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Original Paper

Phytochemical Indicaxanthin Inhibits Colon Cancer Cell Growth and Affects the DNA Methylation Status by Influencing Epigenetically Modifying Enzyme Expression and Activity

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Key Words
Colorectal cancer · Chemoprevention · Phytochemicals · Indicaxanthin · Epigenetics · DNA methyltransferase · Molecular modeling · Betalains

Abstract
Background: Recently, we have shown anti-proliferative and pro-apoptotic effects of indicaxanthin associated with epigenetic modulation of the onco-suppressor *p16^{INK4a}* in the human colon cancer cell line CACO2. In the present study, the epigenetic activity of indicaxanthin and the mechanisms involved were further investigated in other colorectal cancer cell lines. ***Methods:*** LOVO1, CACO2, HT29, HCT116, and DLD1 cells were used to evaluate the potential influence of consistent dietary concentrations of indicaxanthin on DNA methylation, and the epigenetic mechanisms involved were researched. ***Results:*** Indicaxanthin exhibited anti-proliferative activity in all cell lines but HT29, induced demethylation in the promoters of some methylation-silenced onco-suppressor genes involved in colorectal carcinogenesis *(p16^{INK4a}*, *GATA4*, and *ESR1)*, and left unchanged others which were basally hypermethylated *(SFRP1* and *HPP1)*. In apparent contrast, cell exposure to indicaxanthin increased DNMT gene expression, although indicaxanthin appeared to be an inhibitor of DNMT activity. Indicaxanthin also increased the expression of genes involved in DNA demethylation. Finally, an in silico molecular modelling approach suggested stable binding of indicaxanthin at the DNMT1 catalytic site. ***Conclusions:*** Our findings contribute to new knowledge in the field of phytochemicals and specifically suggest dietary indicaxanthin as a potential epigenetic agent to protect colon cells against tumoral alterations.

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Introduction

Epidemiological studies provide evidence that diet can play essential roles in reducing the risk of chronic diseases such as cancer [1]. Colorectal cancer is one of the most common forms of malignancy and the second leading cause of cancer-related death in the Western world [2]. 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 in preventive dietary and lifestyle practices [3]. A number of epidemiological data demonstrated that plant chemicals, consisting of a wide variety of biologically active compounds, have protective and therapeutic effects on several chronic diseases, including cancer [4]. In particular vegetables and fruits are excellent sources of cancer-preventive substances, and a number of cell culture and animal model studies have evaluated the ability of specific edible plants to prevent cancer [5]. Essentially, the concept of cancer chemoprevention by phytochemicals is to arrest or reverse the progression of premalignant cells towards full malignancy using physiological mechanisms. It is now well known that cancer initiation and progression are driven by changes in the expression of multiple genes via both genetic and epigenetic alterations [6]. Several experimental data show that a hallmark of cancer is global DNA hypomethylation alongside hypermethylation of specific regions, mainly within promoters of tumor suppressor genes [7]. For instance, it was demonstrated that methylation-associated loss of expression of the transcriptional factors $p16^{INK4a}$, GATA, and ESR1 is an early event in colorectal carcinogenesis [8–10].

DNA methylation in mammals is primarily mediated by the three well-characterized DNA (cytosine-5)-methyltransferases (DNMT) DNMT1, DNMT3A, and DNMT3B. These enzymes catalyze the transfer of a methyl group from the ubiquitous methyl donor S-adenosyl-L-methionine to the fifth position of a cytosine pyrimidine ring [11]. On the other hand, enzymes involved in methyl group removal have remained elusive until recently. In particular, it is known that enzymes, including ten-eleven translocation (TET) family enzymes, exist that can modify 5-methylcytosine, producing 5-hydroxymethylcytosine. The latter, as a key intermediate in demethylation pathways, may be a substrate of enzymes with demethylating activity, including methyl-CpG-binding domain protein 4 (MBD4) and growth arrest and DNA-damage-inducible protein alpha (GADD45A) [12]. 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 [13]. For instance, in epidermoid carcinoma cells, epigallocatechin-3-gallate (EGCG) decreased global methylation and inhibited DNMT activity as well as the expression of DNMT1, DNMT3A, and DNMT3B [14].

Betalains are water-soluble, nitrogenous pigments found only in some plants in the order Caryophyllales, with red beetroot *(Beta vulgaris)* root and cactus pear *(Opuntia ficus indica)* fruit as the main dietary sources. In recent years, the properties and bioactivities of indicaxanthin (Ind), a betalain highly concentrated in the cactus pear fruit, have been researched [15]. This molecule can behave as a radical scavenger and antioxidant [16–18], possesses physico-chemical characteristics allowing its interaction with and location in membranes [19], and shows anti-inflammatory activity in in vitro and in vivo models [20–22]. Ind has been shown to be stable under digestive conditions and is not metabolized by enterocytes, therefore being highly bioavailable in the gut in its native form [23]. Recently, it has been shown that Ind has an anti-proliferative and pro-apoptotic effect on the human colon cancer cell line CACO2. In the same cells, Ind was able to re-activate the expression of the onco-suppressor $p16^{INK4a}$ gene, inducing demethylation of its promoter region [24].

Here, using a panel of colorectal cancer cell lines, we evaluated the effects of Ind on DNA methylation, both at the genomic and the gene-specific level, together with its potential influence on DNMT expression and activity as well as on the expression of enzymes involved in methyl group removal.

