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The triterpenoid Cucurbitacin B augments the anti-proliferative activity of chemotherapy in human breast cancer

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Abstract

Despite recent advances in therapy, breast cancer remains the second most common cause of death from malignancy in women. Chemotherapy plays a major role in breast cancer management, and combining chemotherapeutic agents with non-chemotherapeutic agents is of considerable clinical interest. Cucurbitacins are triterpenes compounds found in plants of the Cucurbitaceae family, reported to have anti-cancer and anti-inflamatory activities. Previously, we have shown antiproliferative activity of cucurbitacin B (CuB) in breast cancer, and we hypothesized that combining CuB with chemotherapeutic agents can augment their anti-tumor effect. Here, we show that a combination of CuB with either docetaxel (DOC) or gemcitabine (GEM) synergistically inhibited the proliferation of MDA-MB-231 breast cancer cells *in vitro*. This antiproliferative effect was accompanied by an increase in apoptosis rates. Furthermore, *in vivo* treatment of human breast cancer orthotopic xenografts in immunodeficient mice with CuB at either low (0.5 mg/kg) or high (1 mg/kg) doses in combination with either DOC (20 mg/kg) or GEM (12.5 mg/kg) significantly reduced tumor volume as compared to monotherapy of each drug. Importantly, no significant toxicity was noted with low dose CuB in combination with either DOC or GEM. In conclusion, combination of CuB at a relatively low concentration with either of the chemotherapeutic agents, DOC or GEM, shows prominent antiproliferative activity against breast cancer cells without increased toxicity. This promising combination should be examined in therapeutic trials of breast cancer.

Keywords

Breast cancer; cucurbitacin B; chemotherapy

Introduction

Breast cancer is the second most common cause of cancer-related deaths in women (1). The goal of cancer therapy is to prolong survival while minimizing unwanted side effects. Chemotherapy continues to be an essential component of breast cancer regimens; however, resistance and toxicity represent major challenges, underscoring the need for new treatment

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options. Combining chemotherapeutic drugs with new agents may offer novel treatment options to increase survival and improve quality of life for breast cancer patients.

Docetaxel (DOC) and gemcitabine (GEM) are two chemotherapeutic drugs commonly used to treat breast cancer. The taxane agent DOC, is a microtubule inhibitor and has been commonly used either as a single agent in metastatic disease or in combination with other chemotherapeutic agents in early stages of breast cancer (2,3). GEM is a nucleoside analogue with proven activity in the advanced, metastatic stages of this disease (4,5). Cucurbitacins are triterpenes mainly produced by Cucurbitaceae Juss plants, comprising numerous traditional medicinal Chinese herbs. Cucurbitacin B (CuB) has antiproliferative activity against many cancer types including breast (6,7), pancreas (8–10), liver (11,12), brain (13), bone (14), skin (15) and head and neck (16), as well as, leukemia (17–19). In a human breast cancer orthotopic model, CuB reduced tumor size by 50% with no significant toxicities (7). Moreover, CuB in combination with chemotherapeutic agents in pancreatic, skin and head and neck cancers had additive antitumor activity without increased toxicity (8,9,15,16). This promising activity of CuB prompted us to investigate the effect and feasibility of combining CuB with chemotherapeutic agents, to achieve better tumor control with tolerable toxicity in breast cancer.

Materials and Methods

Cell culture and compounds

The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection and cultured in DMEM(Life Technologies), supplemented with 10% Fetal Bovine Serum (FBS) (HyClone,). CuB was generously provided by CK Life Sciences International (Holdings) Inc. (CKBP002) and also purchased from Sigma-Aldrich (C8499). The two sources of CuB showed similar efficacy and were used interchangeably. DOC and GEM were from Sigma-Aldrich and Eli Lilly, respectively.

