Neoplasia • 7(1):85-90,2004,
www.neoplasia.com

CF101, An Agonist to the A_3 Adenosine Receptor, Enhances the Chemotherapeutic Effect of 5-Fluorouracil in a Colon Carcinoma Murine Model

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Abstract

NF-κB and the upstream kinase PKB/Akt are highly expressed in chemoresistance tumor cells and play a major role in hampering the apoptotic pathway. CF101, a specific agonist to the A_3 adenosine receptor (A_3AR), inhibits the development of colon carcinoma growth in cell cultures and xenograft murine models. Because CF101 has been shown to downregulate PKB/Akt and NF-κB protein expression level, we presumed that its combination with chemotherapy will enhance the antitumor effect of the cytotoxic drug. In this study, we utilized MTT and colony formation assay *in vitro* and a xenograft model in nude mice. It has been shown that a combined treatment of CF101 and 5-fluorouracil (5-FU) enhanced the cytotoxic effect of the latter on HCT-116 human colon carcinoma cell proliferation and tumor growth. Downregulation of PKB/Akt, NF-κB, and cyclin D1, and upregulation of caspase-3 protein expression level were observed in cells and tumor lesions upon treatment with a combination of CF101 and 5-FU. Moreover, in mice treated with the combined therapy, myelotoxicity was prevented as was evidenced by normal white blood cell and neutrophil counts. These results support the notion that CF101 potentiates the cytotoxic effect of 5-FU, thus preventing drug resistance. The myeloprotective effect of CF101 grants the molecule an added value and suggests its development as a supportive treatment to 5-FU.

Neoplasia (2004) **6**

Keywords: A_3 adenosine receptor agonist, colon carcinoma, 5-FU, NF-κB, PKB/Akt.

Introduction

Colorectal cancer is one of the most common human malignancies and is considered as the leading cause of cancer deaths. Surgery is the primary treatment option; however, depending on the tumor stage and the involvement of lymph nodes, 50% of patients will experience metastatic disease progression [1]. Chemotherapy, mainly 5-fluorouracil (5-FU) and leucoverin, is given to patients both in the adjuvant setting and upon occurrence of metastasis. The mechanism of action includes the metabolism of 5-FU to 5-fluoro-2′-deoxyuridine monophosphate. The latter inhibits the activity of thymidylate synthase, thereby decreasing dTTP pools, leading to inhibition of DNA synthesis and G_1/S cell cycle arrest [2]. As a single agent, 5-FU is only modestly active, producing a response rate of 15% in advanced colorectal cancer. The standard protocol today is its administration with leucoverin, which increases the cytotoxic effect of 5-FU and avoids resistance to its thymidylate synthase–inhibitory effects [3]. Adverse events upon 5-FU treatment, which include damage to the bone marrow, skin, mucous membranes, intestinal tract, and central nervous system, and cardiotoxicity, are frequently recorded [4,5]. Today, additional combinations of 5-FU with oxaliplatin, irinotecan, paclitaxel, interferon-α, or suramin are given as experimental protocols [6–9].

A_3 adenosine receptor (A_3AR) belongs to the family of the G_i protein–associated cell surface receptors and it is highly expressed on the membrane of various tumor cell types [10–12]. CF101, a synthetic A_3AR agonist, exerts a differential effect on tumor and normal cells. It inhibits *in vitro* the growth of various solid tumor cells and suppresses the development of melanoma, colon, and prostate carcinoma in experimental murine models [13–15]. A major mechanism involved with the antitumor effect of CF101 is deregulation of the NF-κB signal transduction pathway. It was found that CF101 inhibits the expression of PKB/Akt and NF-κB, followed by a decrease in the binding of NF-κB to its DNA consensus sequence [16].

As a result, the transcription of gene products such as cyclin D1 and c-Myc is downregulated [17,18].

It has been shown earlier that in tumor cells, high levels of NF-κB and the upstream kinase PKB/Akt are known to act as inhibitors of apoptosis, thus limiting the effect of chemotherapy and leading to the development of drug resistance [19,20].

