Paricalcitol Enhances the Chemopreventive Efficacy of 5-Fluorouracil on an Intermediate-Term Model of Azoxymethane-Induced Colorectal Tumors in Rats

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Abstract

Colorectal cancer is a common cancer with high mortality rate. Despite being the standard anti–colorectal cancer drug, 5-fluorouracil (5-FU) exhibits only limited therapeutic benefits. Herein, we investigated whether paricalcitol, a synthetic vitamin D analogue with potential antitumor properties, would enhance the chemopreventive efficacy of 5-FU on an intermediate-term (15 weeks) model of colorectal tumors induced by azoxymethane (AOM) in rats. After AOM injection, 5-FU was administered during the 9th and 10th weeks (12 mg/kg/day for 4 days, then 6 mg/kg every other day for another 4 doses), whereas paricalcitol (2.5 μg/kg/day; 3 days/week) was given from the 7th to the 15th week. At week 15, the animals were euthanized and their resected colons were examined macroscopically and microscopically. Quantitative RT-PCR was used to measure the transcription activities of Wnt, β-catenin, DKK-1, CDNK-1A, NF-κB, and COX-2 genes, and ELISA was used to quantify the protein levels of β-catenin, COX-2, HSP90, and VEGF. IHC was additionally used to measure β-catenin, HSP90, and inducible nitric oxide synthase (iNOS). Compared with their individual therapy, combination of 5-FU and paricalcitol showed more significant reducing effect on numbers of grown tumors and large aberrant crypt foci. Mechanistically, paricalcitol and 5-FU had cooperated together to repress the expression of procancerous Wnt, β-catenin, NF-κB, COX-2, iNOS, VEGF, and HSP-90 more, and to upregulate the expression of antitumorogenesis DKK-1 and CDNK-1A, compared with their monotherapies. Our findings suggest that combined use of paricalcitol with 5-FU exhibits an augmenting chemopreventive effect against colorectal tumors, and might potentially be useful for chemoprevention in colorectal cancer patients. Cancer Prev Res; 9(6); 491–501. ©2016 AACR.

Introduction

According to the World Health Organization’s International Agency for Research on Cancer, colorectal cancer represents the third most frequent cancer in men and second in women, ranking as the fourth leading cause of cancer-related deaths worldwide (1). The limited therapeutic efficacy of its current chemotherapy represents the most important challenge in colorectal cancer management. With this aspect, 5-fluorouracil (5-FU)-based therapy, either alone or in combination with other cytotoxic agents (e.g., irinotecan, leucovorin, or oxaliplatin), remains the standard approach (2–4). Overall, 5-FU still exhibits limited efficacy with low tumor response rate ranging between 7% and 17% with its monotherapy and 35%–39% when given with other chemotherapeutic agents (3, 4). More importantly, although the recent addition of new targeted agents, such as bevacizumab and cetuximab, to the standard chemotherapy provides hope for more effective therapy in advanced colorectal cancer, they have shown only modest benefit and are subject to both primary and secondary resistance, like traditional chemotherapy, which ultimately leads to treatment failure (4). Thus, development of potential alternative or combinational colorectal cancer chemopreventive and therapeutic strategies is a paramount medical demand. To that end, vitamin D and its analogues are the most attractive agents in this setting (5, 6).