Measurement of cellular proliferation

MDA-MB-231 cells (10^4 cells per well) were placed into 96-well plates and treated with either diluent control(PBS) or drugs as indicated in Fig. 1 legend. Cell proliferation was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to the manufacturer's instructions. The obtained values of 50% effective dose (ED_{50}) were expressed as the concentrations that corresponded to a reduction of cellular growth by 50% compared to control cells. Drug synergy was determined by the isobologram and combination index (CI) methods (CalcuSyn software, version 1.1.1 1996; Biosoft)(20). The isobologram method is a graphical representation of the pharmacologic interaction formed by selecting a desired fractional cell kill and plotting the required individual drug doses on their respective x and y axes. A straight line connects the points; and the observed dose combination of the two agents that achieves a particular fractional cell kill is plotted. Combination data points that fall on the line represent an additive drug-drug interaction, while data points that fall below or above the line represent either synergism or antagonism, respectively. A CI of 1 indicates an additive effect between two drugs, whereas a $CI < 1$ or $CI > 1$ indicates synergism or antagonism, respectively.

Apoptosis analysis

Apoptosis was measured using Annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol.

Murine orthotopic model and tumor treatment

All animal experiments were in accordance with the guidelines of Cedars-Sinai Research Institute and the National Institute of Health. Five-week-old female *nu/nu* athymic mice were obtained from Harlan Sprague-Dawley. MDA-MB-231 cells (10^6)in 0.2 mL Matrigel (Basement Membrane Matrix, High Concentration; BD Biosciences) were injected orthotopically in two mammary fat pads of each nude mouse (n = 5 mice/group). Treatments were initiated one day after cell implantation and were given intraperitoneally (i.p.). Tumors were measured with Vernier calipers every 3 days, and tumor volumes were calculated using the formula: $(\text{length} \times \text{width} \times \text{depth}) \times 0.5236$ (14). Mice were weighed twice a week. At sacrifice, tumors were harvested, weighed, fixed in 10% neutral-buffered formalin, washed and placed in 70% ethanol embedded in paraffin wax and stained with hematoxylin and eosin (H&E), TUNEL (apoptotic index) and ki-67 (proliferation index). Inhibition rate of tumor growth was calculated using the formula:
tumor growth inhibition rate $(\%)=(1-M_T/M_C) \times 100$, where M_T and M_C are the mean normalized tumor masses of treatment and control groups, respectively.

Toxicity studies

Serum chemistry and blood count analysis—Heparinized blood samples (40 μl/mouse) were obtained on day 0 and at the end of treatment (day 36) by submandibular bleeding. Final blood results were obtained by the Hemagen Analyst® Benchtop Chemistry System (Hemagen Diagnostics, Inc. Columbia, USA).

Colony formation assay—Bone marrow cells from femurs of all groups (3 mice/group) were plated in triplicates in a 6-well plate at 2×10^4 per well in semi-solid 1% methylcellulose medium (MethoCult GF M3434, StemCell Technologies) containing Stem Cell Factor, IL-3, IL-6, and erythropoietin. Number of colonies (BFU-E: Blast-forming unit-erythroid; CFU-GM: Colony-forming unit-granulocyte/macrophages; CFU-GEMM: Colony-forming unit granulocyte/ erythrocyte/ monocyte/megakaryocyte) were counted on day 10.

Exploration of internal organs—After sacrifice, internal organs were grossly examined for metastatic disease and suspicious lesions were excised, and examined for histopathology as described above for the primary tumors.

Statistical analysis

All in vitro experiments were repeated at least three times. Statistical significance was determined by Student's *t* test (two-tailed) when comparing two groups or ANOVA when comparing more than two groups of data set. *P* values < 0.05 were considered statistically significant.

Results

CuB in combination with either DOC or GEM synergistically inhibits proliferation of MDA-MB-231 cells

The effect of CuB, DOC and GEM on the proliferation of MDA-MB-231 cells is shown in Figs. 1A–C. The calculated ED50s were 5×10^{-8} mol/L for CuB, 10^{-8} mol/L for DOC, and 10^{-7} mol/L for GEM. Combinatorial treatment of CuB with either DOC or GEM showed significant growth inhibitory activity (Figs. 1D, E). Synergistic growth inhibition (CI < 0.9) was observed with the combination of DOC (10^{-9} and 5×10^{-9} mol/L) and CuB (10^{-9} or 10^{-8} mol/L), or with the combination of GEM (10^{-9} mol/L or 10^{-7} mol/L) with CuB (10^{-9} mol/L, 10^{-8} mol/L, or 5×10^{-8} mol/L) (Figs. 1F, G).

