Induction of Epigenetic Alteration by CPUK02, An Ent-kaurenoid Derivative of Stevioside

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Abstract

Background: Dietary polyphenols, such as those found in green tea and red wine, are linked to antitumor activity. They are known to influence many signaling pathways epigenetically within the human body. In this regard, CPUK02 (15-Oxosteviol benzyl ester) is a new ent-kaurenoid derivative of stevioside and exhibits strong anti-cancer activity in vitro and in vivo. Nowadays, the role of epigenetics in cancer has been the subject of intensive study and DNA methylation targeting represents a relevant strategy for cancer treatment. There are no reports regarding the effects of CPUK02 on epigenetic alterations in colorectal cancer cell line. This study was an attempt to compare CPUK02 with 5-AZA as DNMT inhibitor agent and evaluate whether it can induce its anti-cancer effects via altering the level of DNMT3b mRNA, MGMT and SFRP2 methylation pattern in HCT116 cell line.

Methods: To evaluate DNMT3b expression, DNMT3B mRNA levels in HCT116 CRC cell line were quantified by real-time reverse-transcriptase Polymerase Chain Reaction (PCR) assay after 24 hr of incubation time with CPUK02 and 5-AZA. In addition, the methylation patterns of 2 CpG islands in this cell line were examined by methylation-specific PCR methods.

Results: CPUK02 surprisingly, decreased the DNMT3b mRNA level. The average expression levels of DNMT3b in HCT116 treated with CPUK02 and 5-AZA relative to the GAPDH expression level in control were 0.16 and 0.5%, respectively. Furthermore, CPUK02 could decrease the methylated allele of MGMT and SFRP2 genes in HCT116 after 24 hr.

Conclusion: In this study, positive correlation was found between mRNA expression of DNMT3b and gene promoter hypermethylation after treatment with CPUK02 and 5-AZA. Our data confirmed that CPUK02 like 5-AZA exhibits demethylating properties.

Keywords: 5-AZA, Colorectal neoplasm, DNMT, Epigenetic, Methylation

Introduction

Colorectal Cancer (CRC) is a multistep process with accumulation of genetic and epigenetic errors which causes a normal cell transformation to an invasive tumor cell.

Three distinct pathways including chromosomal instability, microsatellite instability, and CpG island gene methylation pathways have been recognized in colon cancer initiation and progression. Alterations in DNA methylation patterns are the best understood epigenetic cause of the disease which occurs usually via either hypomethylation of global DNA or hypermethylation of tumor suppressor genes. DNA methylation is performed by at least two DNA methyltransferases (DNMTs; 3a and 3b) and maintained by DNMT1. Overexpression of DNMTs has been also detected in a variety of malignancies, including lung, prostate, and colorectal tumors.

DNA hypermethylation is also associated with gene silencing and is often observed in CpG islands of cancer-related genes. Transcriptional silencing by the hypermethylation of CpG islands is an early event in tumor progression. In this regard, researchers suggest that hypermethylation of O6-methylguanine-DNA methyltransferase (O6-MGMT) is a pacemaker for certain gene mutations such as K-ras. On the other hand, aberrant methylation in genes related to WNT signaling...
pathway has crucial roles for cancer progression especially in CRC. Among specific genes in WNT signaling, SFRP2 methylation is considered as a marker with high sensitivity and specificity in serum and stool for colorectal adenomas and CRC screening. 5-Aza-CR and 5-Aza-CdR potentially inhibit the DNA methyltransferases (DNMTs). Nowadays, therapeutic targeting via the DNA methylation machinery has been the subject of intensive study.

Recently, natural compounds, such as curcumin, Epigallocatechin Gallate (EGCG) and resveratrol, have been considered to increase sensitivity of cancer cells to conventional agents and induce tumor growth inhibition via epigenetic mechanisms. One of the plants that has recently attracted the attention of researchers is stevia rebaudiana bertoni and its glycoside compounds. Among stevia derivatives, isosteviol and derivative compounds inhibit human cancer cell growth. Furthermore, only isosteviol potently suppresses both colorectal adenomas and CRC screening. 5-Aza-CR and 5-Aza-CdR potentially inhibit the DNA methyltransferases (DNMTs). Nowadays, therapeutic targeting via the DNA methylation machinery has been the subject of intensive study.

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Cell line
The human colon cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified 5% (v/v) CO_2 incubator.