Materials and Methods

Cell Lines and Treatment

All colorectal cancer cell lines used for the experiments (CACO2, LOVO1, DLD1, HT29, and HCT116) were obtained from the American Type Culture Collection (Rockville, Md., USA). All of them were epithelial adherent cells of human colorectal adenocarcinomas. They were cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 2 mM L-glutamine, 1% nonessential 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.

Anti-Proliferative Evaluation Assay

Cell sensitivity to Ind was evaluated by MTT assay. For the experiments, exponentially growing tumor cells were seeded into 96-well culture plates (Corning Costar Inc., Corning, N.Y., USA) at a density of 1.0×10^4 cells/cm^2, incubated for 24 h, and then treated with Ind (10–200 μM) for 48 h. Cells were considered suitable for the experiment at passage that did not exceed the number 20. 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 3 h at 37°C. Then, the medium was discarded, and the formazan blue formed was dissolved in DMSO. The absorbance at 560 nm of MTT-formazan was measured in a microplate reader (GloMax®-Multi Microplate Reader; Promega Corporation, Madison, Wis., USA); the values of cell viability are expressed as percentages of control (untreated cells).

Quantitative Methylation Analysis of Long Interspersed Nuclear Element 1

Genomic DNA from all the cell lines analyzed was extracted following a phenol-chloroform protocol. A NanoDrop spectrophotometer (Labtech International, Uckfield, UK) was used to quantify DNA concentration and purity by absorbance measurements at 260 and 280 nm. Up to 2 μg of genomic DNA was then converted with sodium bisulfite solutions and incubated at 50°C for 4.5 h. Following bisulfite conversion, the DNA was recovered and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's specifications [25]. Long interspersed nuclear element 1 (LINE-1) methylation, a surrogate for genomic DNA methylation status, was measured using a quantitative PCR approach as described previously [26].

Gene-Specific Methylation Analysis

The methylation status of specific genes was quantified by COBRA (combined bisulfite restriction analysis) using the PCR primers listed in table 1 [27]. The PCR conditions were as follows: 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, optimum annealing temperature for the primers (table 1) for 30 s, and 72°C for 1 min. The PCR products were digested with specific restriction enzymes, as shown in table 1. The digested PCR fragments were then separated on 5% polyacrylamide gels. The percentage of methylation was calculated by densitometric analysis of the band using TotalLab software.

Quantification of DNMT and Demethylase Gene Expression

RNA from different cell lines, treated with 0 or 100 μM Ind, was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A reverse transcription was performed from RNA to obtain cDNA using qSCRIPT cDNA SuperMix (Quanta Biosciences, Gaithersburg, Md., USA) through a single cycle: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

The cDNA was used as a template for the subsequent quantitative real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, Life Technologies, Paisley, UK). Relative changes in gene expression between control and treated samples were determined with the ΔΔCt method using the expression of the ribosomal subunit 18S as the reference gene. Final values are expressed as fold of induction.

Table 1. Primer sequences, annealing temperatures, and restriction enzymes for the quantification of methylation of gene-specific promoter regions by COBRA

Gene	Primer sequence	Annealing temperature, ° C	Restriction enzyme
p16 forward p16 reverse	GGTTTTTTTTAGAGGATTTGAGGGATA CTACAAACCCTCTACCCACCTAA	62	*Sau*3AI
GATA4 forward GATA4 reverse	GAGTTTGGATTTTGTTTGTT GTGATGTTTTAGGGGTTT	60	*Rsa*I
ESR1 forward ESR1 reverse	GGGATGGTTTTATTGTATTAGATTTAAGGG CTATTAAATAAAAAAAAACCCCCCAAAC	58	*Dpn*I
SFRP1 forward SFRP1 reverse	GTTTTTTAAGGGGTGTTGAGT CAAACTTCCAAAAACCTCC	59	*Eco*RI
HPP1 forward HPP1 reverse	TGTGTGTGAGTTGAAGTAGGGT ACCCAATTTTCCAAATACAACCATCA	68	*Taq*I

DNMT Activity Assay

Total DNMT activity was analyzed by a cell-independent assay using the EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Epigentek, Farmingdale, N.Y., USA). Nuclear proteins (10 μg) were extracted using Cell Extraction Buffer (Invitrogen) from CACO2 cells. Ind was added at different concentrations to the appropriate wells containing the nuclear protein extracts, according to the manufacturer's specifications. Absorbance at 450 nm was measured in a microplate reader (GloMax®-Multi Microplate Reader) and the inhibition induced by Ind was calculated following the formula:

$$\text{DNMT inhibition (\%)} = 100 - [(OD_{test}/OD_{ctr}) \times 100],$$

where OD_{test} is the average of optical density measurements after exposure of the nuclear lysate to Ind and OD_{ctr} is the average of optical density measurements with no exposure of the nuclear lysate to Ind.

