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Curcumin improves MMC-based chemotherapy by simultaneously sensitising cancer cells to MMC and reducing MMC-associated side-effects

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ABSTRACT

Mitomycin C (MMC) potently suppresses tumour growth. However, its use is limited by its severe toxicity to the kidney and bone marrow. The purpose of this study is to investigate whether the chemoprevention agent curcumin can reduce MMC-associated side-effects and improve MMC efficacy in a breast cancer xenograft model. We first determined the effectiveness of combined MMC and curcumin to inhibit *in vitro* cell growth and to regress *in vivo* tumour outgrowth. We then investigated the mechanisms associated with MMC/curcumin-induced cell death by examining the effect of MMC/curcumin treatment on apoptosis, the activation of caspase-3, 8 and 9 and the expression of bcl-2 and bax. We also evaluated the ability of curcumin to alleviate MMC-associated side-effects by comparing the levels of creatinine/blood urea nitrogen (Cr/BUN) and glutamic oxalacetic transaminase/glutamic pyruvic transaminase (GPT/GOT) in serum between animals receiving MMC alone and MMC/curcumin. Curcumin significantly sensitised MCF-7 and MDA-MB-231 cells to MMC-induced cell death and improved MMC's ability to regress MCF-7 xenograft. MMC and curcumin together synergistically enhanced apoptosis in MCF-7 cells and the apoptosis most likely resulted from both the activation of caspases and modulation of bcl-2/bax expression. Most importantly, the inclusion of curcumin in MMC treatment decreased MMC-caused severe side-effects evidenced by significant improvement in the kidney function. Enhancing the tumoricidal effect of MMC, curcumin greatly reduces MMC-associated severe side-effects. Therefore, the combination treatment of MMC and curcumin may be of significant therapeutic benefit in treating breast cancer.

1. Introduction

Mitomycin C (MMC), as a chemotherapy drug, can effectively inhibit tumour growth by causing DNA damages.[1] However, the clinical dosage of MMC[2] also causes severe renal toxicity.[3,4] These side-effects have limited the use of MMC in cancer chemotherapy.[5]

Recently, the chemoprevention agent curcumin (diferuloylmethane) has gained significant attention because it is relatively non-toxic[6] and is capable of normalising the levels

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of tumour-related enzymes when it is used with the chemotherapy drug cisplatin.[7] However, it has not been investigated whether curcumin can improve MMC-based chemotherapy.

There is strong evidence that tumour growth is not only a result of uncontrolled proliferation but also of reduced apoptosis,[8] and thus inducing cancer cell apoptosis has been one of the key strategies in anticancer therapy. Apoptosis requires a family of proteases called caspases that are divided into initiator caspases (caspase-8 and 9) and executioner caspases (caspase-3, 6 and 7).[9,10] In addition to caspases, members of the bcl-2 family also play an important role in the regulation of cell survival/apoptosis by either serving as anti-apoptotic (bcl-2, bcl-xl, mcl, ect.) or pro-apoptotic (bax, bak, bid, ect.) proteins.

Recent studies have shown that MMC[11,12] and curcumin[13,14] induces apoptosis by both activating caspase-3 and 9 and decreasing bcl-2 level. However, it is not clear whether a combination of MMC and curcumin can synergistically provoke cancer cell death. Specifically, it is of great interest to determine whether curcumin possesses the ability to reduce the effective dose of MMC, thus allowing safe use of MMC in cancer treatment. In this study, we show that curcumin significantly improves MMC tumoricidal effect in both *in vitro* and *in vivo* experimental models. Most importantly, we find that the inclusion of curcumin significantly alleviates the severe side-effects caused by MMC in MCF-7 breast cancer xenografts model.

