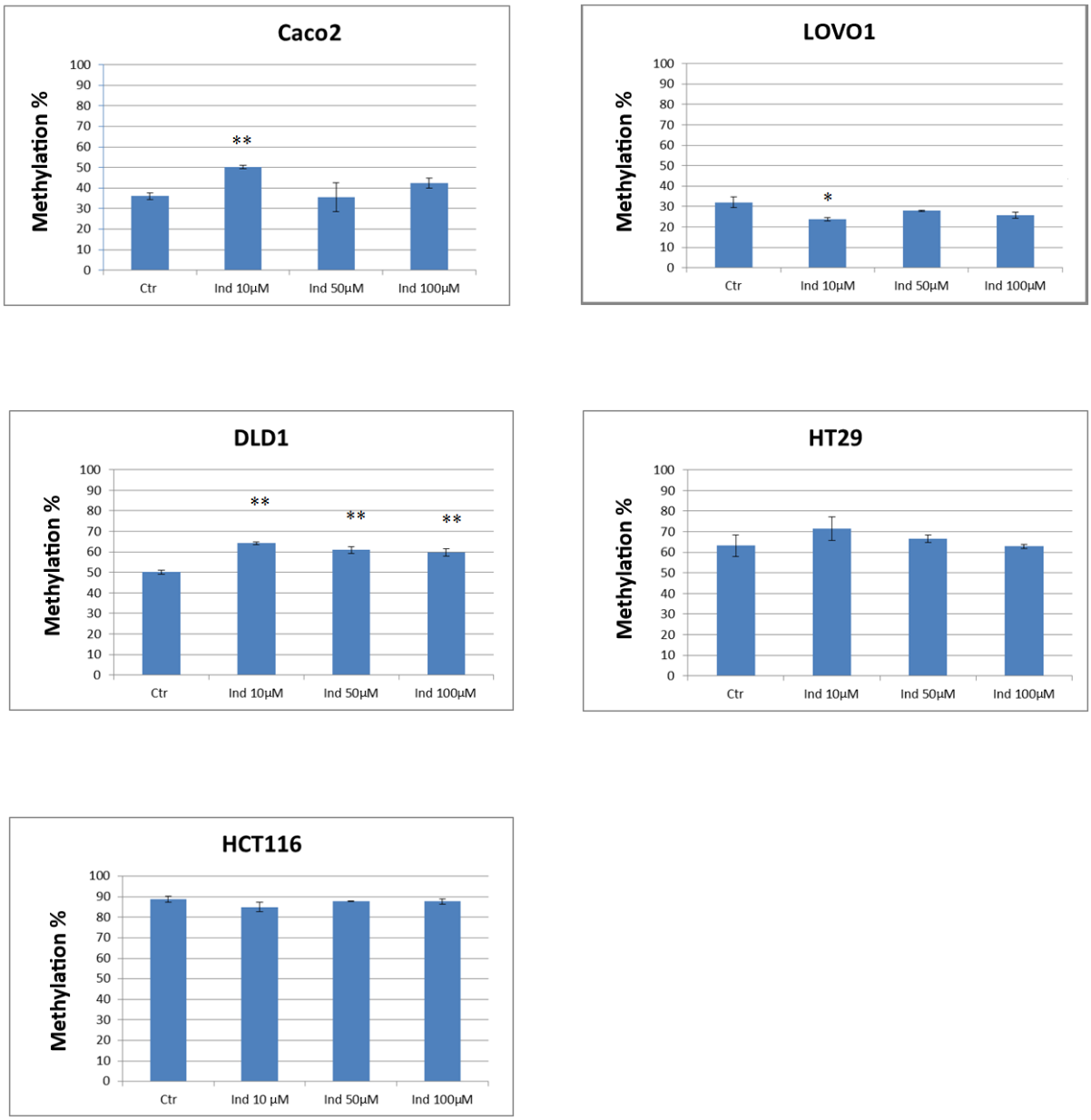


Fig. 25 - Histogram shows global DNA methylation assessed on the basis of LINE-1 amplification. % of DNA methylation is calculated as mentioned in methods. RNA was extracted from the different cell lines (Caco2, LOVO1, DLD1, HCT116 and HT29) treated with 0µM, 10µM, 50µM, 100µM Ind for 48h. (*)p Value <0,05 (**) p Value <0,001

As show in Fig. 25 low concentration of Ind induces a significant increase of LINE-1 methylation in Caco2. Instead Ind 10µM induced a significant decrease of LINE-1 methylation in LOVO1. All concentrations of Ind induce alteration of LINE-1 DNA methylation in DLD1 cells. Methylation of HT29 and HCT116 was not significantly altered.

4.9. Ind influence on gene methylation in different colorectal cancer cell lines

The previous observations showing an effect of Ind on DNA methylation patterns (MESAP), LINE-1 methylation and *p16*^{INK4a} methylation suggest that Ind may affect the methylation status of other genes implicated in colon carcinogenesis.

For the sake of clarity all genes studied are represented in one graph for each cell line. Some genes failed to amplify in some of the cell lines, therefore, no data is presented.

Fig. 26 shows the methylation percentage of each gene studied using COBRA assay. Genome-wide (LINE-1) methylation for each cell line is also included in order to aid the subsequent discussion.

Interestingly each gene in each cell line is differentially methylated. Apart from *SFRP*1 and *HPP1* which show, in every cell line, a high level of methylation.

In Caco2 cell line Ind, at high concentration, induces a significant demethylation of *p16*^{INK4a} (18%), *GATA4* (26%) and *WNT5A* (37%). Demethylation is also induced by 10 µM Ind in *NOD2* gene (7%). It should be noted that all these genes are more than 60% methylated in control conditions. The low level of *DNMT3B* gene methylation is significantly increased after 100µM Ind treatment (10% more than control) (Fig. 26A).

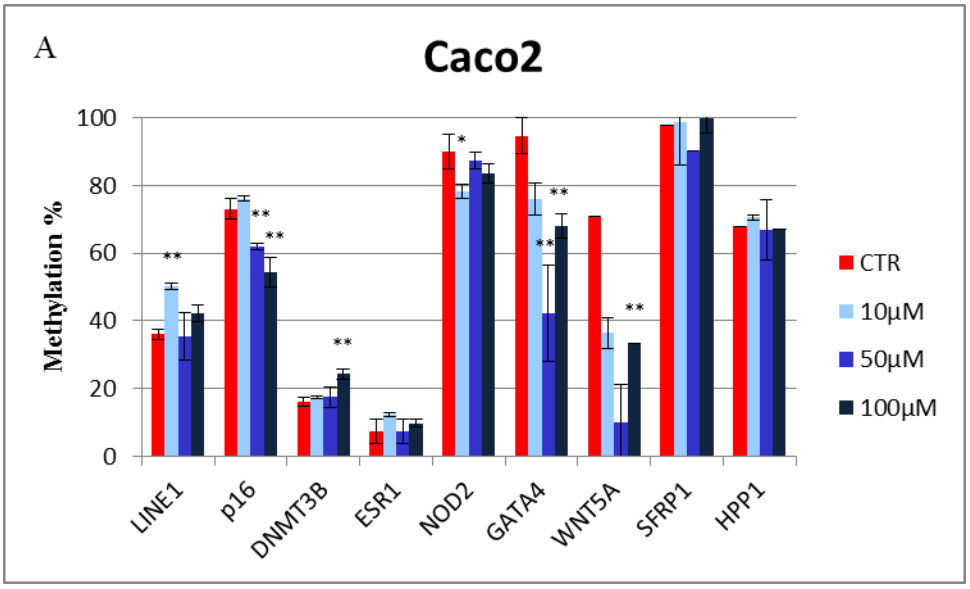
It is important to notice that methylation change have to be differently considered on the basis of original methylation status. For example, a little methylation change in a low methylated gene is heavier than the same methylation change in higher methylated gene.

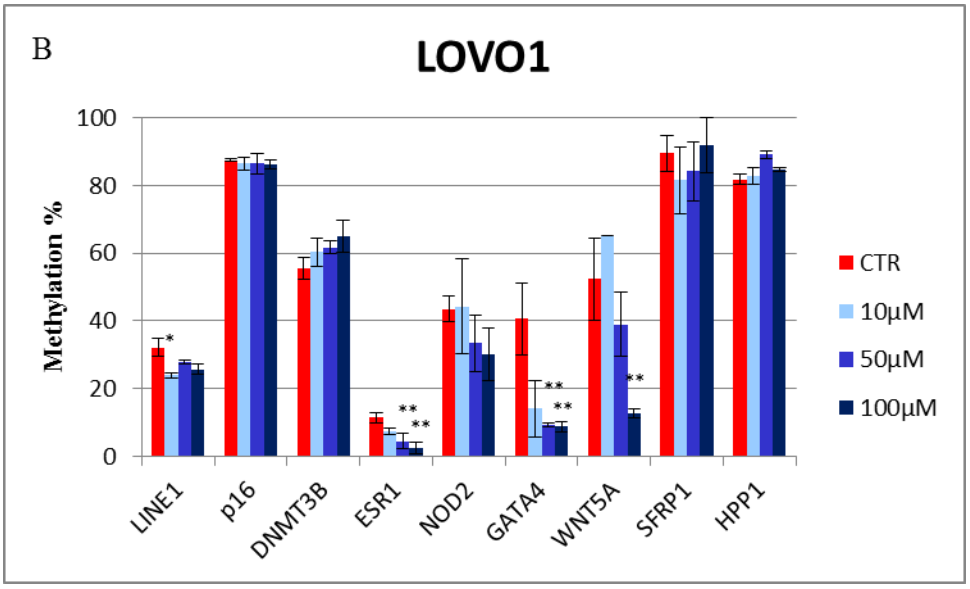
In LOVO1 cell line, Ind at higher concentrations induces a demethylation of *ESR1* (6%), *GATA4* (31%) and *WNT5A* (40%). No significant alterations in methylation were observed in the other genes (Fig. 26B).

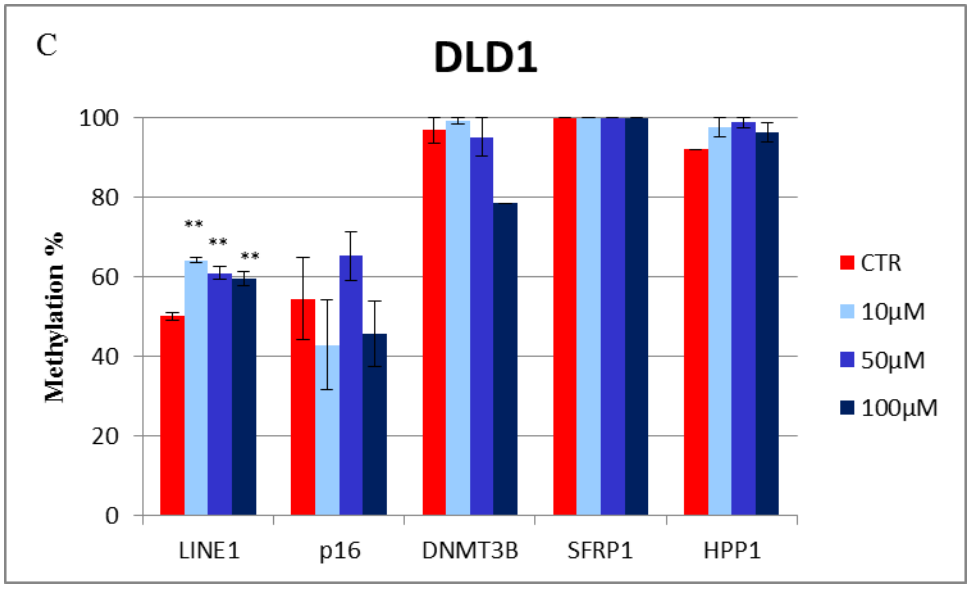
All the genes investigated in DLD1 cells are highly methylated, and no effect of Ind was observed (Fig. 26 C).

In HT29, the *ESR1* gene is strongly demethylated after Ind (50µM and 100µM) treatment (40%). Demethylation of the in $p16^{INK4a}$ gene (14%) was also observed (Fig. 26 D).

In HCT116 cells Ind at higher concentrations induced a significant demethylation of $p16^{INK4a}$ (20%) and *WNT5A* gene (15%), in contrast it increases methylation of *WNT1* (14%) and *ESR1* (6%). Ind has a heavy effect (even for a 6%) changing methylation status in *ESR1* gene just because it is only 11% methylated at control conditions (Fig. 26E).