Interestingly, in parallel to its anticancer effect, CF101 acts as a myeloprotective agent, through the induction of granulocyte colony-stimulating factor (G-CSF) production [21]. Moreover, recent studies showed that A_3AR agonists exert cardioprotective and neuroprotective effects [22]. Based on

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Received 3 June 2004; Revised 30 June 2004; Accepted 6 July 2004.

DOI 10.1593/neo.04364

the above molecular mechanism of CF101 and its protective effects on normal body systems, the present study was undertaken to examine the ability of CF101 to enhance the cytotoxic effect of 5-FU on HCT-116 colon carcinoma cells both *in vitro* and *in vivo*. The data obtained in this study show that CF101 potentiates the cytotoxic effect of 5-FU by downregulation of the NF-κB signaling pathway. In addition, CF101 prevents myelotoxic effects of 5-FU by rescuing white blood cells (WBCs) and neutrophils.

Materials and Methods

Drugs

The A_3AR agonist known generically as 1-deoxy-1-[6-[[(iodophenyl)methyl]amino]-9*H*-purine-9-yl]-*N*-methyl-(-D-ribofuranuronamide) (CF101), a GMP grade, was synthesized for Can-Fite BioPharma by Albany Molecular Research, Inc. (Albany, NY). A stock solution of 10 mM was prepared in DMSO, and further dilutions in RPMI medium for *in vitro* studies or in PBS for *in vivo* studies were performed. RPMI, fetal bovine serum (FBS), and antibiotics for cell cultures were purchased from Beit Haemek (Haifa, Israel). Primary antibodies included rabbit polyclonals against the human cell growth–regulatory proteins phosphorylated PKB/Akt (p-PKB/Akt), NF-κB, cyclin D1, and caspase-3. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and served as primary antibodies.

The chemotherapeutic agent 5-fluorouracil (5-FU) was purchased from ABIC (Israel). 3-[4,5-yl]-2,5-Dephenyltetrazolium bromide for the MTT assay was purchased from Sigma (St. Louis, MO).

Tumor Cells

HCT-116 human colon carcinoma cells were used. Cells were maintained in RPMI medium supplemented with 10% FBS, 200 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were transferred to a freshly prepared medium twice weekly.

MTT Assay

To determine the cytotoxic effect of 5-FU on HCT-116 colon carcinoma cells, different drug concentrations in the range of 0.625 to 10 μM were used. EC_{50} was found at a concentration of 2.5 μM. The efficacy of a combined treatment of 5-FU + CF101 *vs* 5-FU alone was examined in the HCT-116 colon carcinoma cell line. Cells (5×10^4 ml^{-1}) were incubated with 1.25 and 2.5 μM 5-FU in 96-well microtiter plates. After 48 hours, CF101 at a concentration of 10 nM was added to the culture system. At the end of the incubation period (72 hours total), MTT assay was used. MTT stock solution (5 mg/ml) was added (1:10) to the culture system and incubated for 4 hours. Then the culture medium was removed and MTT solvent (0.05 N HCl in isopropanol) was added to the culture in an amount equal to the original volume. Absorbance of the converted dye was measured at 570 nm.

Colony Formation Assay

Exponentially growing HCT-116 cells were seeded at a concentration of 1500 cells per 2-cm Petri dish and treated with 5-FU (2.5 μM) or 5-FU + 10 nM CF101. Medium was exchanged every 3 days. After 10 days, the cells were fixed and stained with Giemsa (diluted 1/10 in PBS). Colonies containing more than 50 cells were counted. For each treatment, three Petri dishes were scored and the study was repeated three times.