2. Material and methods

2.1. Reagents and cell culture

MMC was purchased from the International Chemical and Nuclear Corporation (Ontario, Canada). Curcumin was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Annexin V-FITC and propidium iodide (PI) were obtained from Sigma–Aldrich (MO, USA). The antibodies against caspase-3, caspase-9, caspase-8, bcl-2 and bax were obtained from Cell Signaling Technology (MA, USA). Human breast cancer MCF-7 and MDA-MB-231 cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and 0.01 mg/ml insulin at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Cell growth inhibition test

The inhibition of cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.[15] Various concentrations of MMC with or without curcumin then added to MCF-7 or MDA-MB-231 cells for varying length of times followed by the addition of MTT for another 4 h. After removal of culture solution, the remaining MTT formazan crystals were dissolved with DMSO and measured at 490 nm with a microplate reader. The percentage of inhibition was calculated as followed by the formula: Inhibition ratio (IR) $(\%) = \left(1 - \frac{OD_{sample}}{OD_{control}}\right) \times 100\%$.

2.3. Analysing the effect of combination drug treatment

The combination effect of two drugs was quantified by determining the combination index (CI), in accordance with the classic isobologram[16]: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where Dx is the dose of one drug alone required to produce an effect; $(D)_1$ and $(D)_2$ are the doses of drugs 1 and 2 required to produce the same effect when they are used in combination. From this analysis, the combined effects of the two drugs can be summarised as follows: the CIs of $<$, $=$ and $>$ 1 represent synergistic, additive, and antagonistic effects, respectively.

2.4. DNA gel electrophoresis assay

Genomic DNA was extracted from cells according to manufacturer's instructions of cellular genome extraction kit (TIANGEN BIOTECH, Beijing, China) and electrophoresed on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide (EB) (Sigma-Aldrich, MO, USA) for 1 h. DNA ladders were visualised and photographed under transmitted ultraviolet light.

2.5. Flow cytometric analysis

MCF-7 cells (10^6/mL) were cultured in 6-well plates. It reached 70–80% confluence after seeding cells for 6 h. The media containing FCS and insulin were not changed, and then treated with MMC 2.5 μM, curcumin 40 μM or MMC 2.5 μM plus curcumin 40 μM for 48 h. Cells were subjected to annexin V-PI dual-staining assay according to the manufacturer's protocol. Stained cells were analysed by fluorescence activating cell sorter (FACS) (Becton Dickinson, CA, USA) and the percentage of apoptotic cell population was determined using ModFit LT 3.0 software (Becton Dickinson, CA, USA).

2.6. Western blot analysis

Cells were directly lysed in lysis buffer containing 2 M sodium chloride, 10% NP-40, 10% SDS, 1 M Tris–Cl, 1 g/L phenyl-methylsulphonyl fluoride (PMSF), 0.1 g/L aprotinin and 0.01 g/L leupeptin. The cell lysates were subjected to SDS–PAGE and then blotted onto PVDF membranes. After membrane was blocked with BSA for 1 h, the protein expressions were detected using primary antibodies (1:1000) and secondary antibodies (1:800) conjugated with horseradish peroxidase and enhanced chemiluminescence (ECL) reagents (Pharmacia, Buckinghamshire, UK). Quantitative analyses of Western blots were carried out using Alpha Ease FC (FluorChem FC2) software. The density ratio of proteins to GAPDH as spot density was calculated using the analysis tools.

2.7. Tumour xenograft and treatment

Female nu/nu athymic mice (7 weeks of age) were obtained from Academia Sinica (Shanghai, China). MCF-7 cells are the growth of oestrogen-dependent human breast cancer cells. MCF-7 cells (1×10^7/ml) were inoculated into the mammary fatpad (m.f.p.) of mice and 17β-estradiol was intraperitoneally injected before inoculation.[17,18] Once palpable tumours developed (about 2 weeks), the mice were randomized to 9 groups ($n = 8$). Treatment groups included curcumin (100 mg/kg),[19] MMC (1 mg/kg, 1.5 mg/kg and 2 mg/kg) and combination of curcumin (100 mg/kg) and MMC (1 mg/kg, 1.5 mg/kg and 2 mg/kg). All drugs were administrated

intraperitoneally. Untreated groups were divided into normal and control groups in which animals were injected with physiological saline. After 4 weeks of treatment, blood was collected from the eyes and animals were sacrificed. Tumours were homogenised for Western blot analysis. All procedures conformed to the consideration of animal welfare and were approved by the ethical committee of Shanghai Traditional Chinese Medicine.