B
LOVO1
Methylation %
100
80
60
40
20
0
LINE1
p16
DNMT3B
ESR1
NOD2
GATA4
WNT5A
SFRP1
HPP1
CTR
10µM
50µM
100µM


C
DLD1
Methylation %
100
80
60
40
20
0
LINE1
p16
DNMT3B
SFRP1
HPP1
CTR
10µM
50µM
100µM

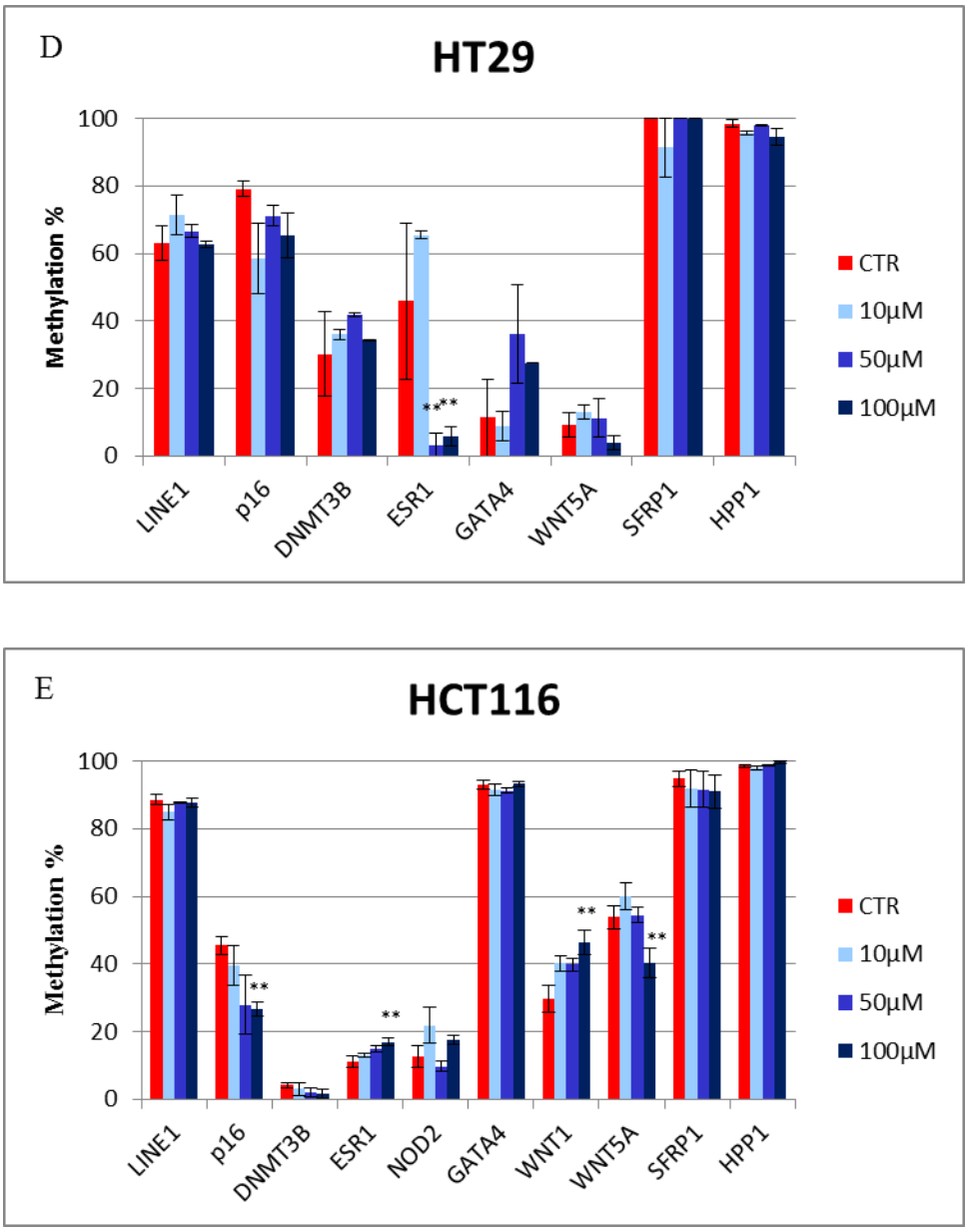


Fig. 26 - DNA methylation values for nine genes and for LINE-1 in each cell line. % of DNA methylation is calculated as mentioned in methods. DNA was extracted from the different cell lines Caco2 (A), LOVO1(B), DLD1(C), HT29(D) and HCT116 (E) treated with 0µM, 10µM, 50µM, 100µM Ind for 48h. (*) p Value <0,05 (**) p Value <0,002

A statistical analysis was performed in order to investigate the relationships between LINE-1 and gene methylation. In Caco2 cells, LINE-1 methylation is highly correlated with *ESR1* (R^2 0.998) methylation. LINE-1 methylation is also inversely correlated to *NOD2* change methylation (R^2 0.935). As well as in Caco2 *WNT5A* methylation is correlated to *GATA4* methylation (R^2 0.942).

Furthermore in HCT116 cell line *ESR1* change methylation is correlated to *p16* change methylation (R^2 0.907). In addition performing statistical analysis between methylation of the same gene in different cell lines, results a inversely correlation between LOVO1 and HCT116 in *ESR1* methylation, and a positively correlation in *WNT5A* methylation between LOVO1 and HCT116.

4.10. Alteration of DNA Methyl-Transferase (DNMT) activity by Ind

Since previous experiments showed demethylating effects of Ind, which raises the possibility that this may be due to an inhibition of DNA Methyl Tranferase (DNMT) activity.

DNMT transfers the methyl group from the donor SAM (S-Adenosyl-Methionine) to cytosine in DNA. If they are inhibited in transferring the methyl group, during a new DNA replication (DNMT3) or even during the maintenance of DNA methylation status (DNMT1), DNA would be inevitably and consequently demethylated.

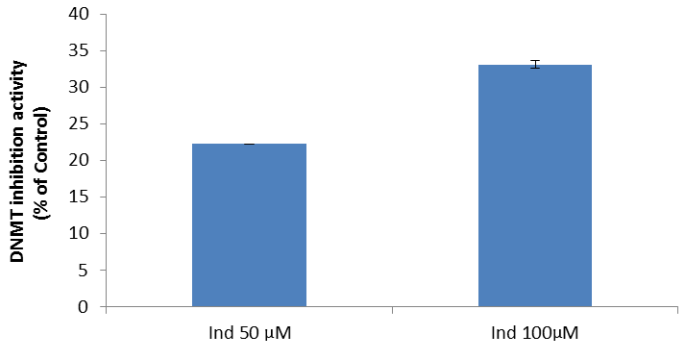
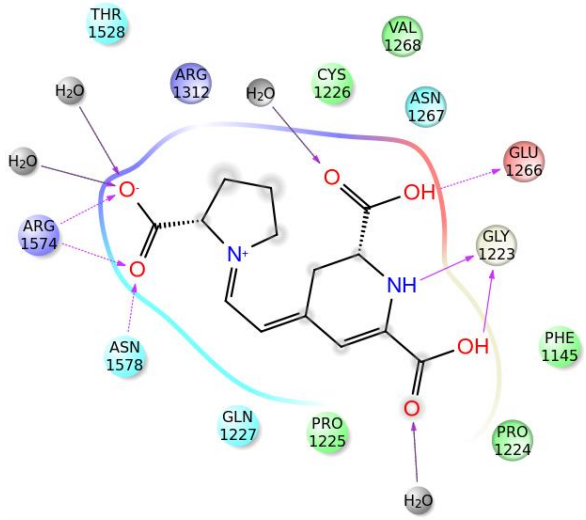


Fig. 27 - % DNMT activity inhibition, with respect to untreated control, tested by EpiQuik ™ DNMT Activity/Inhibition Assay Kit Ultra as reported in method. Values are the mean ±SD of three experiments carried out in triplicate.

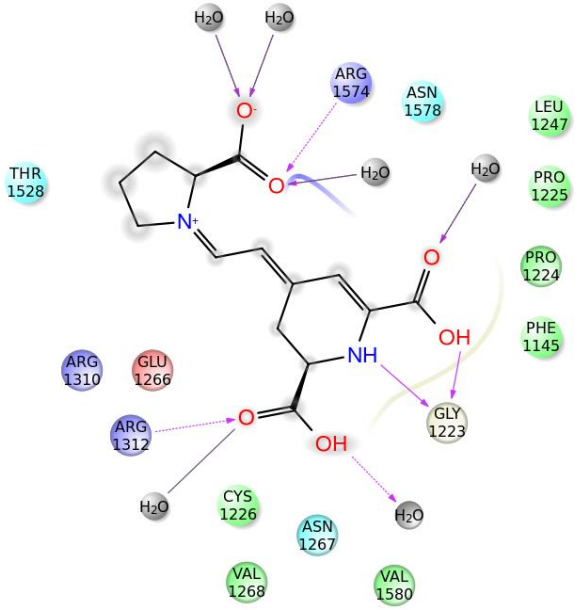
DNMT activity assay performed on Caco2 cells treated with 50 and 100μM Ind for 48h alongside untreated controls, showed a dose dependent inhibition of DNMT activity. With 30% inhibition in cells treated with 100μM Ind. It has to be noted that the assay is not able to distinguish between the activity of the different DNMT enzymes therefore providing a measure of total nuclear DNMT activity (Fig. 27).

4.11.Molecular Modelling interactions between Ind and DNMT1

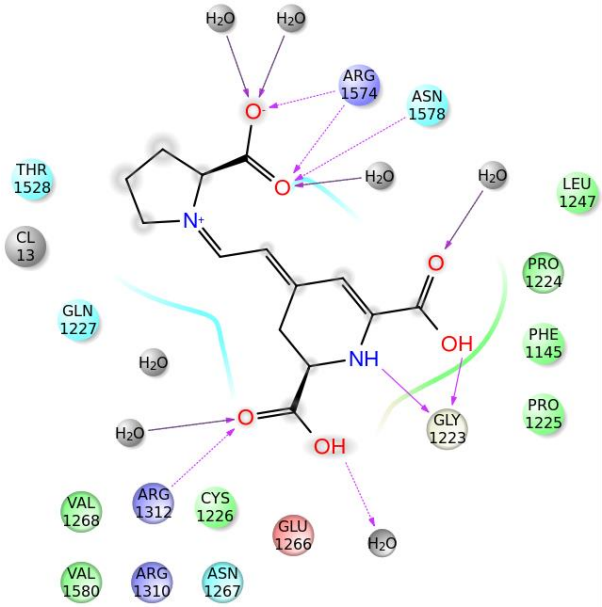
In silico molecular imaging studies performed in Dr. Marco Tutone Lab. (Department STEBICEF - Chemistry Pharmaceutical and Biological industry Section- University of Palermo) shows very interesting data. By adopting the molecular modelling approach of Yoo and Medina-Franco (Yoo et al., 2012) which consisted of refined crystal structure of DNMT1 catalytic domain (residues 1139-1600), induced fit docking and nanosecond-scale molecular dynamics, the interactions between DNMT1 protein and Ind were studied. Ind showed interactions with key residues of the DNMT1 target protein. Results obtained by IFD (Induced Fit Docking) are then confirmed by the molecular dynamics simulation (Fig. 28). The H-bonds between C2-COOH, Arg1574 and Asn1578 were identified as the principal interactions, as well as with Gly1223, Gln 1227, Arg1312, Glu1266 and Thr1528. Last, many hydrophobic interactions stabilize the ligand-protein interactions. After the relaxation period of the system of about 200 ps, equilibration was reached and the most representative snapshots were extracted. Fig. 28 shows some snapshots of the simulation chosen at regular intervals, interactions were analysed.



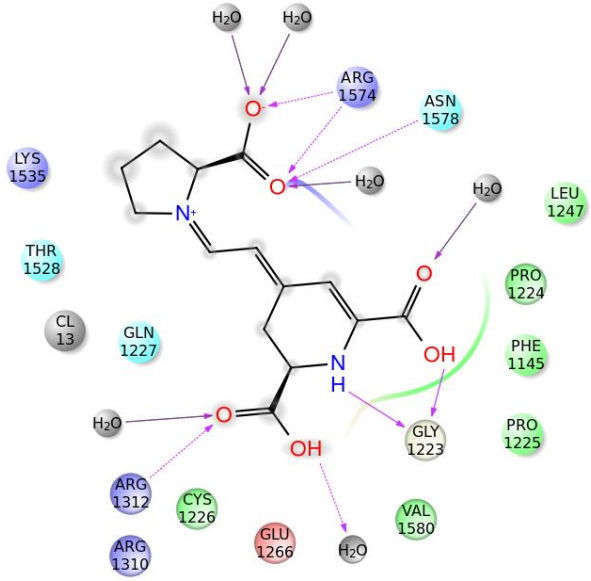
a) snapshot 1



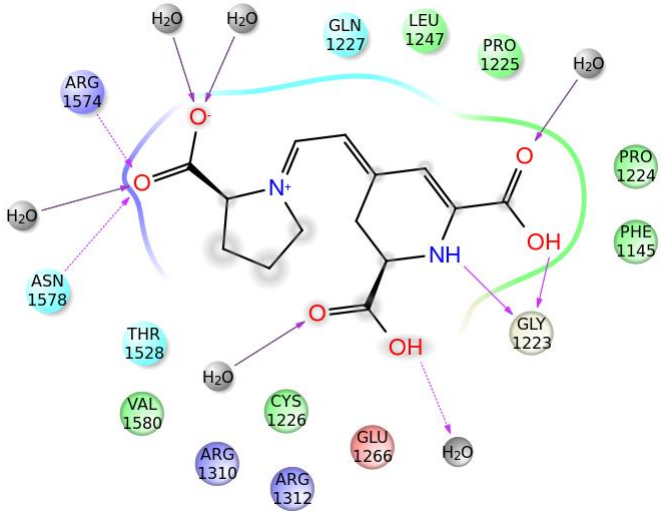
b) snapshot 2



c) snapshot 3



d) snapshot 4



e) snapshot 5

Fig. 28 - Molecular dynamics simulation. After the relaxation period of the system about 200 ps, equilibration was reached and the most representative snapshots were extracted at regular intervals of the simulation.