Treatment with CPUK02
Cells were seeded for 12 hr and then washed with PBS and medium was replaced with serum-free RPMI medium. Finally, non-growing, confluent cells were incubated with CPUK02 and 5-aza dissolved in DMSO to a final concentration of 4.5 µM and 1 µM, respectively for 24 hr. Optimum concentrations were determined according to MTT assay. At the end of the incubation, cells were harvested for RNA and DNA isolation.

Methylation specific-PCR analysis
DNA was extracted from cells according to the standard phenol/chloroform method. The status of promoter methylation of the MGMT-B, and SFRP2 genes was determined by Methylation Specitic PCR (MSP-PCR). The sequences of primers and annealing temperatures used for amplification of the promoter regions of genes are listed in table 1.

The genes promoter methylation status was determined by chemical treatment of DNA samples with sodium bisulfite and subsequent MS-PCR as previously described.

In every reaction, DNA from peripheral blood lymphocytes was considered as a negative control. PCR products were analyzed by electrophoresis on 2% agarose gel.

RNA extraction and semi-quantitative PCR
The total RNA was extracted from the treated cells using the TRIZOL Reagent Kit, according to the manufacturer’s instructions. The RNA concentration was quantified by measuring the absorbance at 260 nm in a spectrophotometer. Ratios of absorption (260/280 nm) of all samples were between 1.8 and 2.0. RNA samples were subjected to electrophoresis through a 1% agarose-formaldehyde gel to verify their integrity. The total RNA samples were stored at -80°C until analysis. The expression of DNMT3b mRNA was determined by semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Two µg of total RNA were reverse transcribed by incubation at 37°C for 1 hr in a 25 µl reaction mixture consisting of 100 U M reverse transcriptase, 8 U RNase inhibitor, 0.5 µg of oligo (dT), 50 mmol/L Tris-HCl (pH=8.3), 3 mmol/L MgCl₂, 75 mmol/L KC1, 10 mmol/L DDT, and 0.8 mmol/L each dNTP. The reaction was terminated by heating at 95°C for 5 min and quickly cooling on ice. Two µl of the RT reaction mixture were used for PCR in a final volume of 25 µl, containing 2.5 µl of 10×PCR buffer, 2 µl of each 2.5 mmol/L dNTPs mixture, 1 unit of Taq DNA polymerase, and 10 pmol/L of each forward and reverse primer. The conditions of PCR amplification were as follows: one cycle at 95°C for 5 min, 33 cycles...
at 95°C for 30 s, 60°C for 40 s and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The PCR-amplified fragments were run alongside molecular weight markers on 2% agarose gels stained with gel red. The experiments were repeated twice. The primers and PCR conditions for DNMT3b, GAPDH are listed in Table 2.

232 and 113 bp amplicons were expected upon performing PCR for GAPDH and DNMT3b, respectively.

Quantitative real-time PCR
Real-time PCR was carried out using the ABI real time PCR 7500 system. The PCR reaction mixture contained 2 μl of cDNA (tenfold diluted), 0.5 μl of 5 mmol/L solutions of each of the forward and reverse primers and 10 μl of SYBR green DNA PCR Master Mix in a total volume of 20 μl. All incubations included an initial denaturation step at 95°C for 10 min and 30 cycles (15 sec at 95°C and 30 sec at 60°C) subsequently. A melting curve analysis was achieved by performing 70 cycles of 10s with a temperature increment of 0.5°C/cycle starting from 60°C. Efficiency of amplification was measured by the slope of a standard curve. Data were analyzed by using the 7500 Software v2.0.1. The relative expression level (fold changes) of DNMT3b gene was calculated by the 2-ΔΔCT formula. GAPDH was also considered as the internal control. Expression levels of target gene were normalized using the GAPDH as the housekeeping control gene.

Statistical analysis
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc Dunnnett multiple comparison tests using SPSS [Version 16; SPSS, Chicago, IL, USA]. The values of p<0.05 were considered statistically significant.