2.8. Colorimetric test of serum creatinine (Cr), blood urea nitrogen (BUN), glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)

Serum Cr, BUN, GOT and GPT were measured by commercially available colorimeter testing kits (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's protocol. Briefly, for Cr and BUN, serum samples were incubated with Cr and BUN standard preparation, trinitrophenol solution and 0.75 M sodium hydroxide at 37 °C for 10 min, and then cooling with tap water immediately. The mixtures were measured at 510 nm and 520 nm, respectively. For GOT and GPT, serum samples were incubated with matrix solution, 2,4-dinitro-phenylhydrazine and 0.4 M sodium hydroxide at room temperature for 5 min. The mixtures were measured at 505 nm.

2.9. Mono- and oligonucleosomes ELISA assay

Tumour tissues were homogenised and lysates were collected to measure nucleosomes with Cell death detection ELISA^{PLUS} kit (Hoffmann-La Roche, Basel, Switzerland) according to manufacturer's protocol. The specific enrichment of mono- and oligonucleosomes in cytoplasm was calculated using the following formula: Enrichment factor = [mU of the sample (dying/dead cells)]/[mU of the corresponding negative control (cells without treatment)], where mU = absorbance [10^{-3}].

2.10. Data analysis

The differences between the mean values were analysed using One-Way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin significantly improves the tumoricidal effect of MMC

MMC-associated severe side-effect has limited its use in cancer chemotherapy.[5] To test this hypothesis, we initially determined cytotoxicity effect of MMC with or without curcumin on MCF-7 and MDA-MB-231 cells. Cells were exposed to various concentrations of MMC in the presence or absence of 50% inhibiting (IC50) of curcumin (40 μM) for 48 h (Fig. 1A), and cell viability subsequently determined by MTT assay. MMC treatment alone led to IC50 in cell number at 5 μM to MCF-7 cells and 15 μM to MDA-MB-231 cells (Fig. 1B). When MMC was used with 40 μM of curcumin, we found that IC50 of MMC was reduced to 1.5 μM in MCF-7 cells and to 2.5 μM in MDA-MB-231 cells (Fig. 1B), representing a 330% and 600% improvement in IC50 of MMC, respectively. The CI value of IC50 of MMC in the presence or absence of 40 μM curcumin is 0.833 in MCF-7 cells and 0.75 in MDA-MB-231 cells, respectively. These results suggest that curcumin can synergistically improve tumoricidal effect of MMC in human breast cancer cells.

3.2. Curcumin and MMC synergistically induce apoptosis in MCF-7 cells

To determine the effect of MMC/curcumin treatment on cells apoptosis, we detected apoptosis of MCF-7 cells by performing DNA fragmentation experiment. Moderate DNA ladder