Effect of CuB in combination with either DOC or GEM on apoptosis in MDA-MB-231 cells

Annexin V/PI staining was used to measure the percent of apoptotic cells in response to the drug treatments (Fig. 2). After 24 hrs of exposure to either CuB, DOC or GEM, 9%, 13% and 4% of the cells were in the early stages of apoptosis, respectively, compared to 5% of control cells. Apoptosis increased in the cells exposed to the combination of CuB with either DOC (16%; $p = 0.049$) or GEM (17%; $p = 0.01$). The percent of apoptotic cells increased with prolonged (48 hrs) exposure to GEM in combination with CuB (71 % of the cells, data not shown).

CuB in combination with either DOC or GEM markedly inhibited the growth of MDA-MB-231 breast tumors in an orthotopic murine model

Next, the ability of the combination of CuB with either DOC or GEM to enhance the growth suppression compared to monotherapy of either DOC or GEM was examined *in vivo*. MDA-MB-231 cells were orthotopically implanted into the mammary fat pads of nude mice and mice were treated with either single agents, drug combinations or diluent control. The experiment ended on day 36 due to excessive tumor size in the control group.

CuB and docetaxel *in vivo* studies

The mean tumor volume was reduced in all treatment groups as compared to control mice: 42% with CuB (low dose), 55% with CuB (high dose), 69% with DOC single agent, and by 90% and 94% with the combination of DOC and either low dose or high dose CuB, respectively ($P < 0.01$) (Fig. 3A). DOC in combination with CuB either at high or low dose caused significant reduction of tumor size as compared to single agent treatment groups of either CuB low dose, CuB high dose or DOC ($P < 0.05$). Interestingly, the inhibitory effect of DOC in combination with either high dose CuB or low dose CuB was not statistically different ($p = 0.3$).

CuB and gemcitabine *in vivo* studies

The mean tumor volume was reduced in all treatment groups as compared to diluent control mice, by 46% with CuB (low dose), 62% with CuB (high dose), 71% with GEM single agent, 97% and 94% with the combination of GEM and either low or high dose CuB, respectively ($P < 0.01$) (Fig. 3B). Gemcitabine in combination with CuB either at high or low dose caused significant reduction of tumor size as compared to single agent treatment groups of CuB (low dose), CuB (high dose) or GEM ($P < 0.05$). The inhibitory effect of GEM in combination with either high-dose or low-dose CuB was not statistically different ($p = 0.27$).

In vivo toxicity of therapies

At the conclusion of the study, all murine cohorts were sacrificed and autopsies were performed; livers, spleens and kidneys were excised. After histological review of all tissue samples, none of organs showed signs of toxicity. All treatment groups continued to gain weight during the period of the study (median 13%, range 10–40%) except for the DOC monotherapy (lost 12% body weight). The latter appeared to lose weight secondary to gastrointestinal toxicity (colon dilation). Toward the end of the study, two mice in the DOC monotherapy were sacrificed secondary to failure to thrive. All treatment groups, which received DOC either as monotherapy or in combination, were found to have dilated colons on autopsy. The degree of colon dilation was less severe in the combination treatment groups.

Myelosuppression was the most common side-effect. Leukopenia occurred in the GEM combined with high-dose but not with low-dose CuB. Liver toxicity occurred with both

DOC and GEM when combined with high-dose but not with the low-dose CuB (Table 1). In order to examine underlying bone marrow toxicities, we preformed colony formation assays in semi-solid methylcellulose medium (Fig. 4). Colony numbers of all treated animals, either with mono- or combination regimens, showed significantly decreased colony counts compared to control mice ($p<0.001$). Interestingly, BFU-E, CFU-GM and CFU-GEMM colony numbers from mice treated either with single agent DOC, GEM or CuB were similar to those from mice treated with the low dose combination (all $p \geq 0.18$). In contrast, counts for BFU-E and CFU-GM were significantly decreased in mice treated with the high dose CuB combined with either DOC or GEM compared to mice who received either the single compound or the low dose CuB combined with either chemotherapeutic drug (BFU-E: $p = 0.009$; CFU-GM: $p < 0.001$). In addition, CFU-GEMM colony numbers were significantly decreased ($p < 0.05$) in mice treated with the CuB/GEM high combination, while in all other treatment groups CFU-GEMM colony numbers were comparable.