Snapshot 1 (Fig. 28a) Ind assumes a rolled-up conformation due to the fact that the two exocyclic double bonds are in *cis-cis conformation*; four water molecules are involved in the binding pocket, interacting with the carboxyl groups; H-bonds are established between Arg1574, Asn1578 and the C2-COOH, between Glu1266 and C11-COOH, two H-bonds between Gly1223, C13- COOH and the adjacent nitrogen atom. The catalytic residues (Pro1225, Cys1226, Gln1227) are very close to the ligand, confirming the prior IFD results. In snapshot 2 (Fig. 28b) Ind flipped out its conformation *trans-trans* which leads to a new H-bond of C11-COOH with Arg1312 and the loss of the H-bond interaction with Glu1266. During the rest of the simulation (Fig. 28c and Fig. 28d) Ind maintained the flipped out conformation and the H-bond interactions with the previous identified residues. Only at the end of simulation (Fig. 28e) Ind comes back to the *cis-cis* conformation, but C11-COOH did not establish any H-bond interaction, except with water molecules. An interesting point to be stressed is the copious hydrophobic interactions with the catalytic loop residues. Water molecules are also present in the pocket and this is synonymous of a quite open and

hydrophilic binding pocket, but this last consideration could be due to the presence of three carboxyl groups.

4.12.Ind influence on DNMT1 protein expression in Caco2 cells

The observed inhibition of DNMT activity by Ind may be due to a reduction of the expression of one or more of the DNMT genes or protein. This was investigated for DNMT1 expression in Caco2 cells treated with 50µM and 100µM Ind by Western blot analysis, which showed that Ind did not significantly affect DNMT1 protein expression (Fig. 29).

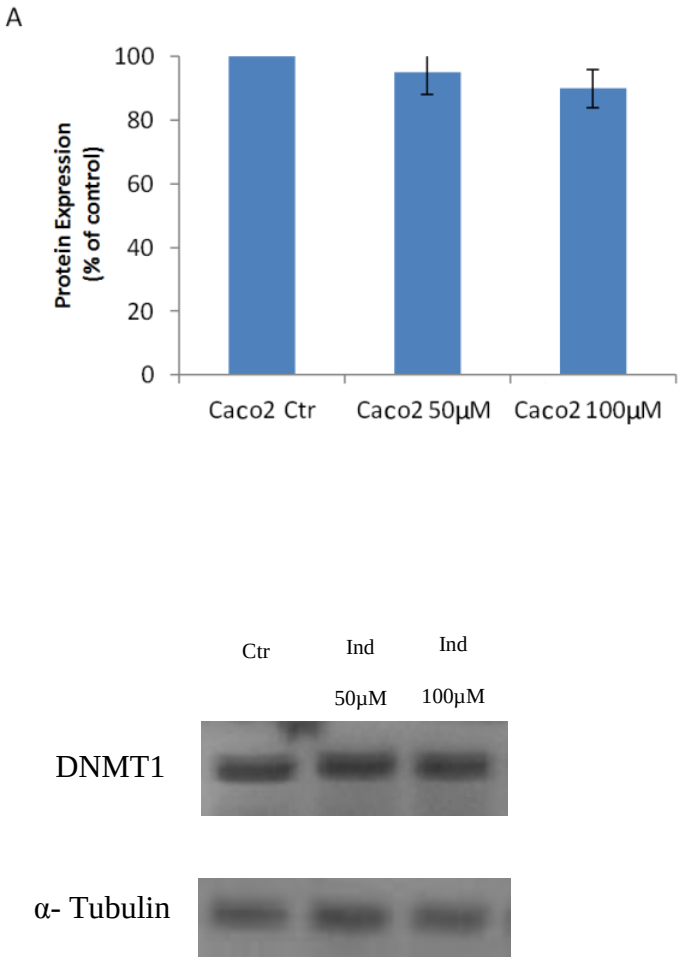


Fig. 29 - Western Blot Analysis of DNMT1 protein extracted from treated and (50 and 100µM) untreated proliferating Caco2 cells for 48h (A) analysis of the immunoblots performed with ImmageJ software (B). Representative images of three experiments with similar results.

The expression of DNMT1 has been further studied by real-time PCR, focusing only on 100µM Ind treatment and 10µM Azacytidine, which showed that in Caco2 cells Ind did not inhibit *DNMT1* mRNA expression (Fig. 30).

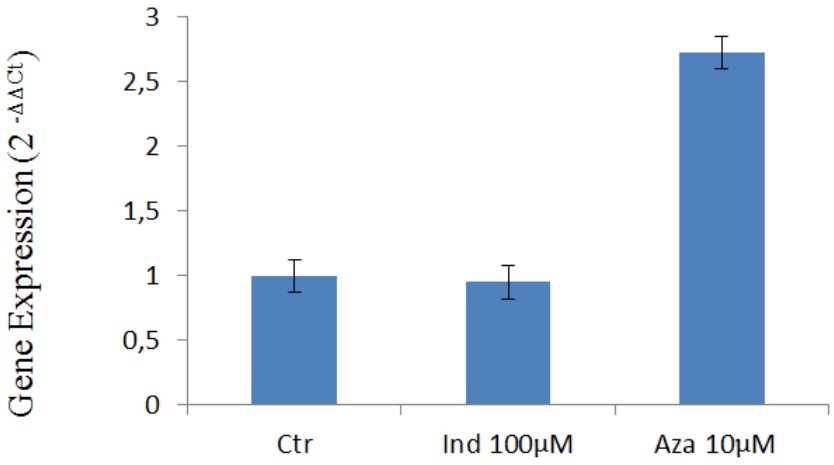


Fig. 30 - DNMT1 gene expression by Real-Time PCR analysis. RNA was extracted from treated 100µM Ind and 10µM Azacytidine and untreated proliferating Caco2 cells after 48h.

The effect of Ind on *DNMT3A* and *DNMT3B* expression was also investigated by real-time PCR. The results showed that Ind 100 µM increased *DNMT3A* mRNA expression by 2.5 fold in Caco2 cells (Fig. 31A) but a weak effect on *DNMT3B* expression (1.2 fold) was observed (Fig. 31B).

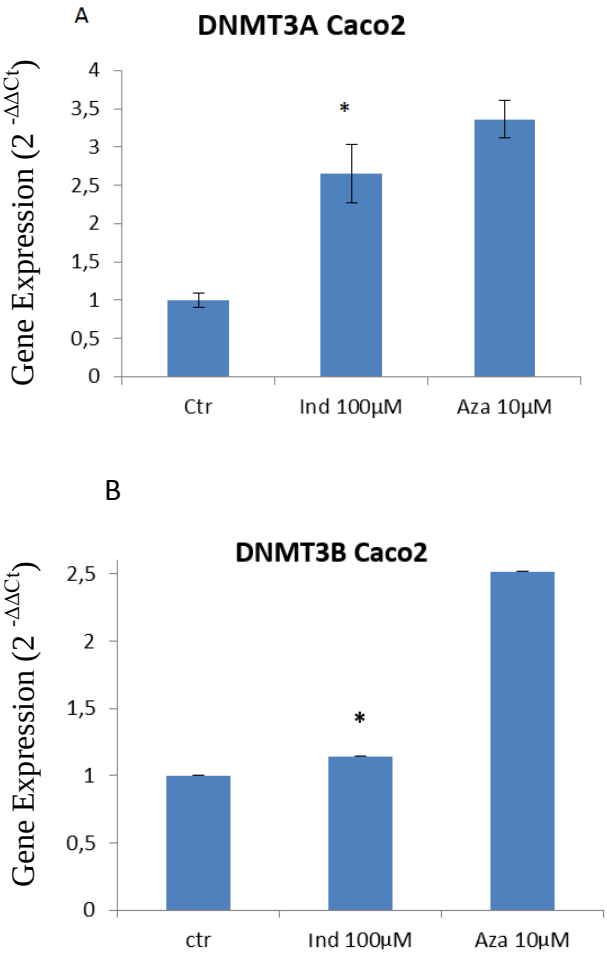


Fig. 31 - DNMT3A (A) and DNMT3B (B) gene expression by Real-Time PCR analysis. RNA was extracted from Caco2 cells after 48h treatment with 0µM (control), 100µM Ind and 10µM Azacytidine Y axis express (*) p Value <0,05

4.13.DNMT expression in colorectal cancer cell lines

Since from previous experiment Ind is able to induce effect on DNA methylation in the different colorectal cancer cell lines, the effects of Ind on the expression of the DNMTs was investigated in those cell lines (LOVO1, DLD1, HT29, HCT116)

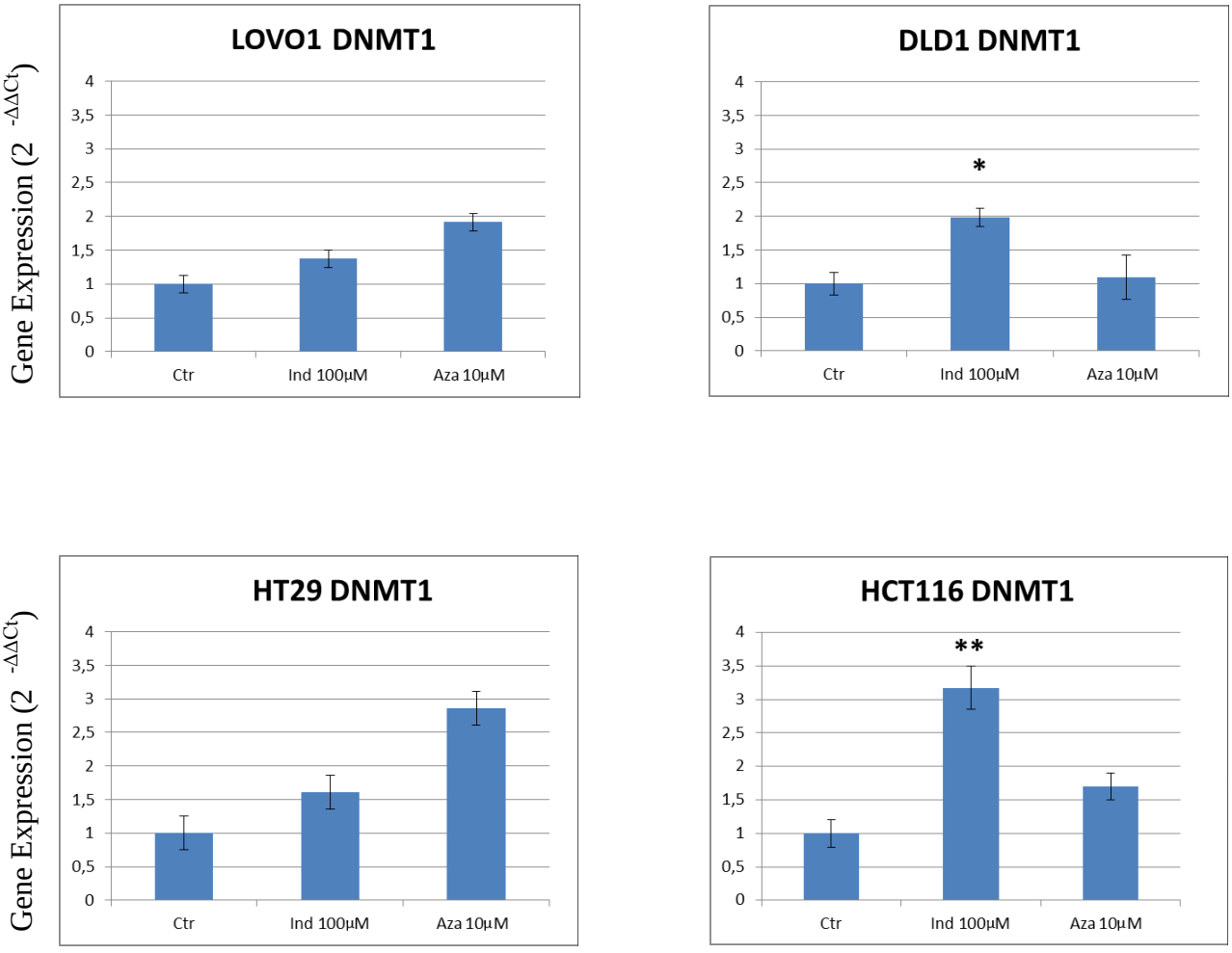


Fig. 32 - DNMT1gene expression by Real-Time PCR analysis. RNA was extracted from the different cell lines (LOVO1, DLD1, HCT116 and HT29) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, Y axis express $2^{-\Delta\Delta Ct}$ *(*) p Value <0,05 (**) p Value <0,002*

Fig. 32 shows Ind (100µM) increased *DNMT1* mRNA expression in DLD1 and HCT116 cell lines by 2 and 3.1 fold, respectively after 48h treatment.