Protein Analysis by Western Blot Analysis

To assess the effect of the combined therapy (5-FU and CF101) on the protein expression level of some cell growth–regulatory proteins, HCT-116 cells were cultured in 10-cm tissue culture plates (5×10^4/ml) with 2.5 μM 5-FU for 72 hours. After 48 hours, CF101 at a concentration of 10 nM was added to the culture system. At the end of the incubation period, cell samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer, pH 7.5, 150 mM NaCl, NP 40). The trypsinized cells were washed again with ice-cold PBS, harvested by centrifugation, and subjected to lysis in TNN buffer. Cell debris was removed by centrifugation for 10 minutes at 7500*g*. The supernatant was utilized for WB analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were blocked with 1% bovine serum albumin and incubated with the relevant primary antibody (dilution 1:1000) for 24 hours at 4°C. Blots were then washed and incubated with the secondary antibody for 1 hour at room temperature. Bands were recorded using a color development kit (Promega, Madison, WI).

In Vivo Studies

All the experiments were performed in accordance with the UKCCCR guidelines (Workman et al., 1998) and Can-Fite Animal Care and Use Committee (Petach-Tikva, Israel). Nude male Balb/C mice, aged 2 months and weighing an average of 20 g, were obtained from Harlan Laboratories (Jerusalem, Israel) and maintained on a standardized pelleted diet and supplied with tap water.

The effect of 5-FU in combination with CF101 on the growth of human HCT-116 colon carcinoma cells in a xenograft model was assessed. Cells (2×10^6) were subcutaneously injected to the flank of nude/BalbC mice. When tumors reached a size of ~150 mm^3, the mice were divided randomly into three groups and treatment was initiated. Each group contained 10 mice and the experiment was repeated three times.

The following treatment protocol was utilized:

1. Control group—vehicle only
2. 5-FU—one cycle of intraperitoneal 5-FU (25 mg/kg), given once a day for five consecutive days

3. 5-FU + 10 μg/kg body weight CF101—CF101 treatment was initiated 24 hours after the last 5-FU injection until study termination.

Tumor size (width [*W*] and length [*L*]) was measured twice weekly and was calculated according to the following formula:

$$\text{Tumor size} = \frac{W^2 L}{2}$$

To test the myeloprotective effect of CF101, blood samples were withdrawn 48 hours after the initiation of CF101 treatment.

At the end of the study, tumor lesions from the three groups were excised and homogenized (Polytron; Kinematica), and protein was extracted. WB analysis was carried out to determine the expression level of various cell growth–regulatory proteins.

Blood Cell Counts

WBC counts were carried out in a Coulter counter and neutrophil counts were performed on smear preparations stained with May Grunvald-Giemsa solution.

Statistical Analysis

The efficacy of the various agents in the *in vitro* and *in vivo* study was evaluated using the Student's *t* test. The criterion for statistical significance was $P < .05$.

Results

Effect of 5-FU, in Combination with CF101, on the Growth of HCT-116 Colon Carcinoma In Vitro and In Vivo

5-FU exerted an inhibitory effect on the growth of HCT-116 cells *in vitro* as was measured by the thymidine incorporation assay. The EC_{50} of 5-FU was detected at a drug concentration of 2.5 μM (Figure 1*A*). The combined treatment of 5-FU + CF101 yielded higher cell growth inhibition in comparison to the chemotherapy alone (Figure 1*B*). In cells treated with 2.5 μM 5-FU alone or in combination with 10 nM CF101, colony formation was inhibited by 32.5 ± 4.87% and 57.3 ± 8.59%, respectively (Figure 2, *A* and *B*). In a different set of experiments, we showed that treatment with 5-FU + CF101 yielded a higher inhibitory effect (48 ± 7.2%) on the growth of HCT-116 human colon carcinoma xenografts in nude mice in comparison to 5-FU alone (29 ± 4.35%, $P < .001$) (Figure 3).

CF101 Prevents the Myelotoxic Effect of 5-FU

Mice treated with 5-FU alone exhibited a 42.9 ± 0.6% decline in the number of WBC and a 81 ± 0.9% decline in neutrophil count. The administration of CF101 following 5-FU treatment counteracted the myelotoxic effect of 5-FU, resulting in a 13.7 ± 0.5% decline in the number of WBC and a 29.1 ± 0.1% decline in neutrophil counts (Figure 4, *A* and *B*).