Molecular Modeling of Ind and DNMT Interactions

The crystallographic structure of human DNMT1 in complex with DNA double helix and bound adenosine-homocysteine was extracted from the Protein Data Bank (3PTA). The missing residues (1480–1483) were modelled using Prime [28]. The model was further refined using the Protein Preparation Wizard implemented in Maestro [29] by removing DNA double helix and the water molecules. Only the catalytic domain was saved together with the bound AdoHcy. 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 an OPLS2005 force field. These coordinates were used to create the docking grid with the aim to dock Ind into the catalytic domain. The grid box was centered on the coordinates of the catalytic loop (1224–1235) and other residues which demonstrated to be fundamental for the transferase activity (Ser1230, Gly1231, Glu1266, Arg1310, Arg1312, Arg1462, Lys1535, Thr1528, and Gly1577). A mixed molecular docking/dynamics protocol, called Induced Fit Docking (IFD) [30], with Ind was performed. In an interactive manner, this approach combines ligand-docking techniques with those used to model receptor conformational changes. The Glide docking software package [31] 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 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 [32, 33]. The best scored ligand/protein complex was then submitted to a nanosecond-scale (2-ns) molecular dynamics (MD) simulation using the Desmond

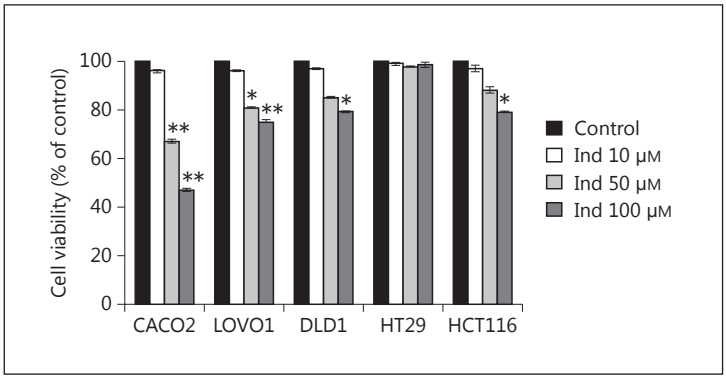

Fig. 1. Effect of Ind on cell growth after 48 h of treatment. The values are plotted as percentages compared to control cells (untreated cells). Each point represents the mean ± SD of 3 independent experiments. For each cell line, asterisks indicate statistically significant values (* $p \leq 0.05$, ** $p \leq 0.001$) in comparison to the control according to Student's t test.

model [34]. 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 [35] (300 K and 1.103 bar).

Statistical Analysis

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

Results

Anti-Proliferative Effects of Ind on Colorectal Cancer Cell Lines

The anti-proliferative effects of Ind were tested by exposing the cells to concentrations from 10 to 100 μM, equivalent to dietarily relevant levels of Ind [23]. The results from the MTT viability assay showed that different cell lines displayed a specific sensitivity to Ind exposure (fig. 1). LOVO1, DLD1, and HCT116 cell proliferation decreased slightly, whereas Ind treatment produced strong effects on CACO2 cell growth, with a decrease of about 50% after cell exposure to 100 μM Ind. It is noteworthy that higher concentrations of Ind up to 200 μM did not induce a greater anti-proliferative effect in any of the cell lines analyzed (data not shown). Finally, HT29 cells were not affected by Ind treatment, and therefore were not included in the further experiments.

Compounds acting on mechanisms controlling cell proliferation may be toxic to normal cells. When 100 μM Ind was incubated with CACO2 cells grown for 15 days after confluence, a condition allowing their spontaneous differentiation, no effect was observed on cell viability, indicating an absence of toxicity for nonmalignant cells [24].

Epigenetic Effects of Ind on Genomic Methylation Status (LINE-1 Methylation)

The methylation status of LINE-1, a transposon in the human genome, has been shown to be a suitable surrogate for global DNA methylation [36]. To investigate changes induced by Ind on global DNA methylation, we evaluated LINE-1 methylation levels after cell exposure to the phytochemical. Figure 2 shows that Ind treatment induced a significant increase in LINE-1 methylation in CACO2 and DLD1 cells, whereas it decreased methylation in LOVO1 cells. LINE-1 methylation in HCT116 cells was not significantly altered by Ind exposure.

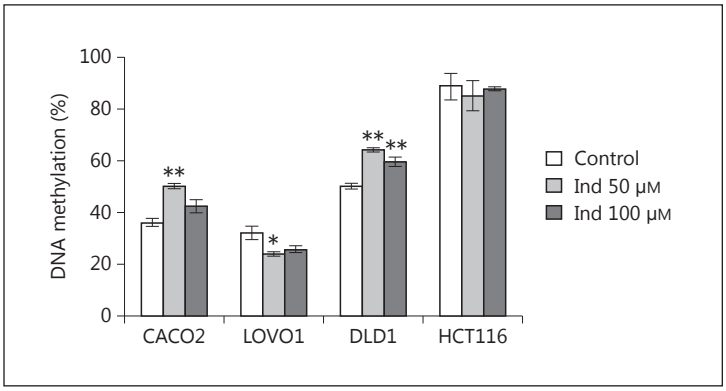

Fig. 2. Effect of Ind on LINE-1 methylation. LINE-1 methylation was quantified in DNA extracted from each cell line treated with 50 or 100 μM Ind for 48 h. Each point represents the mean ± SD of 3 independent experiments. For each cell line, asterisks indicate statistically significant values (* $p < 0.05$, ** $p < 0.001$) in comparison to the related control according to Student's t test.

Influence of Ind on Gene-Specific Methylation in Different Colorectal Cancer Cell Lines

To investigate the effect of Ind on gene-specific methylation, we evaluated the methylation status of promoter regions of several genes involved in colon carcinogenesis [8–10]. Figure 3 shows that the effect of Ind seems to depend on the basal methylation status of each gene. SFRP1 and HPP1 genes showed a high level of methylation in all the cell lines analyzed, which was not affected by Ind treatment. Ind induced a dose-dependent demethylation of *p16^{INK4a}* in all cell lines except for LOVO1 cells, where this promoter is approximately 90% methylated. The methylation status of the *GATA4* promoter region was decreased significantly ($p < 0.001$) in CACO2 (26%) and LOVO1 (31%) cells. The *ESR1* promoter showed a low basal methylation status that was significantly decreased by Ind treatment only in LOVO1 cells.