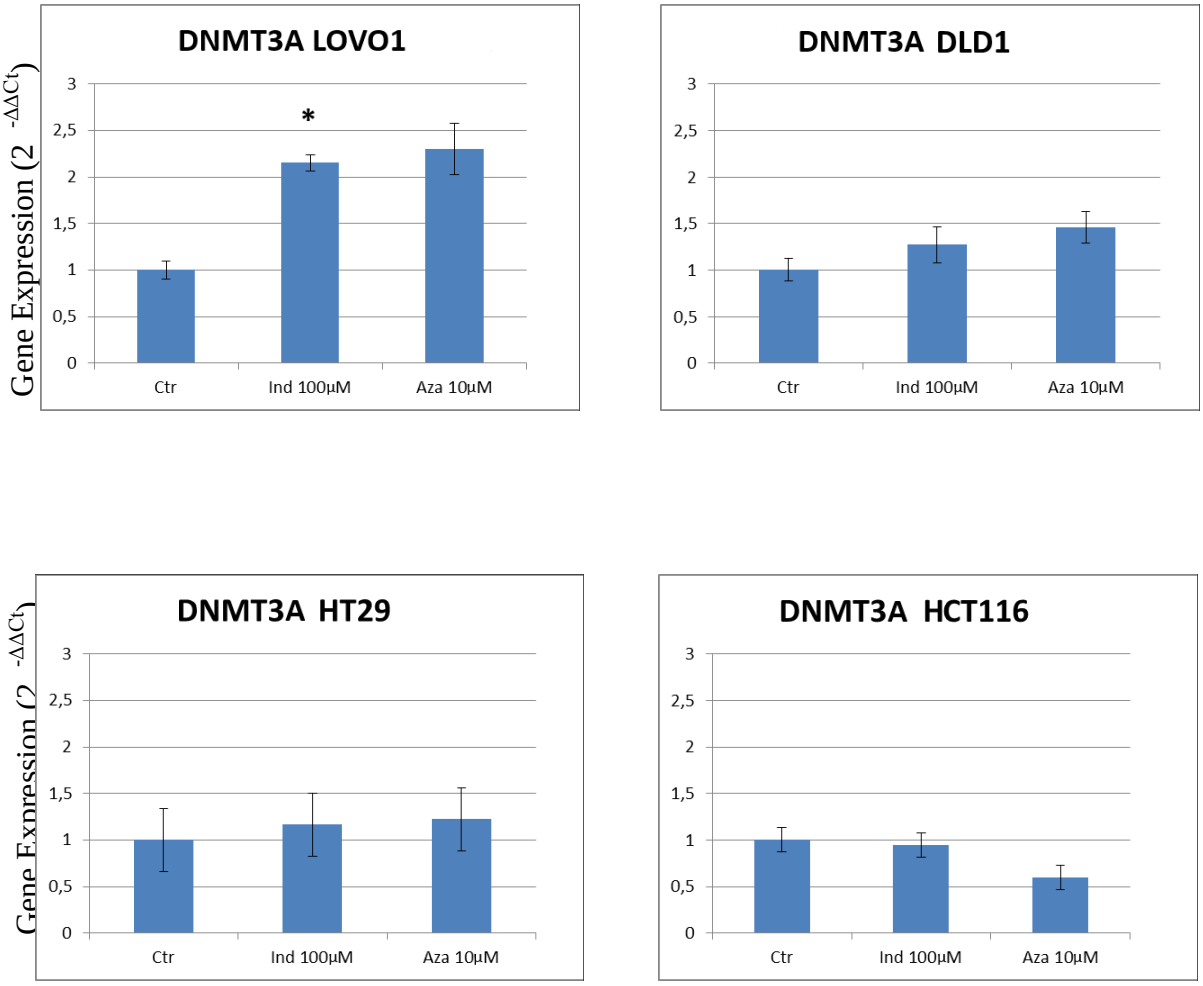


Fig. 33 - DNMT3A gene expression by Real-Time Analysis. RNA was extracted from the different cell lines (LOVO1, DLD1, HT29 and HCT116) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, Y axis express $2^{-\Delta\Delta Ct}$ *(*) p Value <0,05*

DNMT3A expression was only affected by Ind in LOVO1 cells, which showed a 2.2 fold increase in expression. No signicifant effects of Ind were observed in the other cell lines (Fig. 33).

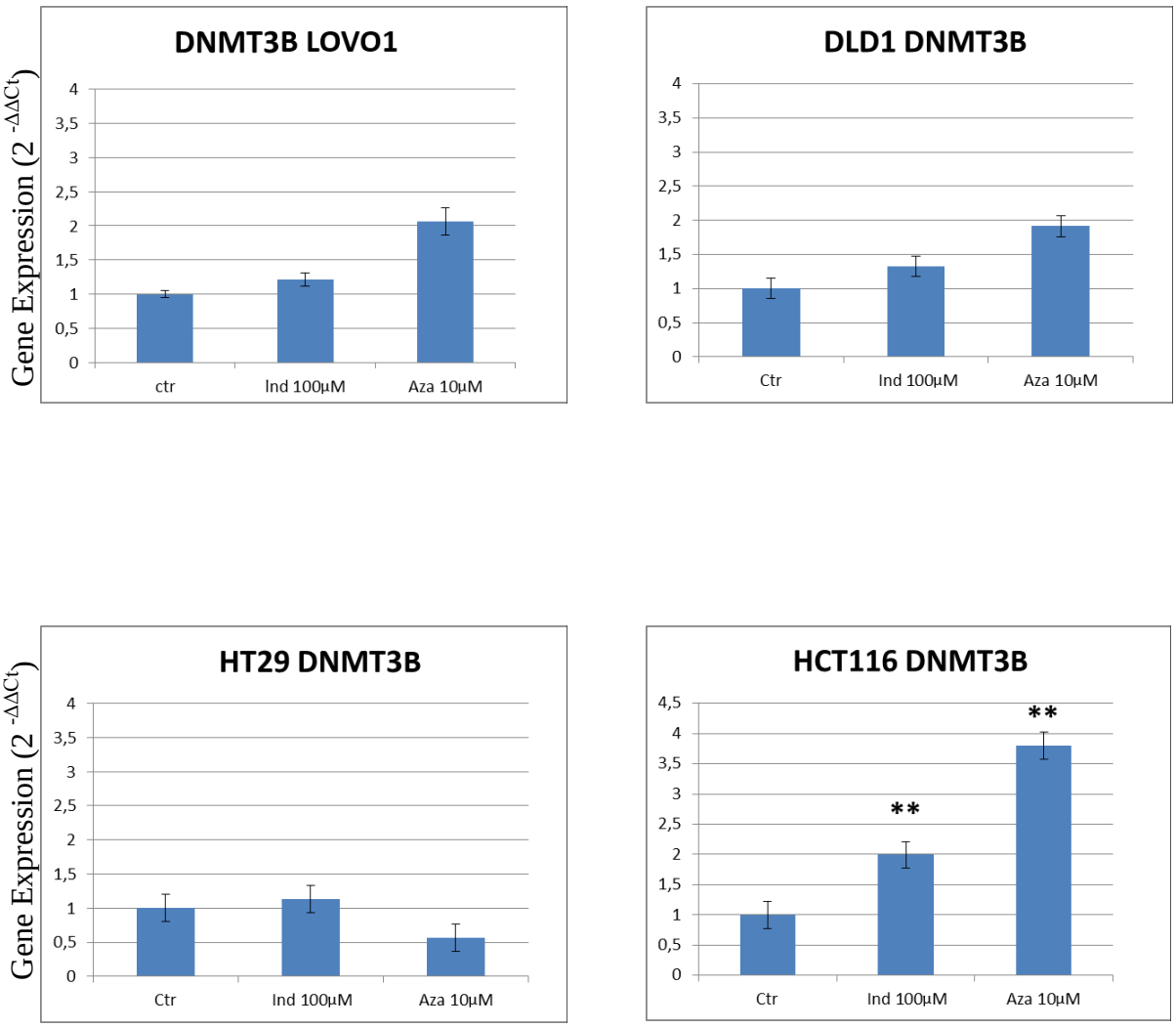


Fig. 34 - DNMT3B gene expression by Real-Time Analysis. RNA was extracted from the different cell lines (Lovo1 DLD1, HCT116 and HT29) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, Y axis express $2^{-\Delta\Delta Ct}$ *(**) p value <0.02*

DNMT3B expression was only affected by Ind in HCT116 cells which showed a 2 fold increase in expression after 48h of treatment. No significant effect were observed in the other cell lines (Fig. 34).

4.14. Ind influence in DNMT3B splice variants expression

It is known that the expression of different DNMT3B splice variants is associated with changes in DNA methylation. Different isoforms of DNMT3B, resulting from alternative splicing and/or alternative promoter usage, have been reported (Wang et al., 2006, Ostler et al., 2007).

The effect of Ind on the expression of all DNMT3B and ΔDNMT3B variants was investigated by real-time PCR. However for clarity, only those results showing a significant change in expression after Ind treatment are shown (Fig. 35 and Fig. 36)

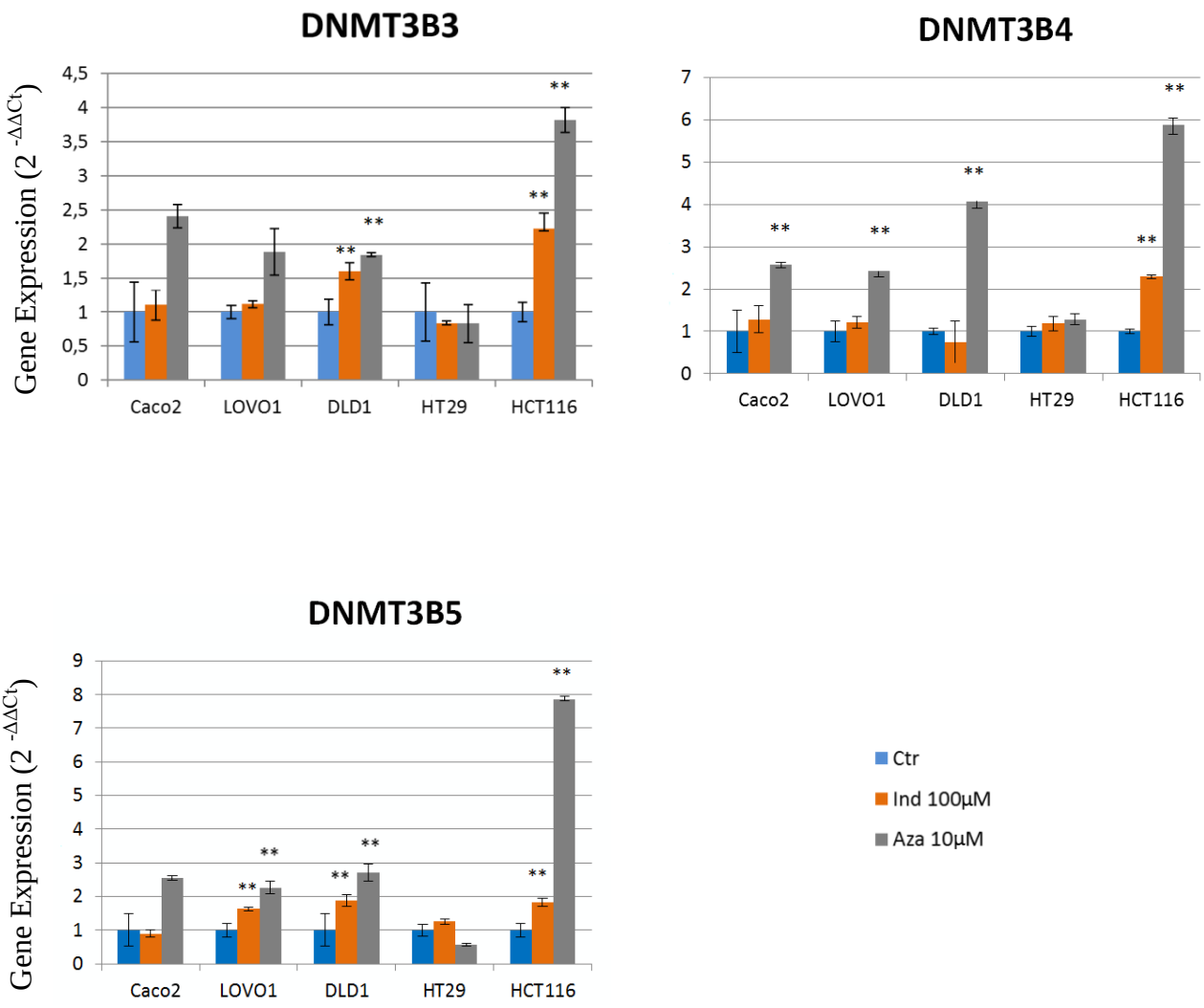


Fig. 35 - DNMT3B splice variants gene expression by Real-Time PCR analysis. RNA was extracted from the different cell lines (Caco2, LOVO1, DLD1, HCT116 and HT29) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, Y axis express $2^{-\Delta\Delta Ct}$ *(**) p value <0.02*

Fig. 35 shows that Ind affected the expression of the DNMT3B transcriptional variants lacking key regions of the catalytic domain (DNMT3B3, 3B4 and 3B5). Ind increased the expression of DNMT3B3 in DLD1 (1.6 fold) and HCT116 (2.2 fold) cells. DNMT3B4 expression was increased only in HCT116 cells by 2.3 fold. The expression of DNMT3B5 was significantly increased by Ind in LOVO1 (1.7 fold), DLD1 (1.9 fold) and HCT116 (1.9

fold) cells. No effect of Ind on the expression of these variant was observed in Caco2 or HT29 cells.