Discussion

DOC and GEM are among the most commonly used chemotherapic agents in breast cancer treatment. As monotherapies, these drugs show varying overall responses; and better outcome can be achieved by combination therapies, although this is often associated with increased toxicity (21–29). Here, we show clear *in vitro* anti-proliferative synergism of CuB in combination with either DOC or GEM. Furthermore, in human breast cancer xenografts grown orthotopically in immunodeficient mice, superior anti-tumor activity occurred with the combination therapies, without increase in toxicity.

Although the structure of CuB and other cucurbitacins is known, the cellular and molecular mechanisms by which these compounds exert their anticancer effects are not well understood. Studies suggest that some cucurbitacin compounds act by inhibiting precursor incorporation into DNA, RNA, and proteins as well as by inhibiting NF-kB activation and iNOS gene transcription (30). Recent investigations using breast cancer cells revealed a number of cellular pathways impacted by CuB including down-regulation of hTERT and c-Myc (31), as well as inhibition of Wnt signaling with reduced nucleus translocation of β-catenin (32). In addition, CuB causes increased radiation sensitivity associated with a G2/M cell cycle arrest in breast cancer cells (33).

Apoptosis is a major mechanism accounting for cell death in response to chemotherapeutic agents (34). We found that both combinations of CuB/DOC and CuB/GEM increased apoptosis in MDA-MB-231 cells compared to single agent treatment. The mechanism by which CuB enhances DOC and GEM induced apoptosis is unknown. STAT3 and NF-κB, have been reported to be inhibited by CuB (8,10,11,35) and may contribute to the induction of apoptosis by CuB. Indeed, a combination of CuB and DOC strongly inhibited STAT3 activation, as well as reduced Bcl-2 expression in head and neck cancer cells (16). In several studies the antitumor effects of CuB were associated with Erk phosphorylation (12,8,16). Erk has been reported to promote apoptosis in response to GEM, in part by increasing Bcl-2 activation, through a p53-independent pathway (36). Additional studies will be required to explore how CuB and either DOC or GEM induce apoptosis.

Similar to our *in vitro* results, our *in vivo* study using orthotopic xenografts of human breast cancer cells in nude mice showed increased anti-tumor effectiveness with the combination therapies. Importantly, we observed no significant additive toxicity when either of the chemotherapeutic agents were combined with CuB (low dose). Neurosensory and neuromotor toxicities are common in patients treated with DOC and other taxanes. Interestingly, when the DOC was combined with CuB, we noted less neurotoxicity in the colon as manifested by dilated colon compared to DOC monotherapy. CuB may have a

protective affect against neurotoxicity of taxanes, but further investigation is warranted. Leukopenia occurred in the combination treatment of CuB high/GEM. However, colony formation assays with bone marrow cells from the treated mice showed lower colony numbers for all treated groups. The discrepancy could be due to modulation in endogenous cytokine levels caused by each agent, which in turn affect the number of white blood cells. An alternative explanation is that CuB high/GEM treatment, but not the other treatments, induced specific changes in the bone marrow microenvironment resulting in leukopenia in the CuB high/GEM treated mice.

Despite recent advances in treatment of metastatic breast cancer, it is still usually an incurable disease promoting the ongoing search for new drug combinations with effective cytotoxicity and good tolerability. In the present study, we show for the first time that the combination of either DOC or GEM with CuB potentiates anti-breast cancer activity without increased side-effects; these findings suggest new combination regimens with potential clinical application for breast cancer.

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Novelty & Impact Statements

Current strategies combining new anticancer drugs with chemotherapy provide clinical benefit for cancer patients. We show that combination of the triterpenoid compound, cucurbitacin B with traditional agents synergistically inhibited proliferation of breast cancer cells in vitro. Furthermore, combination of cucurbitacin B with chemotherapy drugs markedly inhibited tumor growth without increasing toxicity in a murine model of human breast cancer orthotopic xenografts. This promising combination has potential clinical application for breast cancer treatment.