Influence of Ind on the Expression of DNMT and Demethylase Genes

In order to provide insight into how Ind influences DNA methylation, we evaluated the effects of Ind on the expression of the *DNMT* genes and some genes involved in DNA demethylation. The results from the real-time PCR analyses showed that cell exposure to Ind increased *DNMT1* expression in DLD1 ($p < 0.005$) and HCT116 ($p < 0.001$) cells (fig. 4a), while it increased *DNMT3A* expression in CACO2 and LOVO1 ($p < 0.005$) cells (fig. 4b). *DNMT3B* expression was increased only in HCT116 cells ($p < 0.005$) (fig. 4c). Overall, these data indicate that the expression of at least one of the different DNMT enzymes increased after treatment with Ind in all the cell lines analyzed.

The effect of Ind on the expression of members of the TET family, MBD4, and GADD45A was investigated. Figure 5 shows that Ind treatment did not affect TET1 or TET3 expression in any cell line, but significantly increased TET2 expression in LOVO1 cells, MBD4 expression in LOVO1 and DLD1 cells, and GADD45A expression in HCT116 cells. Ind did not significantly affect the expression of any of the demethylase genes in CACO2 cells.

Influence of Ind on DNMT Activity

Using nuclear proteins from CACO2 cells, we further evaluated whether the demethylating effects induced by Ind were associated with inhibition of DNMT activity. Figure 6 shows that Ind inhibited DNMT activity in a dose-dependent manner, with 100 μM Ind leading to a more than 30% reduction in DNMT activity.

Molecular Modeling Interactions between Ind and DNMT1

Following on from the molecular modeling approach of Yoo et al. [37], we carried out an implemented molecular modeling approach which consisted of a refined crystal structure of

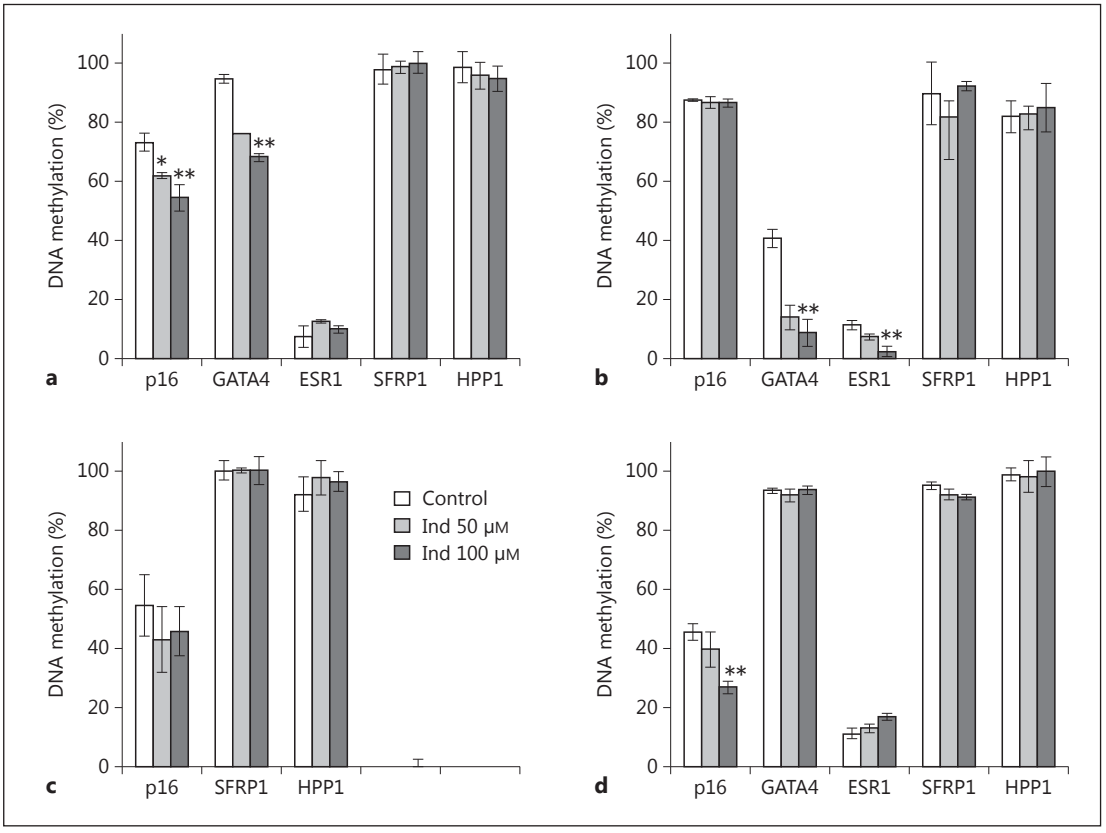

Fig. 3. Effect of Ind on gene-specific, promoter-regional DNA methylation in each cell line. DNA methylation was quantified by COBRA in DNA extracted from cells treated with 50 or 100 μM Ind for 48 h. For each gene promoter, asterisks indicate statistically significant values (* $p < 0.05$, ** $p < 0.001$) in comparison to the related control according to Student's t test. **a** CACO2 cell line. **b** LOVO1 cell line. **c** DLD1 cell line. **d** HCT116 cell line.

the DNMT1 catalytic domain (residues 1139–1600), IFD, and nanosecond-scale MD to study the interactions between DNMT1 and Ind. The IFD protocol allows overcoming the limitation of crystallographic structure, in which the catalytic loop is in an inactive state. In fact, after the first stage, in which the protein was kept rigid, in the second stage ligand boundary residues were able to move, fitting their position and conformation in relation to the flexible ligand.