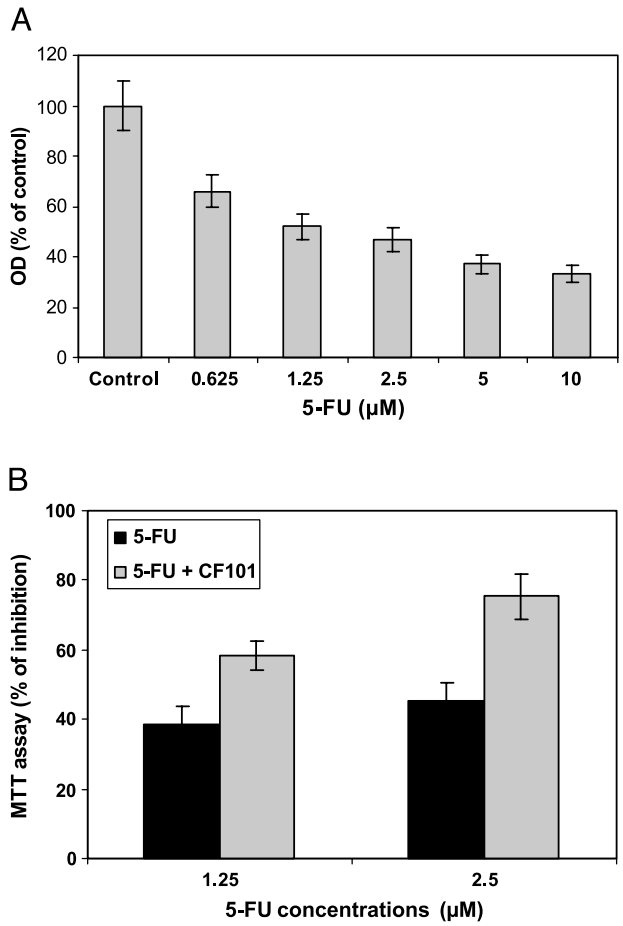

Figure 1. *Effect of 5-FU + CF101 on the growth of colon carcinoma cells in vitro. (A) HCT-116 human colon carcinoma cells were incubated with different 5-FU concentrations for 72 hours. An MTT assay was preformed to detect cell growth. (B) HCT-116 human colon carcinoma cells were incubated with 5-FU (1.25 and 2.5 μM) in the presence or absence of 10 nM CF101 for 72 hours. An MTT assay was preformed to detect cell growth.*

Effect of 5-FU + CF101 on the Expression Level of Cell Growth–Regulatory Proteins in HCT-116 Human Colon Carcinoma Cells and Tumor Lesions

To explore the molecular mechanism involved with the enhancement of the chemotherapeutic effect by CF101, we assessed the expression of the cell growth–regulatory proteins PKB/Akt, NF-κB, and cyclin D1. These proteins are known from our former studies to be modulated upon CF101 treatment. Because caspase-3 is located downstream to PKB/Akt and is upregulated prior to apoptosis, its protein expression level was assessed as well.

A similar protein profile was detected in protein extracts from cell culture and tumor lesions depicting downregulation of PKB/Akt, NF-κB, and cyclin D1 upon treatment with 5-FU + CF101 in comparison to 5-FU. The level of caspase-3 was upregulated in the combined treatment (Figure 5). These results support the notion that CF101 acts *in vitro* and *in vivo* through a similar molecular mechanism to mediate the enhancement of 5-FU effects.