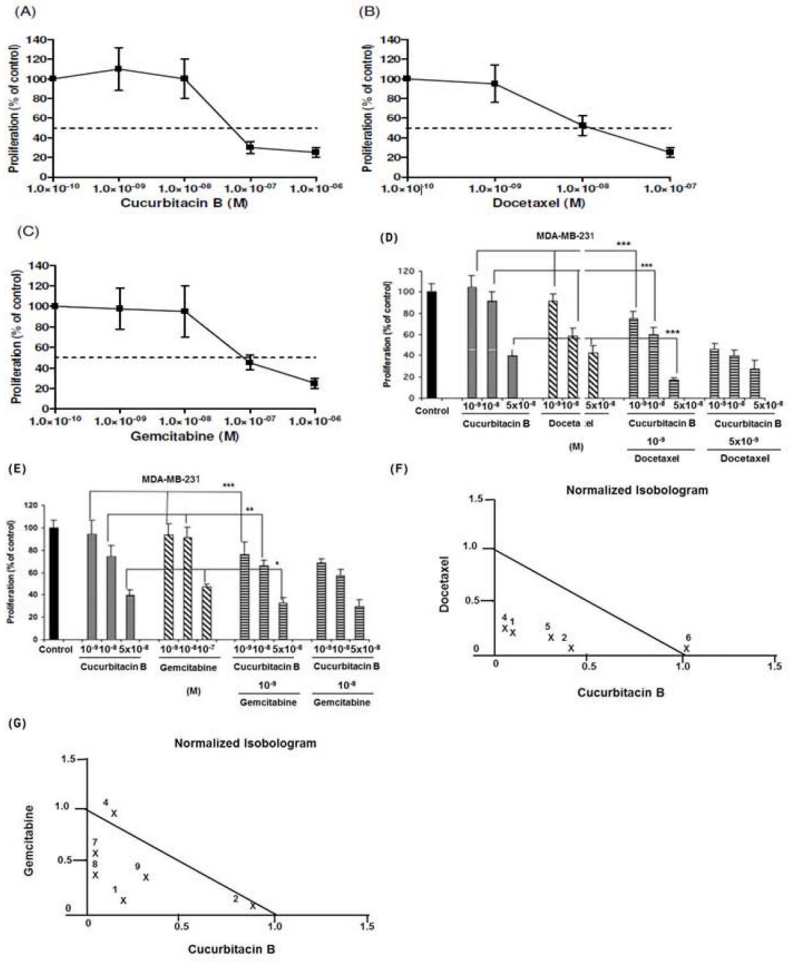

Figure 1. Effect of CuB, DOC and GEM on proliferation of MDA-MB-231 cells *in vitro*

MTT-proliferation assays of MDA-MB-231 cells treated for 96 hrs with different concentrations of the following drugs: Panels (**A**) CuB, (**B**) DOC, (**C**) GEM, (**D**) CuB/DOC or (**E**) CuB/GEM. (**F**, **G**) Normalized isobolograms. Numbers represent different concentrations of Panel (**F**) CuB/DOC, Panel (**G**) CuB/GEM. Panel (**F**) #1: DOC 10^{-9} mol/L+ CuB 10^{-9} mol/L; #2: DOC 10^{-9} mol/L + CuB 10^{-8} mol/L; #4: DOC 5×10^{-9} mol/L + CuB 10^{-9} mol/L; #5: DOC 5×10^{-9} mol/L + CuB 10^{-8} mol/L; #6: DOC 10^{-8} mol/L + CuB 5 x 10^{-8} mol/L. Panel (**G**) #1: GEM 10^{-9} mol/L + CuB 10^{-9} mol/L; #2: GEM 10^{-9} mol/L + CuB 10^{-8} mol/L; #4: GEM 10^{-8} mol/L + CuB 10^{-9} mol/L; #7: GEM 10^{-7} mol/L + CuB 10^{-9} mol/L; #8: GEM 10^{-7} mol/L + CuB 10^{-8} mol/L; #9: GEM 10^{-7} mol/L + CuB 5 x 10^{-9} mol/L; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