There is a compelling body of evidence that calcitriol [1,25(OH)2D3], the active form of vitamin D, may not only reduce the risk of colorectal cancer and other human cancers but also repress the tumor cell resistance toward the cytotoxic effects of 5-FU and other anticancer chemotherapeutic agents, and regulate the activities of various genes that impact cancer cell proliferation, differentiation, apoptosis, angiogenesis, invasion, and drug resistance (5–7). However, such potential antitumor activity of calcitriol is achieved only when it is given in supraphysiologic doses that led to significant hypercalcemia and hypercalciuria side effects that hinder and impede its clinical usefulness for this purpose (7, 8). As a clinically relevant task, synthesis of non- or less calcemic vitamin D analogues has therefore been initiated to achieve or even potentiate the antiproliferative/tumoridal properties of calcitriol but precludes its calcemic side effects (7, 8). In
spite of their well-known vitamin D receptor (VDR)-mediated actions, their precise mechanisms and why specific analogues have superagonistic effects on specific tissues or diseases still have to be deciphered (8). Among these analogues, paricalcitol (19-nor-1α-25-dihydroxyvitamin D₃), a direct acting VDR activator that is clinically approved by the FDA for the treatment of secondary hyperparathyroidism, has recently gained attention on a variety of disease modalities, including cancer, due to its less calcemic effects, wider therapeutic window, and an equipotential activity as calcitriol in several in vivo and in vitro systems (8–11). In cancer patients, paricalcitol may have potential safety and feasibility in women with metastatic breast cancer (12), and in men with advanced prostate cancer (13). Paricalcitol has also demonstrated potential suppressive effects on a variety of human cancer cells and preclinical tumor models such as pancreatic cancer (14), gastric cancer and peritoneal metastasis (10), uterine fibroids (15), androgen-dependent prostate cancer cell model (16), and human leukemic cells (17).

Taken together, paricalcitol may be a potential chemopreventive agent in cancer therapy, especially for patients with low response or who fail in conventional therapies (10, 11, 15). However, there remains insufficient information concerning its benefits on colorectal cancer. Therefore, the current study was aimed to investigate whether paricalcitol would improve and synergize the anti–colorectal cancer effect of 5-FU, and to identify the possible mechanisms underlying such synergy on an intermediate-term model of colorectal neoplasia induced by azoxymethane (AOM) in rats. AOM-induced colorectal tumors and carcinogenesis in rats and mice have been proven as an outstanding rodent model that closely mirrors the phases and features of human colorectal cancer (aberrant crypts foci (ACF)—adenoma–adenocarcinoma and carcinoma sequence) in a time-dependent manner post-AOM injection, and commonly used to assess new chemopreventive and therapeutic strategies and to provide new insights into the pathophysiologic mechanisms and risk factors of human colorectal cancer (18–20). Our findings showed that monotherapy with either 5-FU or paricalcitol resulted in a significant chemopreventive effect on this model; however, their combination exhibited a more significant efficacy to repress the morphologic, histopathologic, and molecular changes that were observed in this model. Further studies are still essential to realize the potential clinical value of this combination in human patients with colorectal cancer.

**Materials and Methods**

**Drugs and chemicals**

Paricalcitol (Zemplar 5 mg/mL vials) was obtained from Abb-Vie Ltd., while AOM and 5-FU were purchased from Sigma-Aldrich. All other chemicals used were of the highest commercial grade.

**Animals, induction of colorectal tumorigenesis, and treatment approach**

All experimental protocols and procedures of the current study were approved by the Institutional Animal Care and Use Committee at the University of Umm Al-Qura (Holy Makkah, Saudi Arabia), and in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. A total of 75 adult male Wistar rats, weighing 220–250 g, were housed in clean, sterile, and polyvinyl cages (5 rats/cage), maintained on standard laboratory pellet diet and water ad libitum, and kept in a temperature-controlled air conditioned environment at 22–24°C with a 12-hour dark/light cycle.

For induction of colorectal tumorigenesis, AOM was dissolved in normal saline and injected subcutaneously at a dose of 15 mg/kg, once weekly for 2 weeks as described previously (18). According to the subsequent treatment schedules, the rats were randomly categorized into the following 5 groups (15 rats/group): group 1 (normal controls): received only normal saline; group 2 (AOM group): AOM-injected rats and left without treatment; group 3 (5-FU group): AOM-injected rats and then treated with 5-FU; group 4 (paricalcitol group): AOM-injected rats and then treated with paricalcitol; and group 5 (5-FU–paricalcitol group): AOM-injected rats and then treated with 5-FU plus paricalcitol combination therapy. In its designated groups, 5-FU was freshly prepared in normal saline and injected intraperitoneally (i.p.) during the 9th and 10th weeks post-AOM injection in a dosage regime similar to that used in the treatment of human colorectal cancer (12 mg/kg/day for successive 4 days, then 6 mg/kg every other day for 4 doses), while paricalcitol was administered in a dosage regimen of 2.5 μg/kg/day i.p., 3 days/week; starting from the 7th week post-AOM injection and continued till the end of the study (week 15 post-AOM injection). The dose of paricalcitol was chosen on the basis of our tested pilot experiments and previously published reports (14).