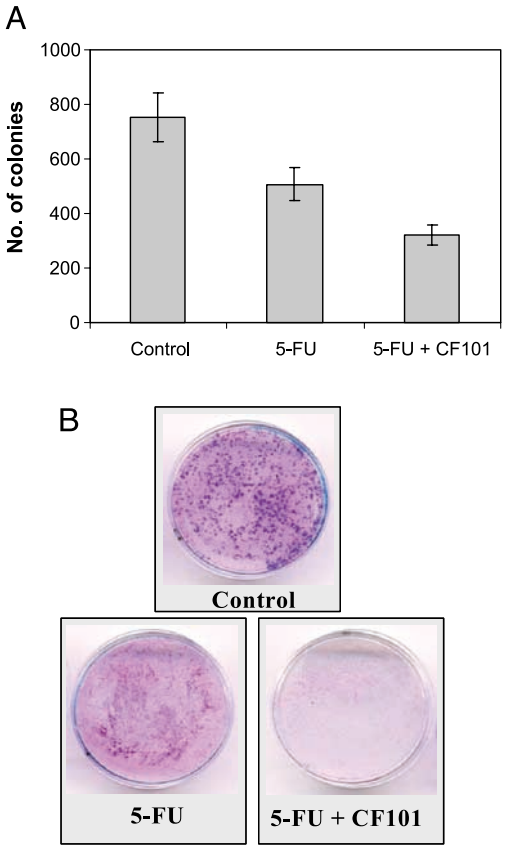

Figure 2. *Effect of CF101 + 5-FU on the development of colon carcinoma colony development in vitro. HCT-116 cells were incubated with 5-FU in the presence and absence of 10 nM CF101. Medium was exchanged every 3 days and the cells were allowed to form colonies over a period of 10 days. At the end of the experiment, the plates were stained with Giemsa and colonies greater then 50 cells were counted.*

Discussion

This study presents data showing that a combined treatment of 5-FU + CF101 potentiates the inhibitory effect of the cytotoxic drug both *in vitro* and *in vivo*.

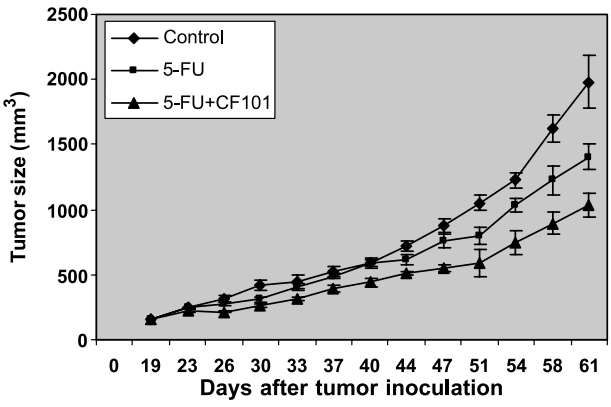

Figure 3. *Effect of CF101 + 5-FU on the growth of HCT-116 human colon carcinoma cells in nude mice. HCT-116 human colon carcinoma cells (2×10^6) were subcutaneously injected to the flank of nude mice. Treatment with 5-FU alone (25 mg/kg, i.p., once daily for five consecutive days), or in combination with CF101 (10 μg/kg, p.o., twice daily), was initiated when the tumor reached a size of ~ 150 mm^3. Tumor size was measured twice weekly.*

In vitro, we utilized two assays: the first entailed the MTT assay, which represents cytotoxic effects; and the second examined the ability of the colon carcinoma cells to form colonies, simulating the establishment of tumor lesions *in vivo*. It was shown that CF101 enhanced the cytotoxic effects as well as the antiproliferative effects of 5-FU. It was further noted that in HCT-116 tumor-bearing mice, the combined treatment of 5-FU + CF101 yielded higher tumor growth inhibition than chemotherapy alone, confirming the ability of CF101 to potentiate 5-FU effects.

To explore the underlined molecular mechanism, we followed up the protein expression level of cell growth–regulatory proteins known to be responsible for tumor cell resistance to chemotherapy.