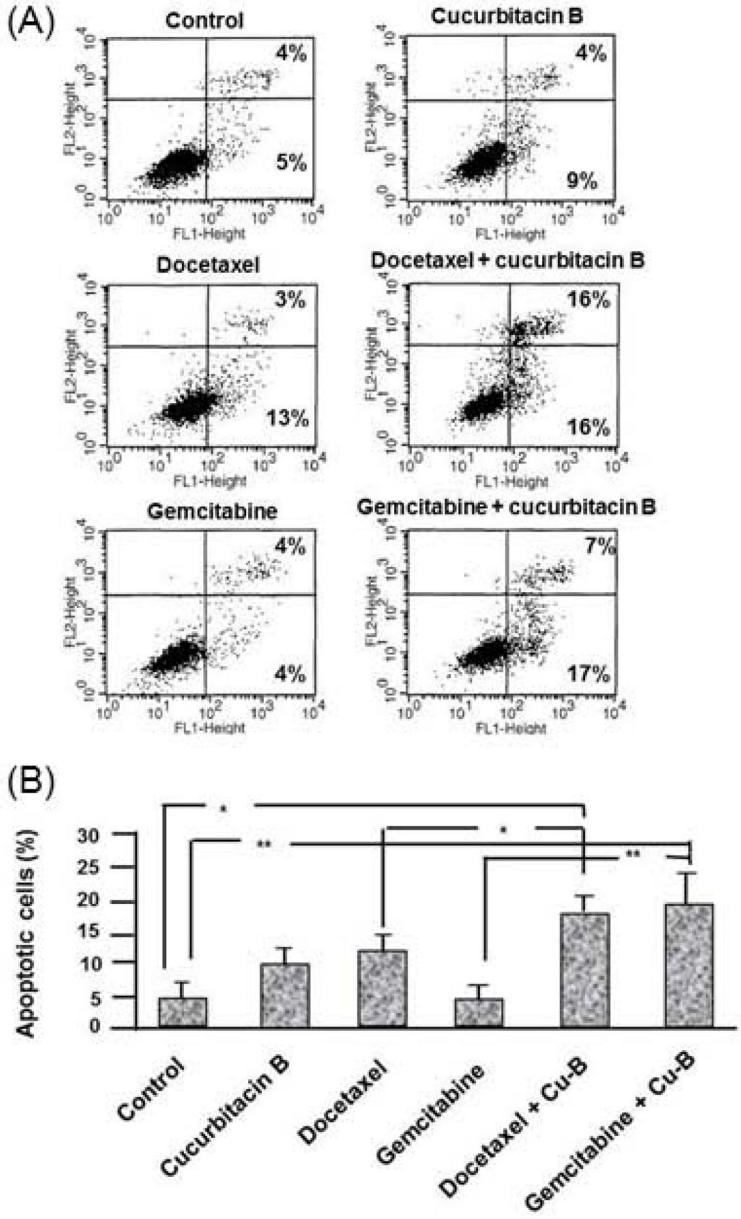

Figure 2. Effect of CuB, DOC and GEM on apoptosis in MDA-MB-231 cells

(**A**) Display of the percentage of cells in early (lower right box) and in late (upper right box) apoptosis. (**B**) Graphic display of the percentage of cells in early apoptosis. *: $p < 0.05$; **: $p < 0.01$.