**Blood sampling and isolation of whole colon**

At the end of the study, rats of the different groups were weighed, fasted overnight, and then euthanized under diethyl ether general anesthesia (Fisher Scientific UK Ltd). After euthanasia, blood sample (4 mL) was collected from the rat’s vena cava into a plain tube, and used to measure the serum levels of calcium, 25-OH vitamin D, kidney function tests (creatinine, BUN, and urea), and liver function enzymes [alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate aminotransferase (AST)] by using Cobas e411 (Roche Diagnostics International Ltd), according to the manufacturer’s instructions. Subsequently, the whole colon from rectum to cecum was gently resected, flushed with PBS, and slit opened longitudinally. The surface area of each isolated colon (length × width in cm²) was measured, and then the whole colon was immersed in 10% (v/v) neutralized formalin for overnight between layers of filter papers with the mucosa on the upper side.

**Quantification of grown tumors and large ACF in the colorectal tissues**

The grown tumors on the mucosa of the isolated colons were blindly counted by the naked eye by two observers. Next, the colon was cut into 3 portions: proximal, middle, and distal segments; and each segment was stained with 0.2% methylene blue solution for 1.5–2 minutes, placed on a microscope slide with the mucosal side upward, and examined under a dissecting microscope to count the small tumors, that were not detected by the naked eye, and large ACF (containing 4 or more aberrant crypts) according to previously published criteria (21). After counting process, a micro-feather scalpel blade was used under the dissecting microscope to excise the colorectal specimens that had tumors and ACF from the surrounding normal tissues to be used for the subsequent histopathologic, molecular, ELISA, and immunohistochemical examinations.
RNA extraction, cDNA synthesis, and qRT-PCR analysis

Colorectal specimens were homogenized in RNAlater solution (Ambion), and then total RNA was isolated by using the Purelink RNA Mini Kit (Applied Biosystems), following the manufacturer’s instructions. The quality and the quantity of the extracted RNA were measured by the Nanodrop equipment (BioSpec-nano, Shimadzu Corporation). Up to 200 ng of the extracted total RNA was employed in the reverse transcription step for cDNA synthesis and by using a high capacity RNA-to-cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR was conducted using the 2−ΔΔCt method on the following target rat genes: Wnt (NM_001105714.1), β-Catenin (AF397179.1), Dickkopf-1 [DKK-1; (NM_001106350.1)], Cyclin-dependent kinase inhibitor 1A (CDKN-1A; NM_080782.3), NF-κB (NM_001008349.1), and COX-2 (AF233596.1). In addition, β-actin (NM_031144.3) was used as an internal reference (housekeeping gene) to standardize the data of the target genes. The nucleotide primer sequences of these seven rat origin genes were summarized in Table 1. All reactions were performed in triplicate and using Power SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Briefly, 10 μL SYBR Green, 7 μL cDNA/ RNase−free water, 1 μL of each primer (5 pmol), and 1 μL cDNA (25 ng) were mixed in each well of the PCR plate, and the amplification was conducted under the following conditions: 40 cycles (15 seconds at 95 °C and 1 minute at 65 °C). Data were analyzed using a comparative threshold cycle (Ct) technique, normalized against the Ct values of β-actin and expressed as fold-change compared with the normal control group.

ELISA

The levels of β-catenin, COX-2, HSP-90, and VEGF were quantitatively measured in the colorectal tissue specimens by ELISA technique. Briefly, the tissue specimens were homogenized in RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotechnology Inc), centrifuged, and then their harvested supernatants were stored in −20 °C until use. During the assays, the total protein concentration in each sample was adjusted to make a final concentration of 500 μg/mL and the concentrations of the candidate proteins were measured by using commercial ELISA kits (CUSABIO) and a fully automated ELISA system (Human Diagnostics), according to the manufacturers’ instructions. All samples were processed in duplicate and the results are presented as ng/mL for HSP-90, pg/mL for β-catenin and VEGF, and pmol/mL for COX-2.

