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# 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 may hamper the apoptotic pathway. CF101, a specific agonist to the $A_3$ adenosine receptor, 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 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays and a colon carcinoma xenograft model. 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 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 on 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 show that CF101 potentiates the cytotoxic effect of 5-FU, thus preventing drug resistance. The myeloprotective effect of CF101 suggests its development as an add-on treatment to 5-FU.**

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## 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 ($A_3$AR) 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 $A_3$AR 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 $A_3$AR agonists exert cardioprotective and neuroprotective effects [22]. Based on the above molecular mechanism of CF101 and its protective

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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 $A_3$AR 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 (Petach-Tikra, 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 ($5 \times 10^4$ $ml^{-1}$) 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 ($5 \times 10^4$/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 (München, Germany) 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 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 ($2 \times 10^6$) 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