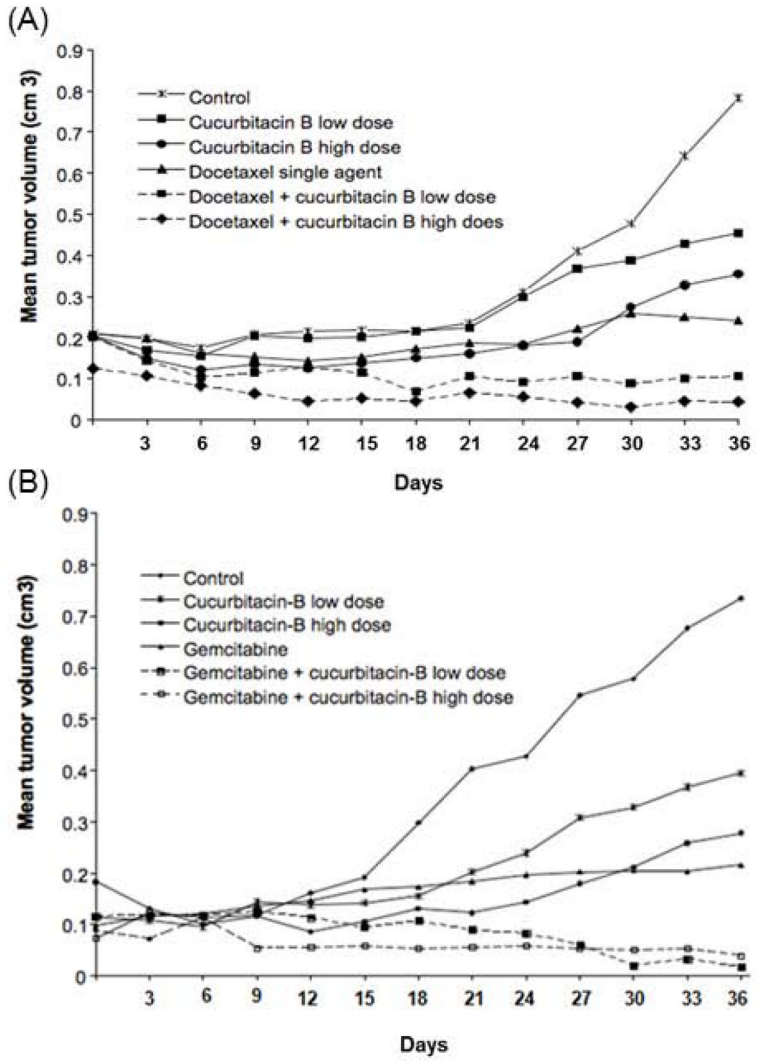

Figure 3. CuB augmented the anti-proliferative activity of DOC and GEM in MDA-MB-231 orthotopic xenografts

MDA-MB-231 cells (10^6) were subcutaneously injected in two mammary fat pads of each nude mouse. Mice were treated as follow: Panel (**A**) CuB/DOC combination: (1) diluent control (PBS, three times/week); (2) low dose CuB (0.5 mg/kg three times/week); (3) high dose CuB (1.0 mg/kg three times/week); (4) single agent DOC (20 mg/kg two times/week); (5) combination CuB (0.5 mg/kg three times/week) and DOC (20 mg/kg two times/week); (6) combination CuB (1 mg/kg three times/week) and DOC (20 mg/kg i.p. two times/week). Panel (**B**) CuB/GEM combination: (1) diluent control (PBS, three times/week); (2) low dose CuB (0.5 mg/kg three times/week); (3) high dose CuB (1.0 mg/kg three times/week); (4) single agent GEM (12.5 mg/kg two times/week); (5) combination CuB (0.5 mg/kg three times/week) and GEM (12.5 mg/kg i.p. two times/week); (6) combination CuB (1 mg/kg three times/week) and GEM (12.5 mg/kg two times/week). Results represent the mean tumor volume (cm^3) of 10 tumors (5 mice) over 36 days of treatment.