Immunohistochemical analysis

Immunohistochemical staining for β-catenin, HSP-90, and inducible nitric oxide synthase (iNOS) was performed on paraffin sections following the conventional protocol. The primary antibodies used were as follows: polyclonal goat IgG antibodies (1:100) against rat HSP-90-α/β (N-17), iNOS (N-20), and β-catenin (C-18; Santa Cruz Biotechnology Inc). Biotinylated anti-goat secondary antibodies (1:200) conjugated with horseradish peroxidase complex (Santa-Cruz Biotechnology Inc) were used, and staining process was developed by DAB chromogen substrate and counterstained with Gill hematoxylin. The intensity of staining was assessed using H-score formula as follows: H-score = ∑Pi (i + 1), where i represents the intensity of positively stained cells (0 = negative; 1 = weak; 2 = moderate; and 3 = strong) and P is the percentage (0%–100%) of positively stained cells (23).

Statistical analysis

Results were expressed as mean ± SD. Comparisons of data between groups were made using one-way ANOVA, with post hoc comparisons using Dunnett multiple comparison test. The difference between data were considered to be statistically significant when P < 0.05, and to be very significant when P < 0.01.

Results

Inhibitory effects of paricalcitol and/or 5-FU on colorectal tumor growth and large ACF formation

In the current study, the chemopreventive effects of mono- and combination therapy with paricalcitol (Pcal) and 5-FU in inhibiting the early colorectal tumorigenesis stages and tumor growth were examined in an intermediate-term model (15 weeks) of AOM-induced colorectal carcinogenesis in rats. As shown in Figs. 1 and 2, in comparison with normal controls (Figs. 1 and 2; panel 1A), rats received AOM and left with treatment developed a significant number of gross tumors and distortions on their colorectal mucosae (Figs. 1 and 2; panel 2A). However, treatment of these AOM-injected rats with either 5-FU (Figs. 1 and 2; panel 3A) or paricalcitol (Figs. 1 and 2; panel 4A) had significantly

Table 1. Primer sequences used in the qRT-PCR for detection of the transcription activities of Wnt, β-catenin, DKK-1, CDKN-1A, NF-κB, COX-2, and β-actin genes including the corresponding genes accession numbers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tbody>
<tr>
<td>Wnt (NCBI: NM_001105714.1)</td>
<td>5’-AGC TGG GTT TCT GCT ACG T1-3’</td>
<td>5’-AAT CTG TCA GGA GGT TCG T1-3’</td>
</tr>
<tr>
<td>β-catenin (NCBI: AF397179.1)</td>
<td>5’-TTC CTG AGC TGA CAA ACC TG-3’</td>
<td>5’-GCA TTA TGG CAT ACA CCA TC-3’</td>
</tr>
<tr>
<td>DKK-1 (NCBI: NM_00106350.1)</td>
<td>5’-ATT CCA GCC TGG TTA CTG CTG-3’</td>
<td>5’-GAA TCG CTG TTA GGA TGG TG-3’</td>
</tr>
<tr>
<td>CDKK-1A (NCBI: NM_080782.3)</td>
<td>5’-AGA AGG GAA CGG GTA CAC AG-3’</td>
<td>5’-ACC CAT AAG AGA GGC AGT TG-3’</td>
</tr>
<tr>
<td>NF-κB (NCBI: NM_001008349.1)</td>
<td>5’-CAG AGC TGG CAG AGA GAC TG-3’</td>
<td>5’-TAG CAA GGA GAC TGC CAC TG-3’</td>
</tr>
<tr>
<td>COX-2 (NCBI: AF233596.1)</td>
<td>5’-AAC CGC TGT ACA AGC AGT GG-3’</td>
<td>5’-GCC GGC ATT TTT TCT TCT CG-3’</td>
</tr>
<tr>
<td>β-actin (NCBI: NM_031144.3)</td>
<td>5’-CGG TCA GGT CAT CAC TAT CG-3’</td>
<td>5’-TTC CAT ACC CAG GAA GGA AG-3’</td>
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decreased the number of the grown tumors and the topographic mucosal alterations. More importantly, the lowest number of grown tumors was observed in rats treated with 5-FU/paricalcitol combination therapy (Figs. 1 and 2; panel 5A). Furthermore, examination of the resected colons of the different studied groups by dissecting microscope following methylene blue staining showed normal mucosal and crypt appearance in the normal control group (Fig. 2; panel 1B), but colons of rats injected with AOM and not treated developed several micro-tumors and ACF (Fig. 2; panel 2B). On the other hand, the number of these micro-tumors and ACF were significantly diminished when these diseased animals were treated with either 5-FU (Fig. 2; panel 3B) or paricalcitol (Fig. 2; panel 4B), and dual therapy with paricalcitol and 5-FU preserved/restored the normal mucosal architecture and was associated with the highest reducing effect on the development of such micro-tumors and ACF (Fig. 2; panel 5B).