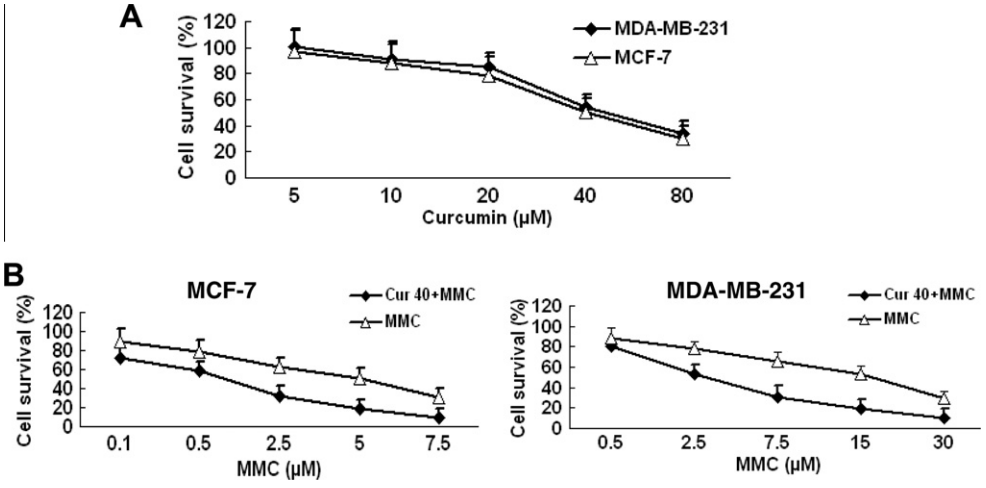

Fig. 1 – Sensitivities of MCF-7 and MDA-MB-231 cells to MMC and MMC plus curcumin. MTT assay was performed to determine cell number as described in Section . (A) Both MCF-7 and MDA-MB-231 cells were incubated in curcumin at 5, 10, 20, 40 and 80 μM for 48 h. (B) MCF-7 cells were treated with 0.1, 0.5, 2.5, 5 and 7.5 μM MMC and MDA-MB-231 cells were treated with 0.5, 2.5, 7.5, 15 and 30 μM MMC with or without 40 μM curcumin for 48 h. Values are means ± SE from three independent experiments.

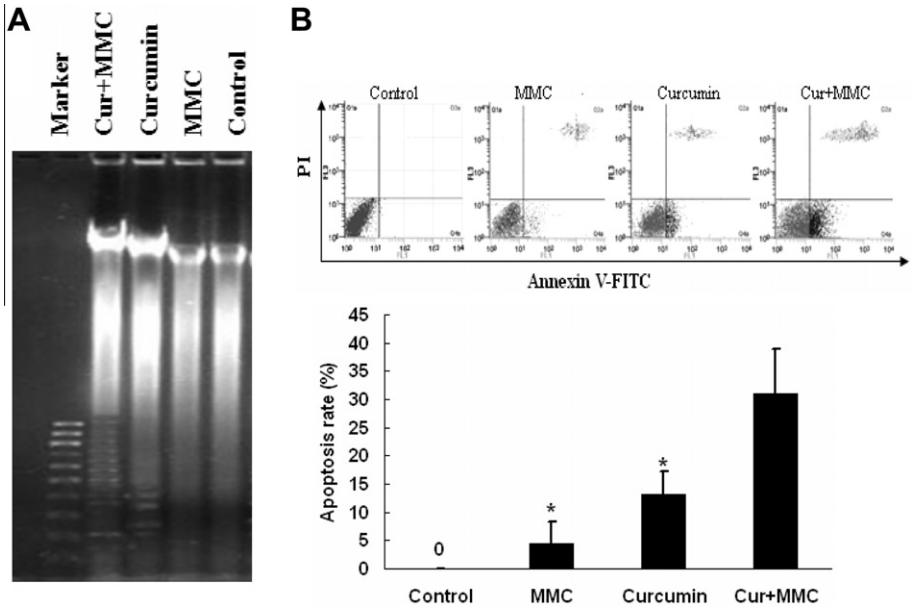

Fig. 2 – Effect of MMC, curcumin or MMC plus curcumin on apoptosis of MCF-7 cells. (A) MCF-7 cells were treated with 2.5 μM MMC, 40 μM curcumin or 40 μM curcumin plus 2.5 μM MMC for 48 h. Genomic DNA isolated from cells and subjected to agarose gel electrophoresis. Gel was stained with EB and DNA fragmentation visualised under UV light. (B) MCF-7 cells were treated with 2.5 μM MMC, 40 μM curcumin or together for 48 h. Cells were subsequently stained for annexinV-PI followed by flow cytometric analysis. Values are means ± SE ($n = 3$). $^{*}p < 0.05$ *versus* MMC plus curcumin.