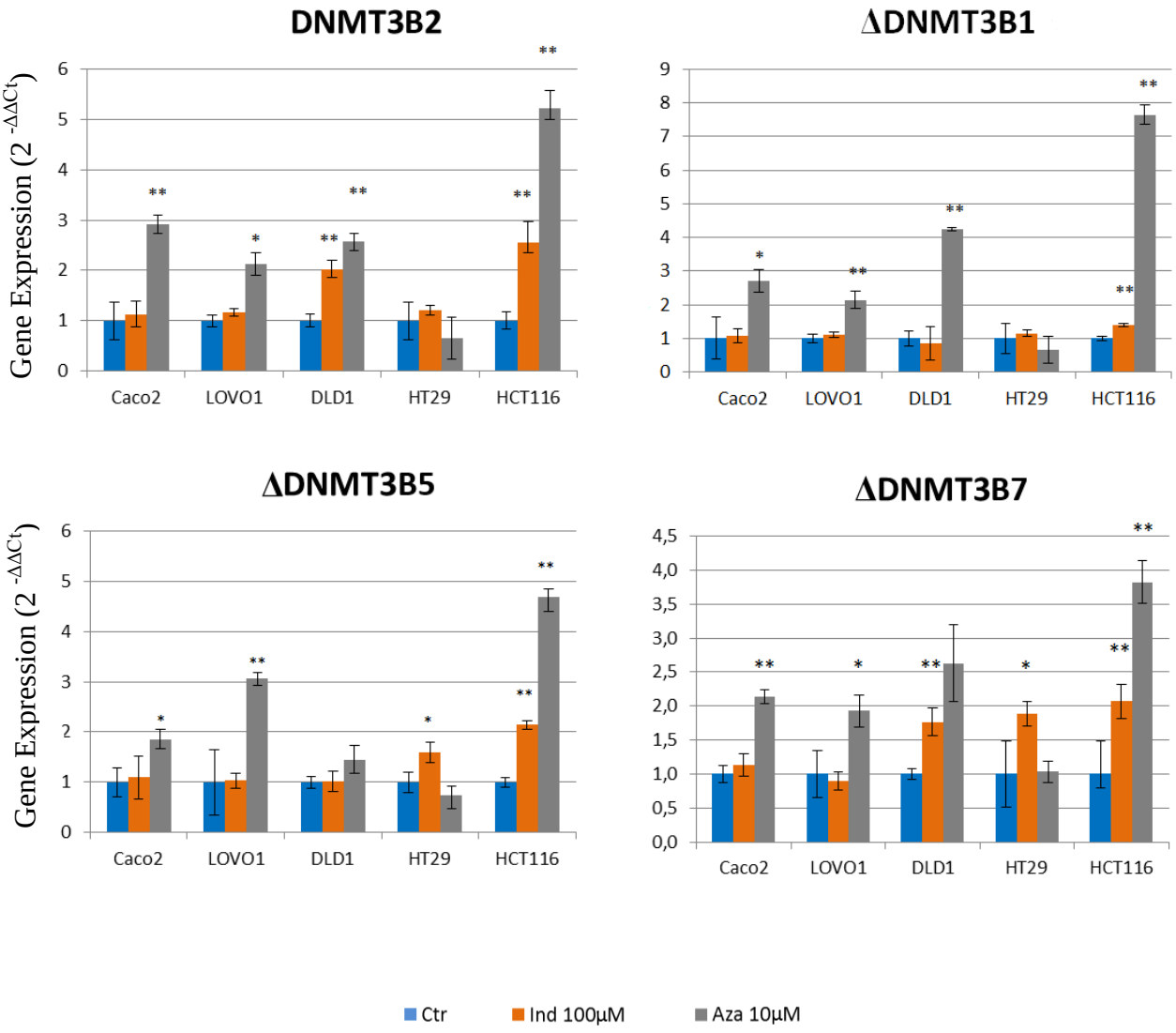


Fig. 36 - DNMT3B splice variant gene expression by Real-Time PCR analysis. RNA was extracted from the different cell lines (Caco2, LOVO1, DLD1, HCT116 and HT29) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, converted in Y axis express $2^{-\Delta\Delta Ct}$ *(*) p Value <0,05 (**) p Value <0,002*

The effects of Ind on the expression of DNMT3B variants lacking the regulatory domain (ΔDNMT3B1, ΔDNMT3B5, ΔDNMT3B7) or containing a mutant version of this domain (DNMT3B2) is shown in Fig. 36 Ind induced a significant increase of DNMT3B2 expression in DLD1 (2 fold) and HCT116 (2.5 fold) cells. ΔDNMT3B1 expression was only significantly increased in HCT116 (1.3 fold) cells while ΔDNMT3B5 expression was significantly

increased in HT29 (1.6 fold) and HCT116 (2.1 fold) cells. The expression of ΔDNMT3B7 was significantly increased by Ind treatment in DLD1 (1.7 fold), HT29 (1.8 fold) and HCT116 (2.1 fold) cells. Azacytidine increases the expression of these DNMT3B transcriptional variants in all the cell lines expected HT29, suggesting that the DNMT3B gene may be subject to its demethylating effect.

4.15.Ind influence on demethylase expression

DNA demethylation may take place as a passive process due to a lack of maintenance methylation during several cycles of DNA replication, or as an active mechanism in the absence of replication.

Several proteins were identified to be involved in this process by a number of different mechanisms that leads to a demethylation of 5-methylcitosine.

In order to assess whether Ind is able to induce an alteration in DNA demethylase mRNA expression, which may explain the effects of Ind on DNA methylation in these cell lines, the effect of Ind on the mRNA expression of members of the TET family, MBD4 and GADD45A was investigated.

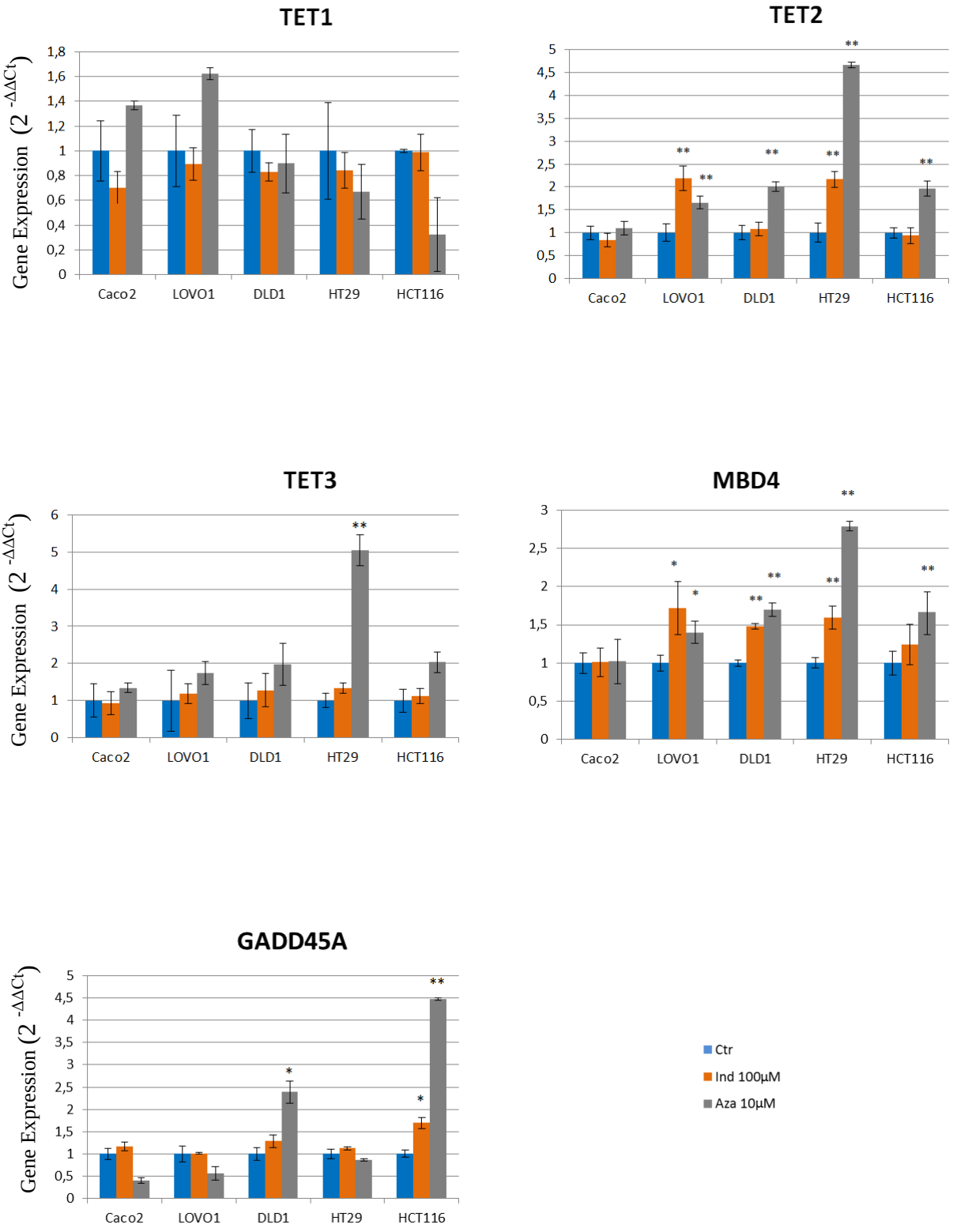


Fig. 37 - DNA Demethylase gene expression by Real-Time PCR analysis. RNA was extracted from the different cell lines (Caco2, LOVO1 DLD1, HCT116 and HT29) treated with 0µM, 100µM Ind and 10µM Azacytidine for 48h, Y axis express $2^{-\Delta\Delta Ct}$ *(*) p Value <0,05 (**) p Value <0,002*

Fig. 37 shows that Ind treatment lead to an increase in TET2 expression in LOVO1 (2.2 fold) and HT29 (2.2 fold) cells, an increase in MBD4 expression in LOVO1 (1.7 fold), DLD1 (1.5 fold) and HT29 (1.6 fold) cells and an increase in GADD45A expression in HCT116 (1.6 fold) cells.

5. DISCUSSION

The designation of “cancer” was first used by Hippocrates in reference to the crab-like appearance of certain tumours. Known as the father of Western medicine, he also coined the phrase, “let food be thy medicine, and medicine be thy food,” demonstrating his respect for the relationship of diet to human health.

Dietary phytochemicals are currently investigated as potential chemo preventive and/or chemotherapeutic agents and mechanisms involved in their effects actively researched. There is experimental evidence that these compounds can counteract cancer-related epigenetic changes, which finally may prevent or revert cell alterations that lead to abnormal cell growth (Fang et al., 2007; Fang et al., 2005)

Additional findings involving the role of numerous phytonutrients in the chemo-prevention of colon cancer are available. The colon is an attractive model for such studies because the incidence of this kind of cancer is inversely related with the consumption of fruits and vegetables (Voorrips et al., 2000) and because given the accumulation and bioavailability of these compounds after ingestion, the potential activity of these dietary components may be higher at the gastrointestinal (GI) tract, due to the direct exposure of the intestinal epithelia to higher doses of dietary compounds compared to most other tissues that often experience lower or much lower levels of exposure.

In this context this study investigated the activity of a singular dietary phytochemical: Indicaxanthin, a betalain from the edible fruit of the cactus *Opuntia ficus indica*. Its intestinal absorption does not require metabolic transformation and results in a high bioavailability in humans (Tesoriere et al., 2008; Tesoriere et al., 2004), molecule uptake by cells has also been observed after ingestion in humans and rats (Tesoriere et al., 2005, Allegra 2013).

5.1. Anti-proliferative effects of Ind in tumour cell lines

The anti-proliferative effects of Ind have been investigated in a number of transformed cells from human tumours of epithelial origin, i.e. HUH-7, HEPG-2, Ha22T, MCF-7 and HeLa, representing cells from liver, breast and cervix cancer, the growth of which did not appear to be, or was less affected in comparison with the colorectal cancer cell line Caco2, at least under comparable assay conditions. Therefore the activity of Ind was also investigated in other colorectal cancer cell lines i.e. LOVO1, DLD1, HT29, HCT116, in order to evaluate the effect of Ind on a broad spectrum of cells derived from the same tissue. Ind has an antiproliferative effect on several of the colorectal cancer cell lines although the magnitude of the effect differs between them. In fact, in the context of colorectal carcinoma cell analysed Ind has a higher anti-proliferative effect in Caco2 and then gradually less powerful in LOVO1 cells, still less powerful in DLD1 and HCT116 cell lines and is ineffective against HT29 cell proliferation. This may be due to the fact that, despite the same tissue of origin, these cells vary widely both in their cytogenetic features i.e. aneuploidy: in fact, Caco2 cells show polyploidy possessing 96 chromosomes in comparison to the other cell lines that present pseudodiploid (DLD1) or hyperdiploid chromosome sets (LOVO1 and HT29) and in their genetic profiles. The mutational profile of these cell lines varied significantly but perhaps the most interesting variation in the context of this study was the mutation of the *RAS* oncogene.