The histopathologic findings (Fig. 2) were also consistent with the macroscopic/microscopic observations and showed the presence of many large ACF (>4 crypt/focus) with hyperplastic and dysplastic features, and multiple tubular adenomas in the colorectal tissues of rats injected with AOM and left without any treatment (AOM group; Fig. 2; panel 2C). However, treatment of these AOM-injected rats with either 5-FU (Fig. 2; panel 3C), paricalcitol (Fig. 2; panel 4C), or 5-FU plus paricalcitol (Fig. 2; panel 5C) had attenuated the development of such large ACF and tubular adenomas; and the highest attenuating effect was observed with 5-FU/paricalcitol dual therapy. Taken together, these results indicate that therapy with paricalcitol not only attenuates colorectal cancer initiation, but is also able to enhance the chemopreventive efficacy of 5-FU in this disease modality.

qRT-PCR findings
To provide mechanistic insights into the observed chemopreventive effects of paricalcitol and 5-FU therapy on this rodent model of the early stages of colorectal cancer, gene expression study was conducted by qRT-PCR to determine the relative mRNA expression patterns of Wnt, β-catenin, NF-κB, and COX-2 pro-oncogenes that have important role in colorectal cancer development and progression, as well as of DKK-1 and CDKN-1A as examples of well-established colorectal cancer-suppressive genes. As illustrated in Fig. 3, there was a significant upregulation in the mRNA expression of Wnt, β-catenin, NF-κB, and COX-2 genes, and a significant decrease in the in the mRNA expression of DKK-1 and CDKN-1A in AOM group, in comparison with normal control group. In contrary, treatment of the diseased animals with paricalcitol and 5-FU had synergistically cooperated to modify the altered mRNA expression patterns of these target genes (Fig. 3).
**ELISA and IHC findings**

To further explore the possible underlying mechanisms that could mediate the observed chemopreventive effects of paricalcitol and 5-FU therapy, we quantitatively measured the protein concentrations of β-catenin, COX-2, HSP-90, and VEGF by ELISA in the colorectal tissue homogenates of the different groups. Compared with the normal control group, the concentrations of β-catenin (Fig. 4A), HSP-90 (Fig. 4B), COX-2 (Fig. 4C), and VEGF (Fig. 4D) were significantly elevated in the colorectal tissue homogenates of AOM group. However, the concentrations of these candidate molecules were significantly reduced when these AOM-injected rats were treated with either paricalcitol or 5-FU.

Interestingly, cotherapy with paricalcitol and 5-FU had resulted in more reduction in the colorectal levels of β-catenin, COX-2, HSP-90, and VEGF proteins compared with paricalcitol or 5-FU monotherapy (Fig. 4).

Data of immunohistochemical assays (Fig. 5) were also in symmetry with ELISA results and demonstrated low expression of β-catenin (Fig. 5; panels 1A and 1F), iNOS (Fig. 5; panels 2A and 2F), and HSP-90 (Fig. 5; panels 3A and 3F) in the colorectal tissues of normal controls. In contrast, a marked expression of either β-catenin (Fig. 5; panels 1B and 1F), iNOS (Fig. 5; panels 2B and 2F), or HSP-90 (Fig. 5; panels 3B and 3F) was observed in the colorectal tissues of rats injected with AOM and left