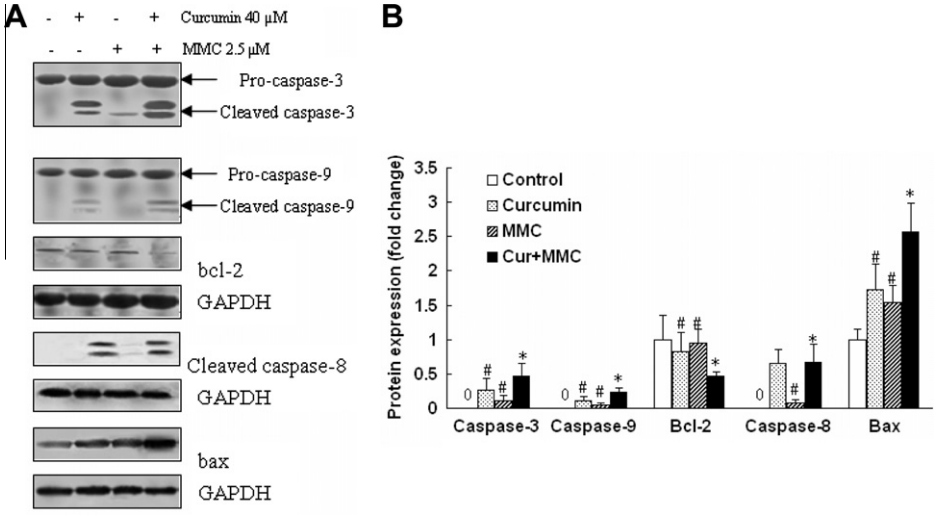

Fig. 3 – Levels of caspase-3, caspase-9, caspase-8, bcl-2 and bax in MCF-7 cells exposed to MMC, curcumin or MMC plus curcumin. MCF-7 cells were treated with 2.5 μM MMC (MMC), 40 μM curcumin (Cur) or 40 μM curcumin plus 2.5 μM MMC (Cur+MMC) for 48 h. Cells were lysed and cell lysates subjected to Western blot to detect the expression of caspase-3, caspase-9, caspase-8, bcl-2 and bax with the respective antibodies. The density ratio of detected proteins to GAPDH was shown as relative expression. Values are means ± SE from three independent experiments. $^{*}p < 0.05$ MMC plus curcumin *versus* control, $^{\#}p < 0.05$ MMC plus curcumin *versus* both MMC and curcumin alone.

was observed with cells treated with curcumin or MMC alone for 48 h while the degree of the DNA ladder was greatly increased in cells treated with MMC and curcumin together (Fig. 2A). To quantitate the number of apoptotic cells, we performed flow cytometry on MCF-7 cells that were exposed to either only drug or together for 48 h. The apoptosis ratio in

cells treated with 2.5 μM MMC, 40 μM curcumin or 2.5 μM MMC/40 μM curcumin was 8.49%, 13.12% and 31.02%, respectively (Fig. 2B). As isobologram analyses showed that CI is less than 1 (CI = 0.833) for MMC/curcumin treatment, these results indicate that curcumin and MMC together synergistically enhance apoptosis in MCF-7 cells.

3.3. *MMC/curcumin treatment induces the activation of caspase-3, 8 and 9, decreases the level of bcl-2 and increases the level of bax*

To investigate the mechanisms potentially involved in MMC/curcumin treatment-induced breast cancer cell apoptosis, we first determined the levels of caspase-3, 8, 9, bcl-2 and bax in MCF-7 cells upon drug treatment. The extent of caspase-3, 8 and 9 activation was greatly elevated in cells treated with MMC and curcumin together (Fig. 3). The ratio of bax to bcl-2 was increased by 5.48-, 3.38- and 2.61-fold in cells treated with MMC/curcumin, compared with the control, MMC alone and curcumin alone, respectively. These results suggest that the combined MMC and curcumin treatment not only induces apoptosis by activating caspases but also by regulating the expression of apoptosis-associated proteins bcl-2 and bax.