Overexpression of PKB/Akt and NF-κB in tumor cells plays a role in cell resistance to chemotherapeutic agents, resulting in failure of the tumor cells to undergo apoptosis. PKB/Akt, a cell survival kinase, is located upstream to NF-κB and is activated in a broad spectrum of neoplasms [23]. PKB/Akt is known to control the NF-κB level by phosphorylating downstream proteins (IKK and IκB), which in turn release NF-κB from its complex [24]. NF-κB then translocates to the nucleus where it induces the transcription of growth factors including TNF-α and the oncogene cyclin D1 [25]. The latter was found to be directly related to chemoresistance. In SW620 colon carcinoma cells overexpressing cyclin D1, resistance to apoptosis induced by cisplatin was observed using microarray analysis [26]. Moreover, inhibition of cyclin D1 expression was shown to modulate the effect of antineoplastic drugs through alterations in the expression

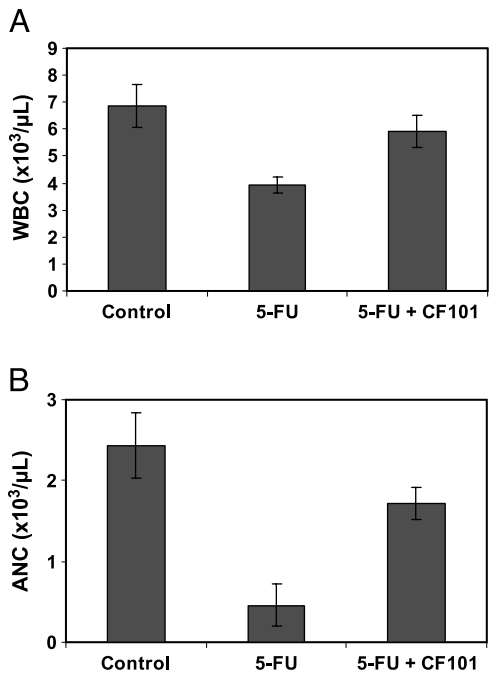

Figure 4. *Effect of CF101 + 5-FU treatment on the number of WBC and ANC in colon carcinoma–bearing mice. HCT-116 human colon carcinoma tumor–bearing mice were treated with 5-FU alone (25 mg/kg, i.p., once daily for five consecutive days), or in combination with CF101 (10 μg/kg, p.o., twice daily, starting 48 hours after last 5-FU treatment). The number of WBC and neutrophils was analyzed.*