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**Figure 2.**
Representative photos of macroscopic and microscopic appearance of colorectal mucosa of (1) normal control group, (2) AOM group, (3) AOM/5-FU group, (4) AOM/paricalcitol group, and (5) AOM/5-FU/paricalcitol group. The colorectal mucosae of the different groups were examined by gross (panel A), dissecting microscopy following staining with 0.2% methylene blue (panel B), and light microscopy at magnifications ×100 and ×200 following staining with H&E (panel C). Black arrowhead, gross tumors observed by naked eye; yellow arrow, large ACF (>4 crypts/focus). Pcal, paricalcitol.
without treatment, particularly in the glandular epithelium, and the combination treatment with 5-FU and paricalcitol was synergistically interacted to diminish the expression of these procancerous proteins in the colorectal tissues of AOM-injected rats (Fig. 5; panels 1E, 2E, and 3E and 3F, respectively).

The biochemical findings

The synthesis of vitamin D analogues, including paricalcitol, was initiated to achieve the therapeutic properties of calcitriol but precluded its hypercalcemic side effects (8, 9). In agreement, the biochemical analyses of the current study (Table 2) did not show any significant differences in the serum levels of calcium, liver function enzymes, and renal function parameters among the different animal groups, confirming the noncalcemic property of the applied paricalcitol dosage regimen, also suggesting the hepato-renal safety of paricalcitol/5-FU combination therapy.

Discussion

5-FU, either alone or in combination with other chemotherapeutic agents, remains the standard drug in the treatment of human colorectal cancer; however, low response rates and development of resistance to 5-FU therapy still represent a major challenge (2–4). On the other hand, the potential antitumor properties of paricalcitol, a synthetic less calcemic vitamin D analogue directly activates VDR, have recently attracted a specific deal of attention (9, 10, 13–16). Herein, an intermediate-term (15 weeks) model of colorectal tumorigenesis was induced in rats by AOM, a commonly used in vivo model for the experimental study of human colorectal cancer at several aspects (18–20); our study was designed to investigate the chemopreventive efficacy of paricalcitol and 5-FU alone and in combination, and whether their cotherapy resulted in an enhanced chemopreventive effect than individual therapy on this model. Interestingly, in comparison with their monotherapy, cotherapy with paricalcitol and 5-FU had resulted in augmenting effects in inhibiting the formation of colorectal tumors in AOM-injected rats.
of preneoplastic large ACF and the grade of cellular dysplasia, and in reducing the number of grown colorectal tumors, which collectively are the main macroscopic and microscopic features during the short- to intermediate term of AOM model (18–20).

Moreover, paricalcitol and 5-FU had also synergized to modulate a number of molecular pathways and candidate molecules that their dysregulations play crucial roles in the development and progression of human and experimental colorectal cancer disease such as Wnt/β-catenin pathway (24, 25), DKK-1 (26–29), CDKN1A (30, 31), NF-κB (32–34), COX-2 (35), iNOS (33), VEGF (35), and HSP-90 (2, 36). In turn, our findings not only support the importance of paricalcitol as an adjuvant agent in cancer therapy (10, 12–16), but also are in harmony with the hypothesis that vitamin D or its analogues improve tumor cell sensitivity and tumoricidal efficacy of 5-FU (6, 7).

Development and progression of colorectal cancer is multigenic and heterogeneous in origin, and also has clinical importance as predictors of disease prognosis and treatment response (37). In this view, aberrant activation of Wnt/β-catenin signaling pathway is highly implicated in the induction and dissemination of most human cancers, including colorectal cancer (24), and significant allocation in the development of 5-FU and multidrug resistance in human cancer therapy (38, 39). Mechanistically, upon binding with their target plasma membrane receptors/coreceptors, Wnt proteins induce a series of downstream signaling events resulting in β-catenin dephosphorylation and stabilization. This allows β-catenin, the main effector protein of this signaling, to translocate into the nuclei wherein it stimulates the transcription of several oncogenes, particularly in cells derived from the intestinal crypts (25, 40). At this point, approximately 80% of all human colorectal cancers have aberrant overactivations in Wnt/β-catenin signal and its downstream components in the colorectal cells (25). On the other hand, the expression pattern of Dkk-1, an inhibitor of Wnt/β-catenin pathway, by blocking Wnt signaling receptor complexes and contributing to colon cancer suppression, is remarkably downregulated in the colonic biopsies of colorectal cancer patients, and its downregulation is disclosed as a biomarker of chemoresistance and poor clinical outcome (25–29).