3.4. *Combined MMC and curcumin treatment suppresses tumour masses by increasing apoptosis in breast cancer xenograft*

To determine the effect of combined MMC and curcumin on *in vivo* tumour outgrowth, we first established MCF-7 breast cancer xenografts. Curcumin significantly enhanced the ability of MMC to reduce tumour masses when MMC was administrated at 1 and 1.5 mg/kg (Fig. 4A). To determine whether MMC/curcumin treatment suppress tumour mass by inducing apoptosis in tumour xenograft, we performed ELISA assay to determine the levels of nucleosome in tumour lysates treated with either saline or drugs. The levels of mono- and oligonucleosomes were increased 2.8-, 3.6- and 3.4-fold by 1, 1.5 and 2 mg/kg of MMC plus 100 mg/kg curcumin over the saline control, respectively while by only 1.1-, 1.4- and 1.7-fold by MMC alone (Fig. 4B).

We next analysed the expressions of caspase-3, 8, 9, bcl-2 and bax in tumour grafts of mice receiving either saline or drugs. The treatment of curcumin or MMC/curcumin induced cleavage of caspase-3, 8 and 9 (Fig. 4C). While either drug alone displayed little effect on bcl-2 or bax levels, the combined treatment of 100 mg/kg curcumin plus 1, 1.5 and