To confirm the interactions between the target DNMT1 and the bound ligand Ind, we also performed a 2-ns MD with the aim to understand the influence of the solvent and whether the involved residues in van der Waals contacts and H-bonds changed during this time. The pose of the target protein and ligand obtained by means of IFD was chosen as the starting point of MD simulation after the system was soaked in water. Some snapshots of the simulation were chosen at regular intervals and the interactions analyzed. After a relaxation period of the system of about 200 ps, equilibration was reached, and the most representative snapshots were extracted. In snapshot 1 (fig. 7a), Ind assumes a rolled-up conformation because the 2 exocyclic double bonds are in *cis-cis* conformation; 4 water molecules are involved in the binding pocket, interacting with the carboxyl groups; H-bonds are established between Arg1574, Asn1578, and C2-COOH, and between Glu1266 and C11-COOH, and 2 H-bonds are established between Gly1223, C13-COOH, and the adjacent nitrogen atom. The catalytic

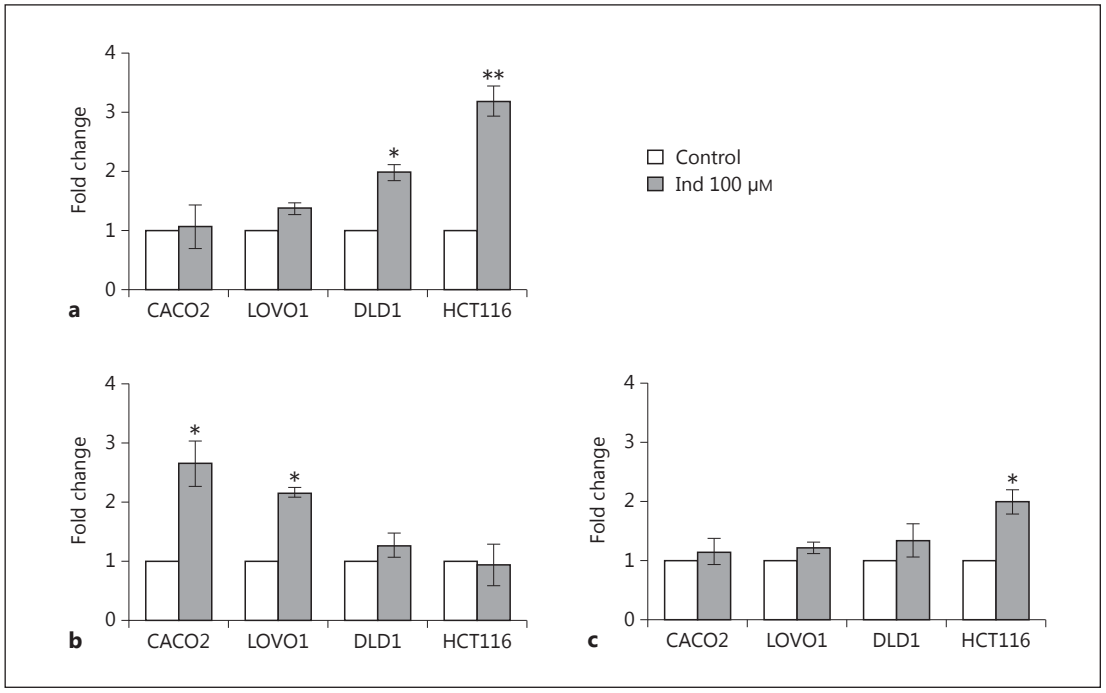

Fig. 4. Effect of Ind on the expression of the DNMT genes. Real-time PCR analysis was performed on CACO2, LOVO1, DLD1, HT29, and HCT116 cell lines treated for 48 h with 100 μM Ind or 10 μM azacitidine. mRNA levels of DNMT1 (**a**), DNMT3A (**b**), and DNMT3B (**c**) genes were evaluated. The values are plotted as fold changes compared to the control (untreated cells); the y-axis expresses $2^{-\Delta\Delta Ct}$. Each point represents the mean ± SD of 3 independent experiments. For each cell line, asterisks indicate statistically significant values (* $p < 0.05$, ** $p < 0.001$) in comparison to the related control according to Student's t test.

residues (Pro1225, Cys1226, and Gln1227) are very close to the ligand, confirming the prior IFD results. In snapshot 2 (fig. 7b), Ind flipped out its conformation *trans-trans*, which leads to a new H-bond of C11-COOH with Arg1312 and loss of the H-bond interaction with Glu1266. During the rest of the simulation (fig. 7c, d), Ind maintained the flipped-out conformation and the H-bond interactions with the previously identified residues. Only at the end of the simulation (fig. 7e) did Ind come back to the *cis-cis* conformation, but C11-COOH did not establish any H-bond interaction, except with water molecules. Interestingly, there are copious hydrophobic interactions with the catalytic loop residues. Water molecules are also present in the pocket, and this is synonymous with a quite open and hydrophilic binding pocket. However, this last consideration could be due to the presence of 3 carboxyl groups. In summary, Ind showed good interactions with key residues of the DNMT1 target protein. The results obtained by means of IFD are thus confirmed by the molecular dynamic simulation. The H-bonds between C2-COOH, Arg1574, and Asn1578 were retrieved as the principal interactions, as well as those with Gly1223, Gln1227, Arg1312, Glu1266, and Thr1528.