Although the overall available data still have discrepancies, Dkk-1 has been found to not only act as an inhibitor of Wnt/β-catenin signaling but also has additional β-catenin–independent tumor suppressor, antiangiogenesis, and antimetastasis actions in colorectal cancer disease (27, 41). Likewise, CDKN1A, a tumor suppressor gene encoding a potent cell-cycle inhibitory factor (CDKN1A, p21, or CIP1), is downregulated or even lost in most colorectal cancer cases (30), and some colorectal
cancer patients have anti-CDKN1A autoantibodies in their colorectal tissues (31). On the basis of these facts, it is conceivable that targeting these crucial colorectal tumorigenesis pathways, through repression of Wnt/β-catenin activity and/or stimulation of Dkk-1 and CDKN-1A, may hold tremendous therapeutic potential in treating colorectal cancer and other cancers, and in enhancing the cytotoxic effects of chemotherapeutic agents (25, 41). Interestingly, data of the current study are in agreement and showed that cotherapy with paricalcitol and 5-FU significantly interacted to repress the overexpressed Wnt and β-catenin, and upregulated the decreased Dkk-1 and CDKN-1A in the colorectal tissues of the chemically induced colorectal cancer model, suggesting a cooperative mechanism between the two drugs that, in part, might be behind their observed tumoricidal effect. In support of our observations, paricalcitol therapy has previously shown to mediate blockade of Wnt/β-catenin signaling (40) and upregulate CDKN-1A (Ref. 14), and 1,25(OH)2D3 (the most active form of vitamin D) is a multilevel repressor of Wnt/β-catenin signaling pathway and its downstream target proinflammatory and oncogenes (42). Furthermore, Aguilera and colleagues (28) reported that in a dose- and VDR-dependent manner, 1α,25-dihydroxyvitamin D3 activates the human DKK-1

Figure 5. Findings of immunohistochemical assays demonstrate the modulatory effects of paricalcitol (Pcal), 5-FU, and their combinations on the protein concentrations of β-catenin (A), HSP90 (B), and iNOS in the colorectal tissues of azoxymethane (AOM)-induced rat colorectal tumors (C). Data are represented as mean ± SD. a, P < 0.01 versus normal controls; b, P < 0.05 versus normal controls; c, P < 0.05 versus AOM group; d, P < 0.05 versus AOM/5-FU group; e, P < 0.05 versus AOM/Pcal group; and f, P < 0.01 versus AOM group.
gene promoter with subsequent high expression levels of DKK-1 RNA and protein in different phenotypes of human colon cancer cells. Furthermore, in vivo therapy with a less calcemic vitamin D analogue in a xenografted model of colorectal cancer in immuno-deficient mice has resulted in antitumor action associated with significant induction of DKK-1 gene transcription and inhibition of Wnt/β-catenin signaling (28).