### Table 1. Primer sequence of SFRP2 and MGMT genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Annealing temperature, °C</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT</td>
<td>MF: GGTCGTTTGTACGTTGC</td>
<td>59</td>
<td>M: 127; U: 127</td>
</tr>
<tr>
<td></td>
<td>MR: TAACCCTTCACCGGATGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF: GTAGGTTTGTATGTTTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UR: TAACCCTTCACGCAAAAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFRP2</td>
<td>MF: TCCTGTTTTTATTCGTTGCTGC</td>
<td>59</td>
<td>M: 138; U: 145</td>
</tr>
<tr>
<td></td>
<td>MR: CATTAAATACCCGCGTCTCGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF: GTTTTGTGGTTTATTTATTTTTGTTTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UR: TCCCCCTAAATACCCACACCTACCCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Primer sequence of DNMT3b and GAPDH genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature, °C</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT3b</td>
<td>F: GGCAAGTTCTCCGAAGGTCCTCT</td>
<td>60</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>R: TGGTGCTGCTTTTCATGATAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CGACCACTTGTCAAGGCTCA</td>
<td>60</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>R: AGGGGTCTCATACGGCGACTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results

### Methylation of SFRP2 and MGMT genes
The methylated and unmethylated sense/antisense primers for MGMT produced a 127 bp fragments. However, the methylated and unmethylated sense/antisense primers for SFRP2 produced 138 and 145 bp fragments, respectively. In this study, it was observed that CPUK02 had a positive effect on SFRP2 and MGMT methylation pattern in HCT 116 cells. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of a product in those lanes marked M indicates the presence of methylated genes in control, cells treated with DMSO as vehicle as well as 5-AZA and CPUK02. Unmethylated lymphocytes (lymphocytes) DNA was used as the negative control.

### DNMT3b expression analysis
The presence of appropriate bands for DNMT3b...
CPUK02 as Inducer of Epigenetic Alteration

(113 bp) and GAPDH (228 bp) amplicons was confirmed by semi RT-PCR. Real time PCR results in figure 2 showed that CPUK02 and 5-aza were able to decrease mRNA level of DNMT3b gene compared with control group in HCT 116 cells. The average expression levels of DNMT3b in HCT116 treated with CPUK02 and 5-AZA relative to the GAPDH expression level in control were 0.16 and 0.5%, respectively.

Discussion

For the first time, the epigenetic role of CPUK02 in HCT 116 cells was shown. CPUK02 as an ent-kaurenoi d compound exhibited strong effect upon DNMT3b mRNA level and methylation status of two important genes; MGMT and SFRP2 in CRC.

Researchers showed that CPUK02 can induce the apoptosis in different cancer cell lines via p53 22. However, there is no data to address the effects of CPUK02 on epigenetic alteration in colorectal cancer. Pierre-Olivier and colleague have found that DNMTs are integral part of the p53 protein network. On the other hand, the levels of DNMTs, especially DNMT3a and DNMT3b, are often increased in various cancer tissues and cell lines such as HCT 116 cells 25. Therefore, this phenomenon may increase hypermethylation of tumor suppressor genes in a variety of malignancies. Alt-
gene in CRC cell line. In this regard, Wodarz et al revealed that methylation rate of MGMT gene in HCT-116 cell line is slower than methylation rate of SFRP2 gene. Also, they showed that MGMT promoter methylation in HCT 116 cell line started with a delay. Given this finding, it can be concluded that methylation rate of MGMT gene will be slower than SFRP2. Therefore, increasing the incubation time during treatment from 24 hr to 48 might help the unmethylated allele of MGMT to appear. This experiment was performed on HCT 116 cells for longer time and the same results were obtained which are seen in ER-negative breast cells.

Recent researchers showed that epigenetic pharmaceuticals could be a replacement or adjuvant therapy for currently accepted treatment methods such as radiation and chemotherapy, or could sensitize cells to the current treatments. In this regard, although 5-Aza is the most potent demethylating agent in clinical usage, general side effects led to efforts for finding new DNA methylation inhibitors with greater potency and low cytotoxicity.

Recently, natural compounds, such as curcumin, EGCG, and resveratrol, have been shown to increase sensitivity of cancer cells to conventional agents via alteration in epigenetic mechanisms, which may lead to tumor growth inhibition. It has been shown that the epigenetic control of the proto-onc genes and the tumor suppressor sequences by conformational changes in histones directly affects the formation and progression of cancer. CPUK02 as a new semi natural compound which exhibits strong anti-cancer effect in in vitro and in vivo models might also manipulate epigenetic pathways and our data confirmed that CPUK02 like 5-AZA exhibits demethylating properties.

**Conclusion**

According to our data, CPUK02 is a promising molecule with epigenetic/anti-cancer properties and it might be a front-runner candidate for new pharmaceutical targets. However, little information exists about performance of CPUK02 and further investigations are needed to introduce it as a new epigenetic drug.

**Acknowledgement**

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**Conflict of Interest**

Authors declare they have no financial disclosure.

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