Discussion

Dietary phytochemicals are currently investigated as potential chemopreventive and/or chemotherapeutic agents, and the mechanisms involved in their effects are actively researched. The colon is an attractive model for such studies, because the incidence of colon cancer is

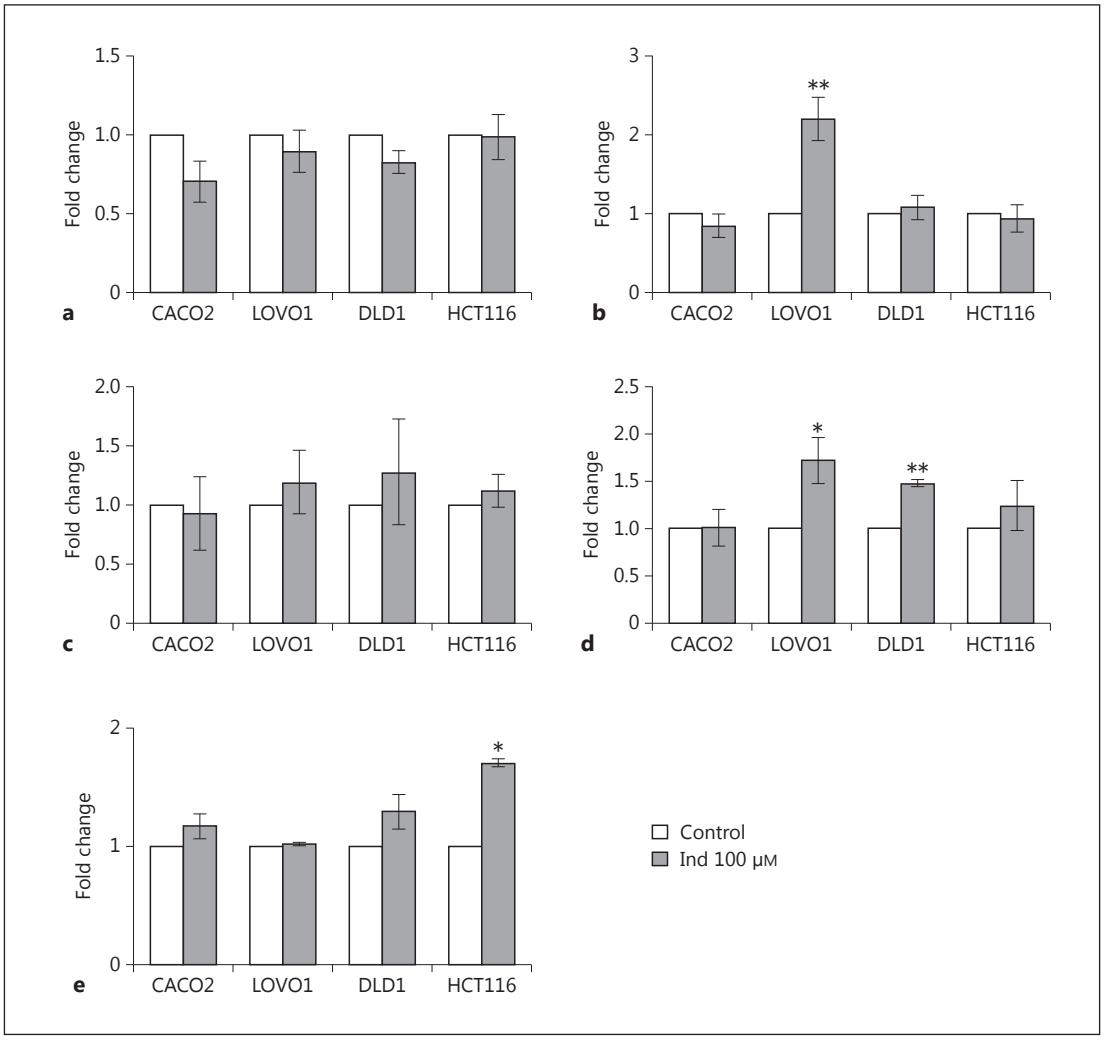

Fig. 5. Effect of Ind on gene expression of enzymes involved in DNA demethylation measured by real-time PCR analysis. mRNA levels of TET1 (**a**), TET2 (**b**), TET3 (**c**), MBD4 (**d**), and GADD45A (**e**) genes from CACO2, LOVO1, DLD1, HT29, and HCT116 cell lines treated for 48 h with 100 μM Ind were evaluated. The values are plotted as fold changes compared to the control (untreated cells); the y-axis expresses $2^{-\Delta\Delta Ct}$. Each point represents the mean ± SD of 3 independent experiments. For each cell line, asterisks indicate statistically significant values (* $p < 0.05$, ** $p < 0.001$) in comparison to the related control according to Student's t test.