Indeed, human colorectal cancer is a life-threatening complication of inflammatory bowel diseases with a complex pathogenesis, in which NF-κB, COX-2, and iNOS may provide a crucial mechanistic link between inflammation and carcinogenesis (32, 33). With this concept, aberrant activation of NF-κB has been shown to regulate the expression of many tumorigenesis genes involved in cellular transformation, proliferation, inflammation, angiogenesis, invasion, metastasis, and numerous other potentially carcinogenic processes (43). NF-κB induces COX-2 gene expression and drug resistance genes (10), and COX-2 upregulation directly correlates with colorectal cancer progression and poor prognosis in human patients (35). iNOS levels and activities are also increased in human adenocarcinomas and in colon tumors chemically induced in rats, to induce chronic inflammation and to create a microenvironment that favors colon carcinogenesis (33). Moreover, angiogenesis mediated by VEGF and other pivotal angiogenic facilitators plays a crucial role in development, neovascularization, progression and metastasis of colorectal cancer and other human cancers, and COX-2 appears to be importantly involved with multiple aspects in this phenomenon, particularly by overexpressing VEGF (35). Coherently, suppression of NF-κB, iNOS, COX-2, and VEGF could represent therapeutic potential in colorectal cancer therapy (35, 43). In harmony, data of the current study showed that paricalcitol and 5-FU alone or in combination significantly decreased the expression of NF-κB, iNOS, COX-2, and VEGF in the colorectal tissues of diseased rats, and their combination was cooperatively acted to further downregulate these tumorigenesis molecules. In agreement with our findings, several lines of evidence have suggested the robust capacity of either vitamin D or its analogues, including paricalcitol, to disrupt NF-κB, COX-2, VEGF, and/or iNOS-dependent inflammation, tumor promotion, and carcinogenesis. For instance, paricalcitol treatment had suppressed COX-2 and NF-κB expression in colon, leukemic, and gastric cancer cells (10, 44), and attenuated VEGF in renal diseases (45). In a constant line, vitamin D has intrinsically shown to inhibit NF-κB activity, reduce NF-κB protein levels, and downregulate a variety of NF-κB target genes in a variety of inflammatory and cancer cell types (46, 47). Mechanistically, activation of VDR by vitamin D or its analogues results in VDR/RXα kinase beta protein (IKKβ) physical interaction and stimulation/stabilization of the NF-κB inhibitory protein α (IκBα), all of which are responsible for blocking NF-κB-binding to DNA and inhibition of its canonical activation pathway (47).

Similarly, earlier in vitro studies on cancer cells revealed that 5-FU exerts direct inhibitory effect on NF-κB and on nitric oxide production mediated by NF-κB (48, 49). Administration of selective inhibitors of NF-κB (34), or COX-2 (50) had previously seen to augment the antitumor effects of 5-FU on colon cancer, and was also associated with suppression of VEGF and the tumor vessel density (50). In short, we may speculate that there was a direct and indirect crosstalk between paricalcitol and 5-FU in inhibiting NF-κB and concomitantly in repressing the colorectal tumorigenesis effects of iNOS, COX-2, and VEGF.

Notably, the potential carcinogenic role of HSP-90 in activation of various oncogenic proteins and angiogenesis is a topic of intense interest, particularly in colorectal cancer, which is why specific HSP90 inhibitors are currently being investigated as potential anticancer drugs (36). More importantly, HSP90 dependence acquired resistance to 5-FU therapy, as well as its potential role as a proangiogenic factor for the induction of VEGF and iNOS for de novo angiogenesis has been reported recently (2). With this concept, treatment of 5-FU-resistant colon cancer cells with selective HSP90 inhibitors had repressed primary colorectal tumor growth, circulation in the blood, and metastatic tumor development (2). Remarkably, our data are consistent and showed a significant increase in HSP90 expression in the AOM group compared with normal controls, and cotreatment with paricalcitol and 5-FU resulted in a more significant repression on HSP90 expression compared with their monotherapy. On the basis of our findings and on the given potential carcinogenic role of HSP-90 in colorectal cancer, we therefore reasoned to hypothesize that paricalcitol could promote the efficacy of 5-FU and the regression of the chemically induced colon cancer partly by downregulating the expression of HSP90, although the current available data on the possible effects of vitamin D or its analogues on this protein in colorectal cancer or other cancers are limited.

In conclusion, this study suggests that paricalcitol augments the therapeutic efficacy of 5-FU on colorectal cancer disease by modulating several pro- and anticancerous molecules that have important roles in the regulation of colorectal tumor cells’ DNA damage, growth, differentiation, inflammation, angiogenesis, and metastasis. These molecular pathways include Wnt/β-catenin pathway, NF-κB, COX-2, iNOS, VEGF, and HSP90. In this context, our findings have therapeutic impact considering ongoing clinical development of paricalcitol/5-FU combination therapy for the treatment of colorectal cancer patients; however, further studies are warranted to...
evaluate this therapeutic combination and also to explore its precise antitumor mechanisms.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.G. El-Shemi, B. Refaat
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Paricalcitol Enhances the Chemopreventive Efficacy of 5-Fluorouracil on an Intermediate-Term Model of Azoxymethane-Induced Colorectal Tumors in Rats

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