Activating point mutations in the genes encoding the RAS subfamily of small GTP binding proteins contribute to the formation of a large proportion of human tumours. RAS proteins are involved in the signal transduction of a number of signalling pathways to regulate various cellular functions such as cell growth, differentiation and apoptosis, as well as cell migration. Constitutive activation of RAS proteins provides a major contribution towards the establishment of the transformed phenotype and the fully malignant state (Downward 2003). Removal of RAS oncogene from human tumour cell lines results in reversal of transformation

(Shirasawa 1993 Chin et al., 1999), suggesting that these tumours can show RAS "oncogene addiction" (Sharma et al., 2006) and making RAS an attractive target for tumour therapy.

"Oncogene addiction" is a term that was first coined by Bernard Weinstein to describe the apparent acquisition of dependency by tumour cells on a single oncogenic activity, despite its plethora of genetic alterations (Weinstein, 2002). This phenomenon has been most clearly illustrated in several different transgenic mouse models of tumorigenesis, and is characterized by the proliferative arrest, differentiation, and/or apoptosis of tumour cells upon the acute inactivation of an oncogene that initially contributed to the tumour phenotype. A profound implication of this hypothesis is that switching off this crucial pathway upon which cancer cells have become dependent should have devastating effects on the cancer cell while sparing normal cells that are not similarly addicted. This, of course, is the discriminating activity required for any effective cancer therapeutic. RAS proteins themselves have so far proved difficult to inhibit selectively using drugs, so attention has shifted to targeting the downstream signalling pathways controlled by RAS, which contain several more tractable enzymes, such as RAF, MEK and AKT protein kinases and the lipid phosphoinositide (PI) 3-kinases (PIK3CA).

Colorectal cancer cells considered in this work differ for mutations in *KRAS*, *BRAF* and *PIK3CA* genes. In particular HT29 has mutation only in BRAF gene, showing a dependence upon or "addiction" to the ERK1/2 pathway (Solit et al., 2006). HCT116 and DLD-1 cells possess the same KRAS ($KRAS^{13D}$) and PIK3CA mutations to which they are addicted for maintenance of their tumorigenic phenotype (Shirasawa et al., 1993). LOVO1 show KRAS mutation as well, to which they are addicted, but they also show a wild type genotype for the *PIK3CA* gene. None of these genes are mutated in Caco2 cells.

Engelbrecht et al., studying proanthocyanidin chemo preventive effects from grape seeds, against Caco2 cells, showed that GSPE exerts its antiproliferative effects by means of

suppression of the important PI3-kinase survival-related pathway (Engelbrecht et al., 2007). In this context it is reasonable to suppose that Ind can act similarly. In fact, it exerted a stronger action on those cells that contain a wild type *PIK3CA* gene (Caco2 and LOVO1). In the cell lines containing mutations in the PIK3CA pathway, Ind had a weaker action, due probably to activation of compensatory signalling by other pathways, for example by the constant activation of the ERK1/2 pathways by RAS mutation (DLD1 and HCT116). Ind had no effect on cells containing mutated BRAF (HT29). It is well known that differences in *KRAS* or *BRAF* mutation leads to a different cell sensitivity, for example, to MEK1/2 inhibitors (Balmanno et al., 2009); in the same way these cells may be less sensitive to Ind due to the different gene mutations they carry.

In this perspective, one might assume for the Caco2 and the LOVO1 a dependence upon PI3-K/PKB signalling pathway, as Engelbrecht et al., supposed PI3-K/PKB signalling pathway as a potential therapeutic target (Engelbrecht et al., 2007).

Therefore it would be interesting for future studies to be directed towards the effect of Ind on the activation of different pathways (i.e. the PIK3CA pathway) in these cells, to further elucidate the biochemical mechanisms responsible for its activity.

Furthermore in light of the different set of chromosomes that distinguishes Caco2 from the other cell lines studied in this work, the higher antiproliferative effects of Ind in Caco2 compared to the other cell lines, suggests that Ind could acts by altering specific genes important for survival. Alteration of these genes in a cell line with significant genomic instability such as Caco2 could be more efficient compared with the other cell lines.

5.2. Pro-apoptotic effects of Ind in Caco2 cell line

Reactive oxygen species (ROS) have been proposed as second messengers in the activation of several signalling pathways leading to mitogenesis or apoptosis (Forman et al., 2002).

Redox-sensitive cysteine residues are known to sense and transduce changes in cellular redox status caused by the generation of ROS (Barford 2004). The effective transmission of information requires specificity, and how ROS signalling occurs with specificity and without oxidative damage remains poorly understood.

The pro-inflammatory micro-environment that drives many tumour types also contains free radicals produced by neutrophils, macrophages, endothelial and other cells. ROS such as $^{\cdot}O_2$, $^{\cdot}OH$, H_2O_2 can react with cellular biomolecules such as nucleic acids, enzymes, carbohydrates, and lipid membranes, causing cellular and tissue damage, which in turn augments the state of inflammation.

In searching for mechanistic aspects of the regulation of cancer prevention and therapy by phytochemicals, generation of high levels of ROS and oxidative stress in cancer cells, with DNA damage and apoptosis, has been demonstrated under certain circumstances. This activity, however, may be potentially harmful for healthy cells (Nair et al., 2007). Sporn and Liby noted that the principal need in the chemo-prevention of cancer remains the discovery of new agents that are effective and safe, that will allow their beneficial use, in a manner that is essentially free of undesirable side effects (Sporn and Libynoted 2005). In this work Ind inhibited growth and promoted apoptosis of proliferating Caco2 cells in a concentration-dependent manner. In addition, measurements of ROS levels in the treated cells showed that these effects did not depend on modifications of the cell redox environment or pro-oxidative activities. Importantly, the data presented also show that Ind did not affect survival of post-

confluent differentiated intestinal-like cells, at least at the assayed concentrations, providing evidence of its safety and suggesting that Ind could exert selective anti-cancer effects acting at the level of modified regulatory mechanisms in the transformed cells.

5.3. Epigenetic effects of Ind in Caco2 cells: reversal of hypermethylation and reactivation of *p16*^{INK4a}

Epigenetic alterations have been identified as promising new targets for cancer prevention strategies as they occur early during carcinogenesis and represent potentially initiating events for cancer development. Changes in DNA methylation have been recognized as one of the most common molecular alterations in human neoplasia and hypermethylation of gene-promoter regions is being revealed as one of the most frequent mechanisms of loss of gene function (Bird 2002).

In general, the majority of the short CpG-rich sequences in gene promoter regions, the CpG islands, are unmethylated in normal cells (Suzuki 2008) while most of other CpG dinucleotides are methylated, mainly where CpG density is low.

Methylation of CpG sequences in the promoter of *p16*^{INK4a} gene, a tumour suppressor gene implicated in cell cycle control and in apoptosis (Esteller 2002), has been reported in approximately 30% of colorectal tumours, and observed in the adenocarcinoma Caco2 cell line (Lind et al., 2004).

Genome analysis performed in Caco2 cell line, showed that Ind treatment produced clear epigenetic effects. These are evident as both a global variation of DNA methylation state and a specific and reversal of the hypermethylation of the onco-suppressor *p16*^{INK4a} gene, latter accompanied by re-expression of mRNA and accumulation of p16^{INK4a} protein. Apparently, whereas changes on the global DNA methylation were observed after cells treated with Ind

concentrations as low as 10 μM, loss of methylation of the *p16*^{INK4a} gene was not apparent after a 48 h incubation with this amount. However, the highly sensitive RT-PCR revealed production of mRNA suggesting that at least a partial gene demethylation and reactivation had occurred even under these conditions.

The *p16*^{INK4a} gene codes for the 16 kDa protein p16^{INK4a} which is a major inhibitor of cell cycle progression (Serrano 1995). The protein inhibits the formation of the cyclin dependent kinase D1-CDK4/6 complex thus preventing phosphorylation of the tumour suppressor complex retinoblastoma protein (RB)/E2F. On phosphorylation of RB, the released transcription factor E2F is allowed to activate S-phase essential genes thus promoting cell cycle progression (Weinberg 1995). In Caco2 cells the observed significant reduction of phosphorylated RB in Ind-treated cells appears to be consistent with a re-expressed and functional p16^{INK4a} protein. In addition, the expression of CDK4 in the Ind treated cells did not appear to be affected, substantiating that re-activation of RB was strongly associated with the p16^{INK4a} –dependent CDK4 inhibition.

A hypo-phosphorylated RB is usually associated with resting cells whose cycle is arrested in the G0/G1 phase (Ludlow et al., 1990, Ludlow et al., 1993). However, when the DNA content was evaluated by flow cytometry, despite the re-expressed p16^{INK4a} and reactivated RB, the transit from G1 to S-phase did not appear to be completely prevented, and Ind treatment of Caco2 cells resulted in a G2/M-phase cell cycle arrest. Compensatory mechanisms involving other cell cycle regulators, which would bring about alteration of the cell cycle dynamics (Santamaria et al., 2007), and/or eventual activity of Ind on other check points of cell cycle, cannot be ruled out. On the other hand, it has recently been reported that p16^{INK4a} over-expression can alter cell cycle distribution of malignant cells, with an S-phase lengthening, even in the presence of a hypo-phosphorylated RB (Chien et al., 2010).

Mechanisms by which Ind affected apoptosis in Caco2 cells were not investigated, and require other molecular studies; however the expression of $p16^{INK4a}$ and activation of RB may be involved in the effects observed. Besides being major controllers of the cell cycle, $p16^{INK4a}$ and RB possess multiple anti-tumour functions (Lu et al., 2012) and their activity in regulating cell death has been shown. Over-expression of $p16^{INK4a}$ has been associated with apoptosis of various transformed cells with down-regulation of the anti-apoptotic bcl-2 protein (Lu et al., 2012, Kataoka et al., 2000), and involvement of RB in apoptosis has been reported (Hilgendorf et al., 2013, Knudsen et al., 1999). Then, with reactivating the expression of the $p16^{INK4a}$ anticancer gene, and allowing the activation of RB downstream, Ind may have the potential of restoring cell mechanisms inducing Caco2 cells to undergo apoptosis.

Targeting epigenetic pattern is an important aspect to take into consideration as an advantage in the study of phytochemicals as chemo-preventive or chemotherapeutic agents; This is not only because epigenetic changes are reversible and can therefore potentially be prevented by specific natural phytochemicals, but it should be noted that epigenetic changes are stable over time.

Interestingly, even transient exposure to Ind in Caco2 cells, may induce long-lasting epigenetic changes in the promoter region of $p16^{INK4a}$ gene. In fact after 5 days $p16^{INK4a}$ promoter region is still demethylated, as this modification is maintained through cell generation.

Alteration of epigenetic gene regulation mechanisms, which persists over time should be an important topic to be considered in the concept of cancer prevention and cancer therapy, as changes in DNA methylation can be transmitted to the next generation of cells. Thus phytochemicals contribute not only to restore methylation patterns altered by malignant transformation events, but also long-lasting maintenance of this epigenetic status.

5.4. Ind Bioavailability

Biotransformation and bioavailability issues have frequently been raised in claiming potential activity of dietary phytochemicals in humans (Visioli et al., 2011, Yang et al., 2008). Clinical trials of natural compounds such as curcumin, lycopene and genistein, as chemo-preventive and therapeutic agents for cancer show poor bioavailability, which raises issues with extrapolation of *in vitro* results to physiological effects (Bemis et al., 2006; Amin et al., 2009).

Furthermore data from literature indicate the effects of several natural compounds depend on their concentrations. Folic acid is an excellent example; it was shown to reduce cancer cell growth, to demethylate and activate methylation silenced tumour suppressor genes at 0.88 mg·L-1 of media in Caco2 colon adenocarcinoma cells. However, a 10-fold higher concentration led to enhanced cancer cell proliferation, with concomitant increase in methylation of tumour suppressor genes (Berner et al., 2010).

In this study remarkable epigenetic effects have been elicited even by a single treatment of colorectal cancer cells (observed through different techniques MSRE and LINE1) with 10 μM Ind, an amount comparable with the level of Ind revealed in human plasma (7 μM) after a single ingestion of a dietary consistent amount of cactus pear fruits (four fruits, Tesoriere et al., 2004). Interestingly, when considering an intestinal volume of 600 mL and the stability of Ind in the gastro-intestinal conditions (Tesoriere et al., 2008), Ind solution in intestinal *digesta* from a single fruit serving size (149 g fruit pulp, yellow cultivar, 14.7 mg Ind) may be similar to the amounts observed to be active in this work (50 - 100 μM).

Moreover the effects of Ind on gene promoter methylation appear more pronounced at higher concentrations suggesting increased consumption may enhance its effects.

Furthermore, a key issue to decide about real beneficial effects of dietary phytochemicals on human health, is their bioavailability, which must be referred not only to the tested compounds but also to their metabolic products (Manach et al., 2005) Many effects exhibited by plant polyphenols *in vitro* are frequently diminished or even lost *in vivo*, due to incomplete absorption and first-pass metabolism (Williamson and Manach 2005).