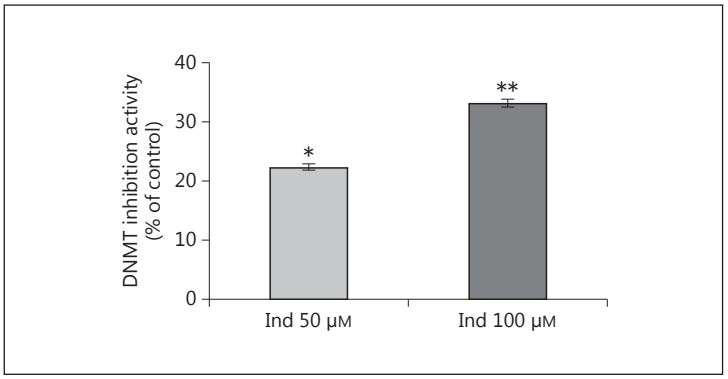

Fig. 6. Effect of Ind on DNMT enzyme activity. Total DNMT activity was analyzed by a cell-independent procedure using nuclear proteins from CACO2 cells. Values represent the mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.001$, according to Student's t test.

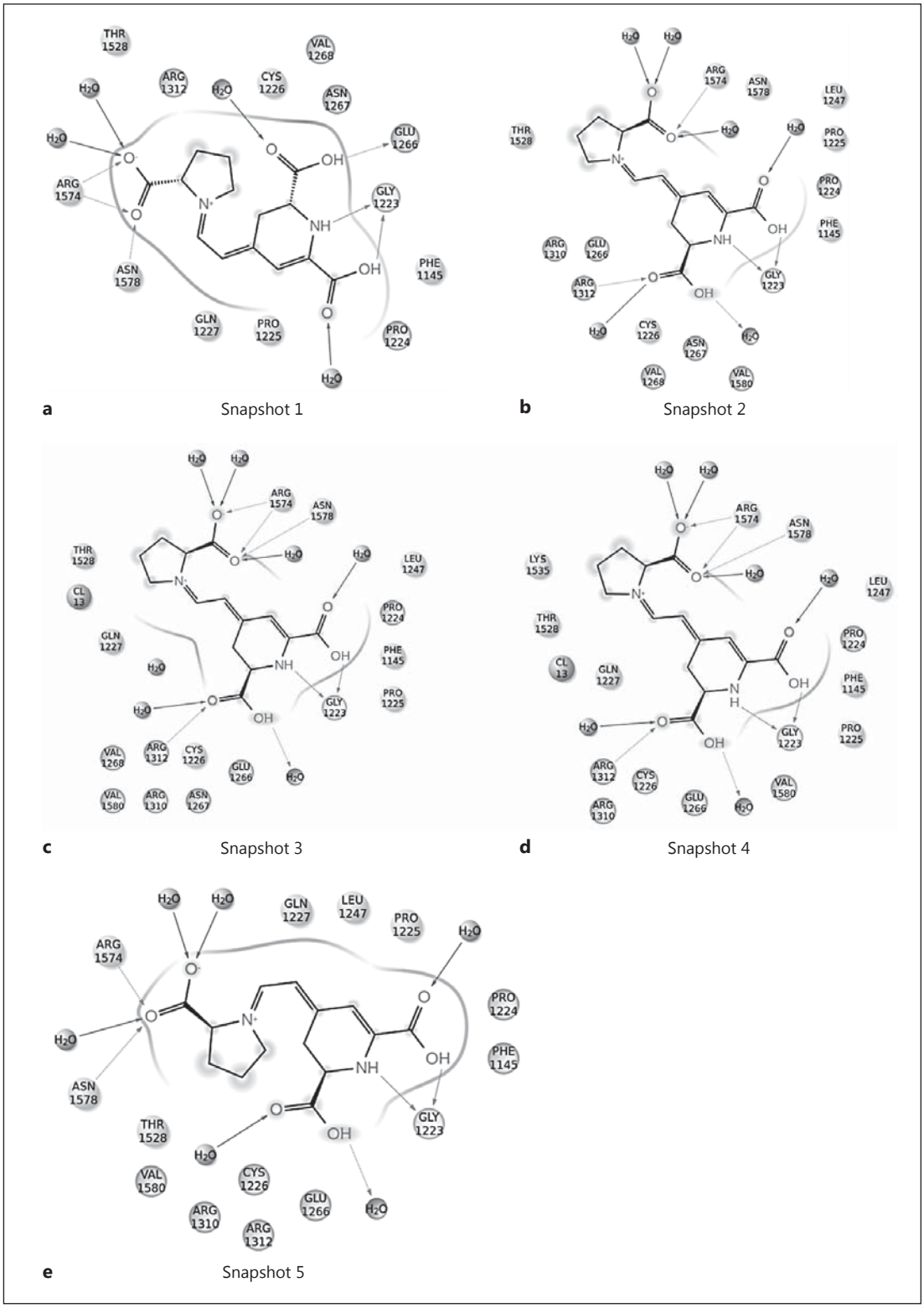

Fig. 7. MD simulation of the interaction between DNMT1 and Ind. After a relaxation period of the system of about 200 ps, equilibration was reached, and the most representative snapshots were extracted at regular intervals of the simulation.

inversely related to the consumption of fruits and vegetables [38]. On the other hand, food components, reaching higher concentrations in the gut lumen than in other tissues, have the potential to produce beneficial effects [39].