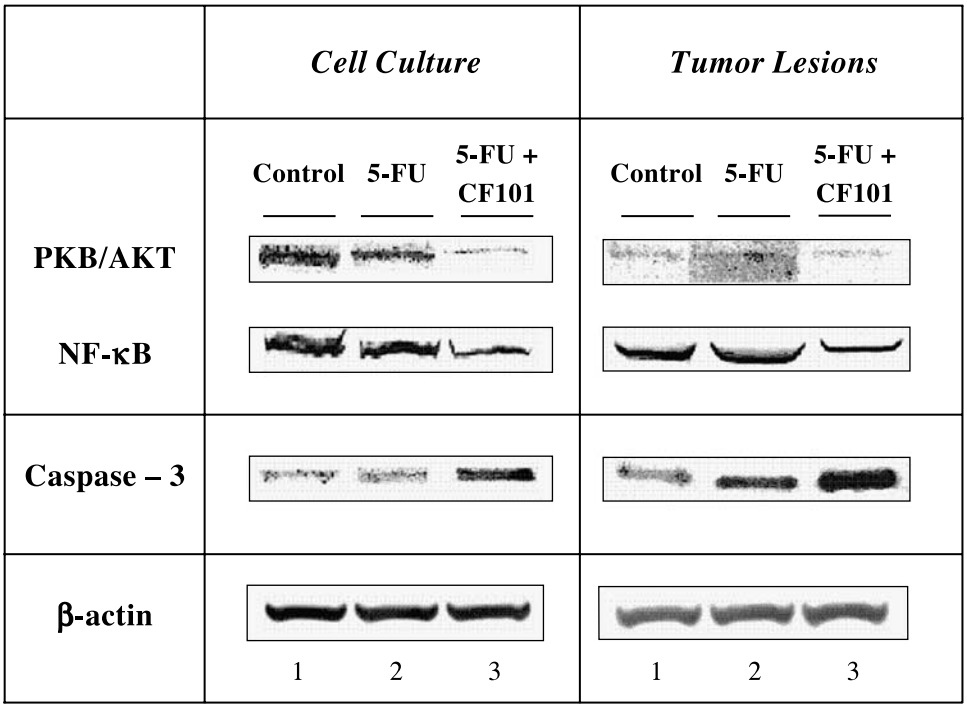

Figure 5. *Effect of CF101 + 5-FU treatment on the expression of cell growth–regulatory proteins in colon carcinoma cells. Immunoblots showing the effect of 5-FU alone, or in combination with CF101, on the expression level of PKB/Akt, NF-κB, cyclin D1, and caspase-3 in HCT-116 cell cultures (left) or tumor lesions (right) are presented. Culture conditions and experimental colon carcinoma model are as described in legends to Figures 1 and 3, respectively.*

level of drug resistant genes. Kornmann et al. demonstrated that suppression of cyclin D1 expression after a stable transfection with cyclin D1 antisense construct in PANC-1 and COLO-357 human pancreatic cancer cells resulted in decreased levels of the chemoresistance gene products MDR-1 and MRP. This led to a significant increase in sensitivity to 5-FU and mitoxantrone [27].

Furthermore, Uetsuka et al. [28] have shown that elimination of NF-κB activity induced apoptosis and reduced chemoresistance to 5-FU. It is thus clear that upregulation of NF-κB is directly and indirectly linked to cell proliferation and inhibition of apoptosis. PKB/Akt controls additional apoptotic pathways including GSK-3β, caspase-9, and caspase 3, which, upon PKB/Akt activation, are modulated, thereby failing to activate pathways leading to apoptosis [29]. Evidence to support the role of PKB/Akt in controlling the resistance of tumor cells to apoptosis came from studies in which transfection of constitutively active PKB/AKT in lung cancer cells reduced topotecan-induced apoptosis [30], whereas inactivation of PKB/AKT enhanced apoptosis induced by SN-38, doxorubicin, and etoposide [31–33].

In the present study, treatment of HCT-116 cells with 5-FU + CF101 resulted in decreased levels of PKB/Akt, NF-κB, and cyclin D1, whereas the apoptotic protein caspase-3 was upregulated. A similar pattern of the response was shown in tumor lesions excised from the 5-FU + CF101–treated mice, confirming that the above signaling proteins are involved with the enhanced chemotherapeutic effect of CF101.

We have recently shown that CF101, given as a monotherapy to melanoma-, colon carcinoma–, or prostate carcinoma–bearing mice, inhibited tumor development through the downregulation of NF-κB and cyclin D1. These data strengthen the results reported in the present study and enlighten the molecular mechanism described above.

Myelotoxicity, mainly granulocytopenia, is dose-limiting in most chemotherapeutic drug treatments, including 5-FU [34]. In this study, we show that CF101, on top of its capability to potentiate the antitumor effect of 5-FU, prevented the decrease in WBC and neutrophils upon 5-FU treatment. Our earlier studies have shown that CF101 induces the production of G-CSF by mononuclear cells. Interestingly, this characteristic of CF101 was found to be mediated through the upregulation of NF-κB—the only transcription factor of G-CSF. This was concluded based on *ex vivo* data showing that in splenocytes derived from CF101-treated mice, upregulation of PI3K, PKB/Akt, IKK, and IkB occurred, leading to increased NF-κB level and G-CSF production.

It thus seems that in the present study, a differential effect of CF101 on tumor and normal cells is observed and is most probably mediated through the ability of CF101 to inhibit NF-κB levels in tumor cells and stimulate it in normal cells (such as G-CSF–producing cells). To conclude, CF101, an orally bioavailable molecule, potentiates the cytotoxic effect of 5-FU toward tumor cells through a mechanism that ''opens the gate'' for apoptosis to occur. Concomitantly, CF101 prevents the myelotoxic effect of chemotherapy by rescuing the number of WBC and neutrophils. CF101 may be suggested as an ''add on'' to 5-FU in the treatment of colon carcinoma.

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