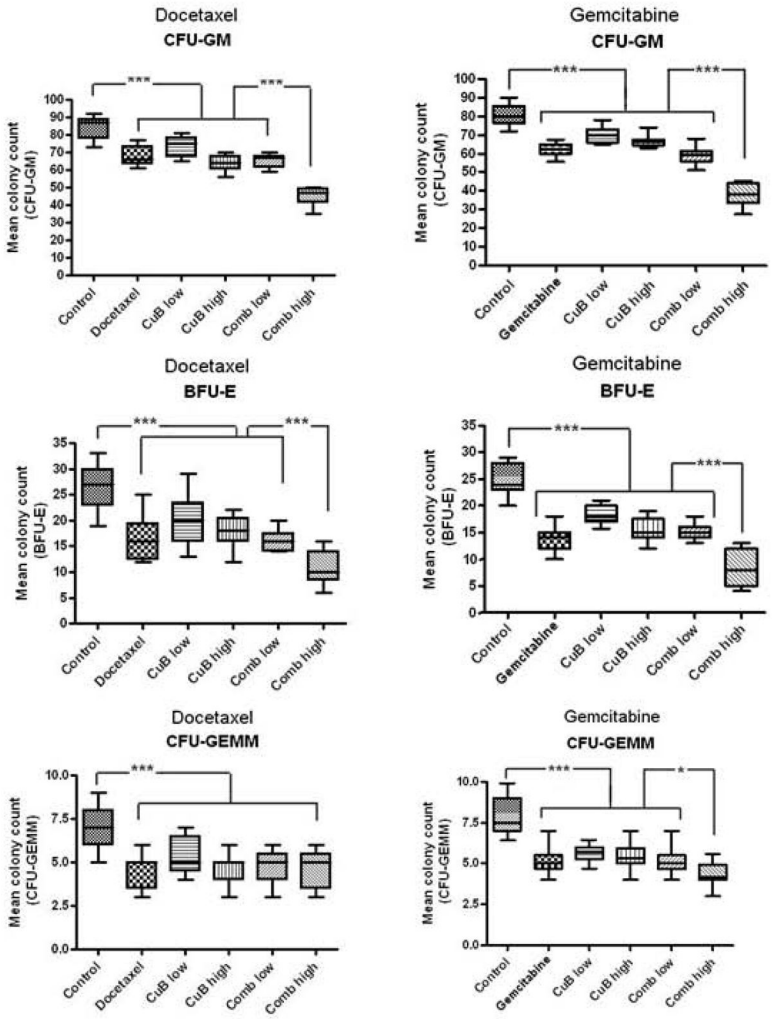

Figure 4. Analysis of bone marrow toxicity after drug treatment measured by clonogenic assays

Bone marrow cells isolated from mice treated either with PBS (control), low dose CuB, high dose CuB, DOC, GEM, or combinations of either DOC or GEM with either low dose or high dose CuB (Comb low, Comb high, respectively; see Fig. 4 for *in vivo* treatment details, before harvest of bone marrow) were plated in triplicates in semi-solid methylcellulose medium. Box and whisker plots display mean colony counts +/−SD of CFU-GEMM, CFU-GM or BFU-E of three mice per treatment group. *, $p < 0.05$; **, $p = 0.009$; ***, $p < 0.001$.

Table 1

	AST	ALT	BUN	WBC	Hgb	PLT
Normal value	54–298 (U/L)	31–46 (U/L)	0–140 (mg/dL)	1.8–10.7 (K/μl)	11–15.1 (g/dL)	592–2972 (k/μL)
Control	125 +/−12	45+/−10	30+/−9	2	12+/−1	829+/−42
Cucurbitacin B low dose	116 +/−13	43+/−3	29+/−5	5+/−1	11+/− 1	811+/−57
Cucurbitacin B high dose	83 +/−58	40+/−9	108+/−38	5+/−2	12+/−1	847+/−259
Docetaxel	109 +/−96	40+/−8	49+/−43	6+/−4	10+/−2	1107+/− 347
Docetaxel + Cucurbitacin B low dose	101 +/−96	46+/−2	60+/− 55	2+/−1	9+/−2	559+/−303
Docetaxel + Cucurbitacin B high dose	423 +/−244*	50+/−9	77+/−28	1+/−1*	11+/13	674+/−404
Gemcitabine	81 +/−24	39+/−6	33+/−28	2+/−1	11+/−2	470+/−412
Gemcitabine + Cucurbitacin B low dose	124 +/−88	51+/−13	36+/−20	1	9+/−4	607+/−341
Gemcitabine + Cucurbitacin B high dose	241+/−56*	36+/−9	26+/−14	4+/−5	8	490+/−240

Mean and standard deviation of 3 mice per treatment group at the end of the study. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BUN: blood urea nitrogen; WBC: White blood cells; Hgb: hemoglobin; PLT: Platelets;

*indicate $p < 0.05$.