Over the past few years, evidence has accumulated that natural products and dietary constituents with chemopreventive potential can have a critical impact on DNA methylation [13]. Previously, we proposed the chemopreventive activity of Ind in CACO2 cells in terms of epigenetic modulation of $p16^{INK4a}$ gene expression [24]. In the present study, anti-proliferative effects and the epigenetic activity of Ind have been investigated in a panel of colorectal cancer cell lines to evaluate the potential of Ind in other transformed cells from the same tissue. An important point is that the amounts of Ind used in our experiments are consistent with the concentrations of Ind in the gut after dietary ingestion. Indeed, Ind concentrations in the intestinal digesta from a single serving of prickly pear fruit may be higher than the amounts observed to be active in our experimental conditions [23].

Here, we showed that Ind inhibited the cell growth of colorectal cancer cells, although the magnitude of the effect changed between the different cell lines tested. The HT29 cell line is the only one insensitive to Ind treatment. These differences may be due to the fact that, despite the same tissue of origin, these cells vary widely both in their cytogenetic features as well as in their genetic and mutational profiles. In particular, the colorectal cancer cell lines considered in this study are different for mutations in *KRAS*, *BRAF*, and *PIK3CA* genes, which are important for maintenance of the tumorigenic phenotype [40–42]. It is well known that differences in *KRAS* or *BRAF* mutation lead to a different cell sensitivity, for example, to MEK1/2 inhibitors [43]; interestingly, HT29 cells are the only ones that contain a mutation in the *BRAF* gene.

The cancer-preventive abilities of several bioactive food components, including resveratrol, genistein, and EGCG, have been linked also to their ability to regulate tumor suppressor gene expression by epigenetic processes [44]. Our data showed that Ind altered global DNA methylation and induced the demethylation of specific gene promoters, with effects depending on the specific cell line investigated, indicating different sensitivities of different cell lines to the phytochemical. In analogy, Chuang et al. [45] and Fang et al. [46] demonstrated that treatment with EGCG induced the demethylation and re-expression of $p16^{INK4a}$ in HCT116 cells but failed to produce similar effects in other cells. Interestingly, Ind induced changes in global methylation status in those cell lines with a moderate level of global DNA methylation (no more than 50%). HCT116 cells showed a high level of global methylation, which was not affected by Ind treatment. Similarly, the effect of Ind on specific gene promoters seems to be influenced by the basal methylation status of each gene. Indeed, SFRP1 and HPP1 gene promoters showed a high level of DNA methylation not affected by cell exposure to Ind. We hypothesize that the presence of highly condensed chromatin structures could make difficult an induction of epigenetic modifications by Ind.

Global loss of DNA methylation at repetitive genomic sequences (DNA hypomethylation) during carcinogenesis has been associated with genomic instability and chromosomal aberrations; in contrast, increased methylation (DNA hypermethylation) of promoter CpG islands leads to transcriptional silencing of tumor suppressors and other genes with important biological functions [47]. Thus, a hallmark of cancer is global DNA hypomethylation as well as hypermethylation of specific tumor suppressor genes [7]. With respect to CACO2 cells, which appeared the most responsive to Ind, an overview of the effects of Ind indicates that the phytochemical could work against the pathophysiological arrangement of tumor progression.

An abnormal overexpression of DNMT has been found in many types of cancer, and a contribution by them to aberrant DNA methylation has been proposed [48]. On the other hand, natural compounds can affect epigenetic patterns through the inhibition of expression

and/or activity of proteins involved in the epigenetic machinery [13, 14]. Here, we showed that cell exposure to Ind increased the expression of at least one of the three DNMT genes, which may provide an explanation for the increase in global methylation after Ind treatment as observed in CACO2 and DLD1 cell lines. However, the observed ability of Ind to act as an inhibitor of DNMT enzymes could help to explain the demethylating effects of Ind at the gene-specific level. It has been shown that different DNMT are responsible for targeting DNA methylation to specific regions of the genome [49]. Thus, methylation induced by DNMT is not an event that is widespread throughout the genome, but it may occur in specific regions as a result of the activity of specific DNMT. The variation in effects produced by Ind on DNA methylation may result from the observed ability of Ind to inhibit the DNMT enzymes coupled with its varying effects, in different cell lines, on the expression of some DNMT enzymes.

Natural nutritional compounds were found to interfere with the enzymatic activity of DNMT through different mechanisms [50]. In silico molecular modeling showed that Ind is able to stably bind DNMT1 at the catalytic site. Although the DNMT activity assay is not specific for a particular DNMT enzyme, the structural similarities of the catalytic sites of the different DNMT enzymes [51] allow us to hypothesize that the binding and inhibitory activity of Ind may not be selectively limited to the DNMT1 enzyme.

It is widely accepted that active demethylation does take place in the genome [52]. 'Active demethylation' mechanisms involve TET proteins and a number of DNA-modifying enzymes such as DNA cytosine deaminases, DNA glycosylases (MBD4), and other DNA repair factors (GADD45). We found that Ind induced an increase in the expression of some genes encoding enzymes involved in DNA demethylation (TET2 and MBD4). These data suggest that the epigenetic effect of Ind could also be mediated by the increased expression of enzymes involved in active DNA demethylation.

Overall, the results of this study indicate that dietary concentrations of Ind have anti-proliferative activity and may affect the epigenetic pattern in terms of an alteration in DNA methylation in colorectal cancer cells. By reducing the DNA hypermethylation of key cancer-related genes, Ind may affect tumor progression. Our investigation shows that Ind may induce epigenetic effects via a complex mechanism that involves modulation of the DNA methylation machinery.

Disclosure Statement

The authors declare to have no conflicts of interest.

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