A number of studies showed that Ind is bioavailable in humans and is not modified during absorption (Tesoriere et al., 2004). This is particularly important because it means that any effect after Ind treatment can be ascribed only to it without the involvement of any metabolites deriving from its metabolism.

5.5. Influence of Ind on global DNA methylation end specific genes methylation in different colorectal cancer cell lines

In this study Ind altered both global DNA methylation and genes-specific promoter methylation in colorectal cancer cell lines. However, marked changes in global DNA methylation are reported in those cell lines with a medium level of global DNA methylation (around 50%). HCT116 cells have a high level of global methylation which was not affected by Ind treatment. This may be explained by a high condensed chromatin structure. It is well known that specific histone modifications are associated with DNA hypermethylation. High DNA methylation levels are recognized by DNA methyl-binding proteins (MBD) which can interact with corepressor-associated enzymes (i.e. HDACs) the latter may, also be directly recruited by DNMT1,(Fuks et al., 2000) suggesting a tight interplay between histone deacetylation and DNA methylation, therefore linking DNA methylation and chromatin regulation (Perissi et al., 2010).

Before most activators of a gene can access their DNA-binding sites, a transition from a condensed heterochromatin (solenoid like fiber) to a decondensed euchromatin (beads on a string) structure appears to take place. Conversely, the acquisition of a more condensed heterochromatin structure is often associated with gene silencing (Chi et al., 2010). This structural restriction of silenced chromatin on gene expression can be overcome by chromatin cofactor complexes, which remodel nucleosomes along the DNA. Histone modifying complexes have to be recruited to relevant genomic locations by DNA-binding proteins, RNAs or protein-RNA complexes that bind to their specific DNA sites as a consequence of their own binding specificities (Gupta et al., 2010) It cannot come from the enzymatic activities per se as neither DNMTs, nor enzymes which modify histones, know which part of the genome needs to be tagged; (Hervouet et al., 2009). In light of complex chromatin regulation, that need specific DNA binding protein to be accessible; depending on the mechanism adopted by Ind inducing epigenetic modification, it appears difficult for this phytochemical to induce alterations in DNA methylation in highly condensed chromatin structures.

Various nutritional natural compounds (including epigallocatechingallate(EGCG), resveratrol, genistein, curcumin, isothiocyanates) were found to interfere with the enzymatic activity of DNMT, HDAC and HAT. DNMT inhibitors work mainly through one of the following mechanisms: non-covalent blocking of DNMT catalytic active site (i.e. EGCG) (Lee et al., 2005), covalent trapping of DNMT through incorporation into DNA (i.e. nucleoside analogues decitabine, 5-Azacytidine), interruption of binding site of DNMT to DNA (i.e. procaine), degradation of DNMT (i.e; decitabine), or suppression of DNMT expression (i.e. miRNAs).

In this study both the inhibitory action of Ind on DNMT (Fig. 27) and its molecular bond with DNMT1 were analysed (Fig. 28). This showed that, as for the well-known natural

product EGCG which inhibits DNMT activity through a specific binding to DNMT1, Ind also induced a reduction of DNMT activity. Furthermore, performing the same analysis carried out for molecular EGCG, Ind is able to stably bind DNMT1 at the catalytic site. Therefore it is reasonable to suppose that Ind may be defined as a DNMT inhibitor, through non-covalent blocking of the DNMT1 catalytic active site.

5.5.1. Comparison between global methylation and gene specific methylation after Ind treatment

It is well known that global loss of DNA methylation at repetitive genomic sequences (DNA hypomethylation) during carcinogenesis has been associated with genomic instability and chromosomal aberrations and was first described about 30 years ago (Goelz et al., 1985). In contrast increased methylation (DNA hypermethylation) of promoter CpG island (CGI) leads to transcriptional silencing of tumour suppressors and other genes with important biological functions (Jones et al., 2007, Kopelovich et al., 2003).

With respect to Caco2 cells, which showed the higher phenotypic effect of Ind on proliferation; an overview of Ind effects on global methylation and on individual genes methylation shows that Ind induces an overall increase in methylation in contrast to a decrease of promoter genes methylation (Fig. 26) except for *DNMT3B* gene which change in methylation status will be discussed later. This overall Ind effect suggests that the phytochemical generally works against the normal arrangement of tumour progression.

5.6. Ind influence on genes methylation in different colorectal cancer cell lines

It is known that the regulation of expression of some genes is governed by complex chromatin structures: an example may be the *SFRP1* gene. It has been shown that treatment of colorectal cancer cells with polyamine analogs that inhibit LSD1, an amine oxidase that demethylates mono-methylated and di-methylated H3K4 as part of a multiprotein co-repressor complex leads to re-expression of *SFRP1* and to an increase of activating histone marks at the promoter of *SFRP1*. Moreover, proliferation reductions were observed when combined treatments were carried together with DNA demethylating agents (Huang et al., 2009). This suggests that the inefficiency of Ind in inducing methylation alteration in some genes can be attributed to the complex chromatin structures of their promoter. It is shown in fact that Ind is ineffective in changing DNA methylation on the promoter of *SFRP*1 and *HPP1*genes that carry a high methylation level, in all cell lines studied.

Following the theory that Ind may act as a DNMT inhibitor, as explained above, it might be reasonable to suppose that it may lead to broad global effect on gene methylation. This global effects could be a concern using an epigenetically active molecule. However it has been shown that different DNMTs are responsible for targeting DNA methylation to specific regions of the genome (Choi et al., 2011), so methylation induced by DNMTs is not an event widespread throughout the genome, but it may occur in specific regions by specific DNMTs. Then methylation alteration induced by Ind may affect particular genes rather than others.

In particular with regard to $p16^{INK4a}$ gene in Caco2 cells using two different methodologies, demethylation induced by Ind was confirmed; moreover HCT116 cell line showed demethylation in that region after Ind treatment. However, $p16^{INK4a}$ in LOVO1 and DLD1 cells was not affected in gene methylation. Previously other studies have demonstrated

that treatment with EGCG induces demethylation and re-expression of $p16^{INK4a}$ in HCT116 cells (Fang et al., 2003), these results could not be confirmed in HT29 cells, concluding that different cell lines have different sensitivities to EGCG (Chuang et al., 2005). Again in analogy to EGCG, Ind effects may be dependent on the cells analysed.

5.6.1. WNT5A Gene

WNT pathways play key roles in colonic stem cell maintenance and epithelial cell proliferation during normal gut development and tissue homeostasis in adults (Gregorieff and Clevers 2005) On the other hand, aberrant WNT signaling is considered one of the most correlated factors in over 90% of both benign and malignant colorectal tumors (Giles et al., 2003). The WNT5A ligand is generally considered an antagonist of WNT pathway. It can act through different membrane receptors and participate in both canonical and non-canonical WNT pathways (Mikels and Nusse 2006), therefore representing an important target for WNT pathway regulation. It has been shown that WNT5A down-regulation is correlated with hypermethylation of its promoter in human colon cancer patients (Hibi et al., 2009). Wang and Chen reported that WNT5A promoter methylation correlated with lowest expression and treatment with genistein increased WNT5A mRNA levels, accompanied by a decrease in WNT5A promoter methylation (Wang Z and Chen H 2010). In this work Ind induced a significant and consistent demethylation in WNT5A promoter region, with the greatest effect in those cell lines which were more sensitive to anti-proliferative effects of Ind. Although mRNA expression was not evaluated, it could represent a starting point for further investigations on the mechanism of action through which Ind exerts its anti-proliferative effect.

5.6.2. GATA4 Gene

GATA factors are a family of transcription regulatory proteins. GATA4 and GATA5 play an essential role in the development and differentiation of the gastrointestinal tract and are suggested to be involved in colorectal cancer development (Molkentin 2000). GATA4/5 would be more likely to behave as tumor suppressor genes since increased expression levels correlate with terminal differentiation in intestinal epithelium (Hoque et al., 2006) and terminal differentiation induced in colorectal cancer cells by sodium butyrate (Molkentin 2000). Loss of GATA4/5 expression due to promoter hypermethylation has been reported in primary colorectal tumours (Akiyama et al., 2003, Fu et al., 2007) and it was demonstrated that methylation of GATA4 is an early event in colorectal carcinogenesis (Hellebrekers et al., 2009). Importantly, GATA proteins are transcriptional activators for a number of proposed antitumor genes. Interestingly, in colorectal cancer cells with epigenetic silencing of GATA4, a series of downstream GATA target antitumor genes are silenced with associated epigenetic silencing marks at their promoters. Both the upstream and the downstream genes are simultaneously reactivated by drug and genetic demethylating strategies, suggesting that promoter methylation of GATA is an interesting target (Akiyama et al., 2003). Therefore demethylation of GATA4 promoter region induced by Ind sheds new perspective in how Ind exerts its effects. In fact further and future analysis on the basis of GATA4 demethylation induced by Ind, investigating the re-expression of GATA4 and consequently re-expression on its downstream targets may promote our understanding of Ind's mechanism of action.

5.6.3. NOD2 Gene

Previous data regarding Ind potential anti-inflammatory effects shows that Ind is able to modulate inflammatory processes at the intestinal level. In fact it is able to inhibit the release of inflammatory mediators like pro-inflammatory cytokines IL-6 and IL-8 and NO and prevents the activation of NOX-1 and nuclear factor κB (NF-kB) in Caco2 cells stimulated by IL-1b. Similar results were obtained *in vivo* in carrageenin induced rat pleurisy (Tesoriere et al., 2013;Allegra et al., 2013)

In light of these data, this study investigated whether anti-inflammatory effects by Ind could be associated with the alteration of methylation of sequences upstream of *NOD2* gene, which encodes a receptor protein involved in inflammatory signalling. When ligands interact with cytoplasmic receptor NOD2 (nucleotide-binding oligomerization domain 2) it triggers host defence pathways, including activation of NF-κB signalling, which lead to inflammatory immune responses. Deregulation of NOD2 signalling is associated with inflammatory diseases, such as Crohn's disease, and Crohn's disease is associated with a higher risk of colorectal cancer (Lubiński et al., 2005).

In this work Ind induced in Caco2 cells a significant demethylation of CG residue upstream of the *NOD2* gene. It should be highlighted that these residues are not in CpG islands; thus it is possible that the effect of Ind in these regions should be similar to the effect of Ind on LINE1 methylation. Surprisingly the effects of Ind on *NOD2* methylation were inversely correlated with the effects on LINE-1 methylation. Ind (10μM) induced demethylation of *NOD2* but increased the methylation of LINE-1.

This study highlights for the first time that methylation of LINE1 and methylation of CG residues upstream of *NOD2* are inversely related; and that they respond in a opposite manner to phytochemicals that affect DNA methylation.

Onset and progression of inflammatory responses are complex and dynamic processes, involving pathophysiological mechanisms with a number of biochemical reactions.

These studies for the first time give an indication that Ind could modulate the inflammatory response by altering epigenetic patterns. However, there is no evidence establishing the influence of the CG residues upstream of the *NOD2* gene on gene expression.

Recent studies have reported that Ind affects COX2 and iNOS gene expression and prevented NFkB activation, suggesting molecule intervention of inflammatory pathways lead to the evolution of the inflammatory response (Allegra et al., 2013). Interestingly it was demonstrated that depletion of NFκB can also trigger DNA demethylation and gene reactivation, illustrating gene-specific epigenetic effects which may further depend on post-translational NFκB modifications (Dong et al., 2008). This demonstrates that cancer-inflammation pathways and transcription factors are able to rewire epigenetic settings and amplify gene expression in an autocrine fashion (Liu et al., 2008).

How Ind carries out its anti- inflammatory functions, through such mechanisms, is not yet clear and further studies are warranted. However the indication that Ind treatment leads to methylation alteration of upstream sequences of genes involved in inflammatory response sheds light to new possible future studies on the effect of Ind in epigenetic modifications that occur in other genes involved in the inflammatory response.

5.6.4. ESR1 Gene

ESR1 gene encodes an estrogen receptor, in particular, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. Estrogen and its receptors are essential for sexual development and reproductive function but are also involved in pathological processes including colon cancer. ESR1 mediates the biological function of the steroid hormone 17 β-oestradiol, which is a critical regulator of growth, differentiation and function in a wide variety of tissues including the colonic epithelium.

ESR1 appears to fulfil all traditional criteria for a tumour suppressor gene. It is expressed in normal tissue, molecularly altered in tumours, not expressed in tumour tissue and cell lines, and finally, it suppresses growth in colon cancer cell lines (Issa et al., 1994). The putative tumour suppressor gene *ESR1*, contains a CpG island (CGI) in exon 1 which, when methylated, leads to gene-silencing. In cultured colon cancer cells, methylation-associated loss of expression of *ESR1* gene results in deregulated growth (Issa et al., 1994). In addition *ESR1* gene methylation appears to be an early event in colorectal tumorigenesis. (Issa et al., 1994). Issa et al., found a partial methylation of *ESR1* gene in normal colonic mucosa and a complete methylation of this island in several uncultured colonic tumours. Moreover they report that ESR CGI methylation arises as a direct function of age in normal colonic mucosa (Issa et al., 1994).

Evidences suggest that individuals may be prone to methylation alterations as a consequence of genetic, dietary or environmental exposures (Ricciardiello et al., 2003). Furthermore it has been demonstrated that supplementation with dietary folate accelerates age-dependent methylation of the *ESR1* CGI in mouse colon (Belshaw et al., 2005).

Thus this gene may be an excellent target in the study of chemo-preventive phytochemicals action. In fact prolonged use of phytochemicals which may alter methylation of *ESR1* CGI, could prevent its abnormal methylation. This methylation has been demonstrated through a "all or nothing" mechanism which means *ESR1* CGI becomes methylated in an allele-specific, rather than a random, manner in the human colon and this can lead directly to aberrant gene silencing (Belshaw et al., 2005). Therefore cancer-preventive abilities of several bioactive food components have been linked to their estrogenic and epigenetic activities like folic acid, zebularine, resveratrol, genistein and EGCG that regulate *ESR1* and other tumour suppressor gene expression by epigenetic processes (Berner et al., 2010).

This study demonstrates that Ind altered methylation of *ESR1* CGI. This finding raises another point in favour of Ind chemo-preventive action. Although further studies on gene expression in all cell lines analysed would help to understand the relationship between methylation and expression in each cell line, as well as further studies on the activation of the mechanisms downstream of ESR1 transcription factor would be interesting to better investigate the role of ESR1 in tumorigenesis as well as Ind chemo-preventive action.

5.7. Ind influences on DNMT expression in colorectal cancer cell lines

Aberrant expression of DNMTs and their isoforms has been found in many types of cancer, and their contribution to aberrant DNA methylation has been proposed. Following this hypothesis, the effect of Ind on the expression of DNMT, was investigated. The expression of DNMT increased appreciably only in some cell lines with respect to only some DNMT genes after Ind treatment. However, previous studies demonstrated that frequent hypermethylation of CGI (CpG islands) in human colorectal tumors did not result from a simple transcriptional

up-regulation of any DNMT, due to the fact that CGI hypermethylation did not correlate with the expression of any DNMT (Eads et al., 1999; Saito et al., 2002; Ehrlich et al., 2006). Thus, other factors regulating the activity of the DNMTs, either by interacting with the enzymes themselves or by regulating access to the DNA substrate, are important to establish an aberrant methylation pattern rather than the mere up-regulation of DNMT levels in the cell.

This work confirms Eads's results, in fact ANOVA analysis used to compare *DNMT3B* gene expression to percentage of methylation both of genes and LINE-1, provide no correlation between expression and methylation in any of the cell lines analysed.

In this respect, in this study it was demonstrated that epigenetic effects of Ind are not mediated by a direct alteration of DNMT expression, rather by an influence of Ind on their activities.

In this perspective, Ind binding the DNMT and altering their methyltransferase function could cause an imbalance of global methylation. Ind could lead, for example, to demethylation and re-expression of important transcription factors. Ind, in fact, was able to induce a significant demethylation of *GATA4*, whose demethylation and possible re-expression, is known to correlate to reactivation of its downstream targets. Although there is no evidence linking *GATA4* demethylation and expression of DNMT the possibility that Ind induces demethylation and re-expression of other transcription factors that downstream targets *DNMT* genes cannot be ruled out.

Moreover the results from the effect of Ind on the expression of DNMT3B splice variants were interesting.

In recent years, reports have fuelled the hypothesis of a link between DNA structure and pre-mRNA processing. The evidence that exons carry elevated nucleosome density, DNA methylation of cytosine, and over-representation of certain histone modifications, relative to

introns raise the intriguing possibility that epigenetic modifications are maintained on DNA to aid the spliceosome in the process of exon definition (Tilgner et al., 2009). In particular several studies have shown that DNA methylation is substantially enriched at exons relative to introns (Luco et al., 2011) and this may guide splicing decisions. Indeed DNA methylation at exons may inhibit binding of proteins influencing pol II elongation dynamics (Shukla et al., 2011). Thus there is a correlation between epigenetic modifications and alternative splicing of pre-mRNA, indeed altered DNA methylation patterns can lead to alternative pre-mRNA splicing.

Here Ind treatment resulted in increased expression (probably indirectly) of some DNMT3B splice variants, raising the possibility that an imbalance in DNA methylation would lead to the altered expression of DNMT3B splice variants.

Although the mechanism by which the different DNMT3B isoforms act is not completely understood and remain largely unknown, their association with change in DNA methylation patterns is intriguing given that most of them are catalytically inactive (Ostler et al., 2007). Some of these variants were reported to be overexpressed in various cancers and to be associated with global changes in DNA methylation (Xie et al., 1999). ΔDNMT3B5 is upregulated in liver and skin cancer cell lines and its overexpression in HCT116 cells results in loss of DNA methylation at centromeric and pericentromeric repetitive elements (Gopalakrishnan et al., 2009).

Wang et al., demonstrated that DNMT3B subfamily, ΔDNMT3B, are involved in regulation of the specific de novo DNA methylation of some promoters rather than others (Wang et al., 2007).

Recent studies have shown that inactive DNMT3B isoforms (DNMT3B3 and DNMT3B4) can form complexes with, and modulate the activity of catalytically competent

DNMT3B or DNMT3A isoforms. However complex formation leads to a strong reduction in DNA binding and DNMT activity, causing a dominant-negative inhibition (Gordon et al., 2013). Saito et al., instead proposed the hypothesis that inactive DNMT3B4 might function by outcompeting active DNMT3B isoforms for targeting to DNA regions, resulting in DNA hypomethylation (Saito et al., 2002).

In addition to affecting intrinsic catalytic properties, it is possible that DNMT3B variants also modulate de novo DNA methylation indirectly through effects on chromatin condensation and modification. Some data suggest that expression of DNMT3B2 or DNMT3B3, but not DNMT3B4, drive chromatin condensation (Gordon et al., 2013).

Overexpression of DNMT3B7, a major splice variant found in numerous tumour types, triggers both global and local changes in DNA methylation patterns as well as chromosomal rearrangements (Ostler et al., 2007). At last over expression of DNMT3B7 in neuroblastoma cells leads to growth inhibition, increase in global DNA methylation and induce a less aggressive clinical phenotype (Ostler et al., 2012).

In light of this alteration of expression of some DNMT splice variants induced by Ind (supposed indirectly), may leads further to an imbalance in the genome DNA methylation pattern.

However, an association between expression level of DNMT3B and the promoter methylation status of some genes has not been established (Sato et al., 2002). On the contrary, several studies have shown that there is no correlation between increased DNMT expression and DNA methylation levels (Eads et al., 1999 Saito et al., 2002; Ehrlich et al., 2006). This study points out the effect of Ind on alteration in DNA methylation pattern; bringing out the complex mechanism underlying regulation of DNA methylation at some genes.

5.8. Ind influence on demethylase expression in colorectal cancer cell lines

It is widely accepted that active demethylation does take place in the genome. There are several studies suggesting different mechanisms through which demethylation at 5-methylcytosine (5mc) may occur. One of them is called “passive demethylation”, it does not involve any active enzymatic processes that alter the base itself; it is pertinent to describing the replication dependent loss of 5mC. Other “active demethylation” mechanisms involving TET protein and a number of DNA modifying enzymes like DNA cytosine deaminases that can introduce genomic mismatches, DNA glycosylases that can excise bases (MBD4), other DNA repair factors (GADD45) and even DNMTs themselves have been suggested to be involved in DNA demethylation.

Down regulation of TET expression has been observed in human breast, liver, lung, pancreatic and prostate cancers (Yang et al., 2013). TET mutations are consistently associated with a decrease in 5hmC (important intermediate in demethylation process), which has been suggested as a potential diagnostic biomarker (Ko et al., 2010) TET2 mutations have been demonstrated to be frequent lesions in myeloid lineage malignancies (Delhommeau et al., 2009).

In this study, Ind induced an increased expression of some enzymes with demethylating activity (TET2, MBD4) relatively only in some cell lines.

Although there are no data in the literature attesting an association between expression of demethylase enzymes and reduced genome methylation, in this study, the regression analysis, based on data of global methylation and demethylase gene expression, showed that there is no correlation between global methylation and expression of demethylase enzymes.

6. CONCLUSION

From this work it appears that the phytochemical Indicaxanthin certainly induces different effects (both epigenetic and biochemical) on colorectal cancer cell lines tested. However Ind does not act according to a simple and clear linear mechanism, rather it acts through a complex mechanism that involves both epigenetic alterations and alterations in gene expression of specific proteins. It is not easy to correlate epigenetic alterations induced by Ind to the phenotype found after Ind treatment i.e. reduction of proliferation, apoptosis and cell cycle arrest, through a well-defined mechanism. The involvement of other molecules that, induced by, or in concert with Ind, lead to the phenotype observed cannot be ruled out.

The effects brought by Ind, are often variable depending on the cell line used, such phenomenon can be attributed to the cell lines themselves; since, they are often maintained in culture for prolonged periods, presumably accrue genetic alterations and undergo genetic drift over time, resulting from the genomic instability inherent in cancer. Moreover, cell lines are subject to the artificial pressures imposed by growth under non physiological conditions. A study on the effects of Ind directly on patients with cancer, with respect to healthy subjects (for instance in a dietary intervention trial), could definitely clarify not only Ind chemo-preventive activity under physiological conditions, but may also rule out all discrepancies in the results observed in this study between different cell lines.

In assessing the effects induced by Ind, a parameter to be considered is the short period of time within which epigenetic effects are detected. Indeed many epigenetic changes require a period of "latency" that is longer than 48h. In this perspective long-lasting treatments (with a continuous administration of Ind) could lead to highlight further epigenetic changes that are not detectable within 48h (more marked changes in the same genes considered in this study or

in other genes); and/or even these modifications could induce further other alterations in gene expression.

Moreover Ind effects should be reviewed in light of the effects induced by 5-Azacytidine (Aza), considered as a positive control in this work.

Aza is a cytidine nucleoside analogue that becomes incorporated into newly synthesized DNA, where it binds DNA methyltransferases (DNMTs) in an irreversible, covalent manner (Santi et al., 1984). The sequestration of DNMTs prevents maintenance of DNA methylation state, leading to DNA hypomethylation (Haaf et al., 1995). Aza is now recognized as a demethylating agent, and its clinical activities are now established against acute myeloid leukemia.

Despite its proven demethylating ability, in this study, treatment with Aza, does not result always in an increased gene expression, and above all it does not lead to the same effects among the different cell lines analysed.

Nguyen et al., demonstrated that while Aza caused DNA hypomethylation, distinct action are established on gene expression (Nguyen et al., 2010). This suggests that in the complex mechanism of gene expression regulation there is not always a simple association: demethylation leads to increased expression. It is possible, for instance, that demethylation could lead to the expression of a negative regulator (or repressor), which eventually leads to a reduction of the expression of downstream genes. Thus as well as a demethylating agent (Aza) may lead eventually to a wide and diverse effect on gene expression, it is reasonable to suppose that Ind, albeit through a different mechanism of action, also leads to a variable response in gene expression.

In conclusion, this study highlights an Ind action against colorectal cancer cells. Although these effects may be ascribed more to chemo-preventive action of the phytochemical rather than a therapeutic activity.

In this respect, “Let food be your epigenetic medicine” could represent a novel interpretation of what Hippocrates said already 25 centuries ago, as food is now known as a conditioning environment in the prevention against cancer.

LIST OF ABBREVIATION

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethycytosine
5mC	5-methycytosine
AM	Active Modification
APC	Adenomatous Polyposis Coli
AR	Active Restoration
Arg	Arginine
Asn	Asparagine
Aza	5-Aza-2'deoxycytidine
BA	Betalamic Acid
CCSC	Colorectal Cancer Stem Cell
CDK	Cyclin-Dependent Kinase
CGI	CpG Island
CKI	Cyclin-dependent Kinase Inhibitors
COBRA	Combined Bisulphite Restricion Analysis
CRC	Colorectal Cancer
Cys	Cysteine
DCFDA	Dichlorodihydrofluorescein diacetate
DDD	Double Digested DNA
DiOC6(3)	3,3'-dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
DNMT	DNA cytosine methyltransferase
E2F	Elongation 2 Factor
EGCG	Epigallocatechin-3-gallate
FAP	Familial adenomatus polyposis
Gln	Glutamine
Glu	Glutamic Acid

Gly	Glycine
GSPE	Grape Seed Proanthocyanidin Extract
HAT	Histone acetiylase
HDAC	Histone deacetylase
HDMT	Histone- demethylases
IFD	Induced Fit Docking
IL-4	Interleukin 4
Ind	Indicaxanthin
LINE-1	Long Interpersed Nucleotide Elements- 1
MeSAP-PCR	Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction
MSRE-PCR	Methylation-Sensitive Restriction Endonucleases Multiplex-Polymerase Chain Reaction
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
PD	Passive Diluition
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-Kinase
PIK3CA	Phosphoinositide (PI) 3-Kinase
pRB	Retinoblastoma protein
Pro	Proline
ROS	Reactive oxigen species
SDD	Single Digested DNA
TDG	Thymine DNA Glycosylase
Thr	Threonine

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