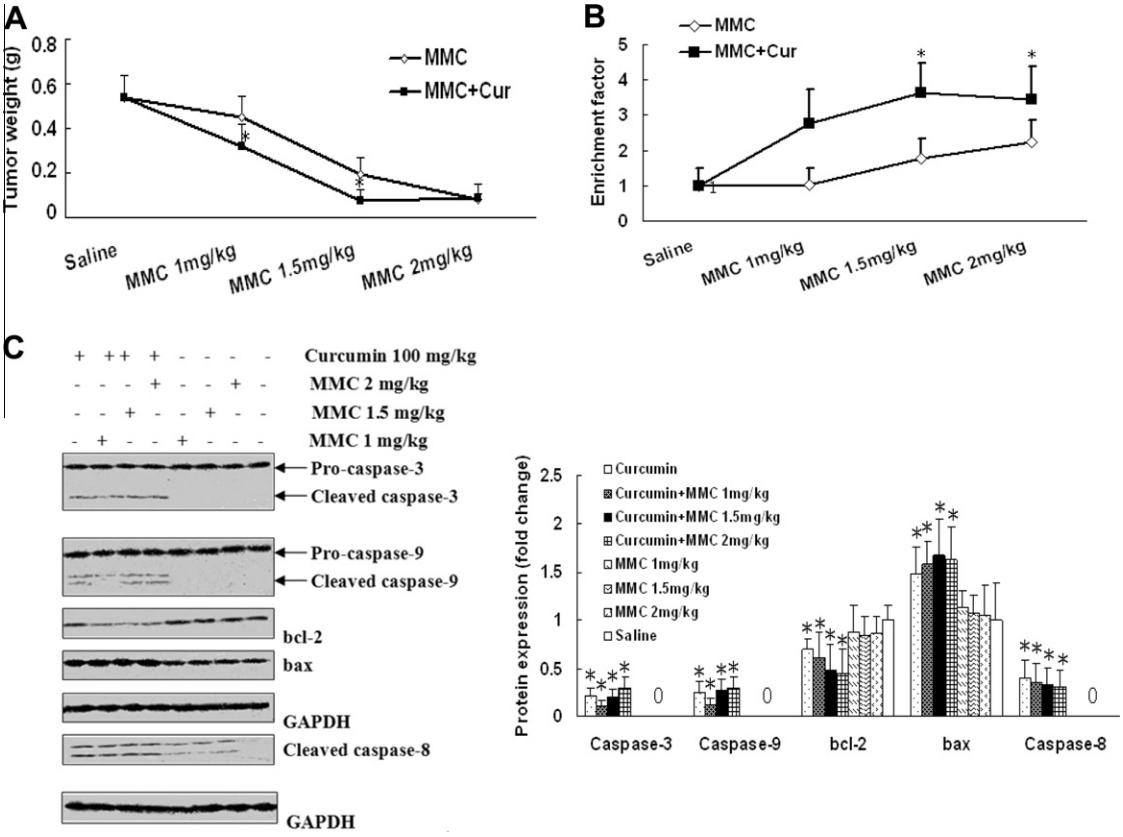

Fig. 4 – Effect of MMC or MMC plus curcumin on tumour outgrowth and apoptosis in MCF-7 xenografts. (A) Mice were first injected with MCF-7 cells for 2 weeks to establish xenograft. Mice were then administrated with 1 mg/kg, 1.5 mg/kg and 2 mg/kg with or without curcumin. After 4 weeks, mice and sacrificed, tumour excised and weighed. *$p < 0.05$ *versus* 2 mg/kg MMC alone. (B) Excised tumours were lysed and lysates subjected to ELISA assay to detect mono- and oligonucleosome as described in Section . The enrichment factor is the ratio of drug treated group to saline group. *$p < 0.05$ *versus* 2 mg/kg MMC alone. (C) Tumour lysates were analysed for caspase-3, caspase-9, caspase-8, bcl-2 and bax with the respective antibodies. The density ratio of detected proteins to GAPDH was shown as relative expression. Values are means ± SE from three independent experiments. *$p < 0.05$ *versus* saline treatment.

2 mg/kg of MMC increased the ratio of bax to bcl-2 by 3.57-, 3.44- and 3.58-fold in comparison with saline treatment. These results suggest that apoptosis induced by MMC/curcumin treatment is contributed by both activation of caspases and the enhancement in the ratio of bax to bcl-2 levels.

3.5. *Curcumin greatly increases the safe dosage of MMC in breast cancer model*

Severe toxicity was apparent in MCF-7 xenograft model when MMC was administrated alone as 1 out of 8 mice receiving 1.5 mg/kg of MMC and 4 out of 8 mice receiving 2 mg/kg of MMC died during treatment (Fig. 5A). Moreover, mice receiving only MMC had significant weight loss (25%, 25% and 52%, respectively at 1, 1.5 and 2 mg/kg MMC; Fig. 5B) and displayed multiple pre-death behaviours such as pale skin and cachexia (data not shown). These observations indicate that high dose of MMC was not tolerable by mice. However, none of the mice died when MMC was combined with curcumin (Fig. 5A). Mice receiving MMC and curcumin together had no apparent weight loss (Fig. 5B) and exhibited healthy appetite (data not shown). These results suggest that curcumin possesses the capability to ease MMC toxicity during MMC-based treatment.

Severe MMC-associated side-effects may be caused by MMC toxicity to the kidney and liver. We thus investigated whether curcumin was able to lower MMC toxicity by analysing the level of Cr/BUN and GPT/GOT in animals receiving only MMC or together with curcumin. MMC dose-dependently increased the levels of Cr and BUN (Fig. 5C) but exhibited little effect on the levels of GPT/GOT (data not shown), indicating that MMC causes severe toxicity to the kidney but not the liver in mice. However, inclusion of curcumin brought the levels of Cr and BUN back to those seen in the control mice (Fig. 5C). These results suggest that curcumin may reduce MMC toxicity by protecting renal function.

4. Discussion

Although the chemotherapeutic agent MMC can effectively inhibit tumour growth, it is accompanied with severe side-effects such as renal toxicity.[5] However, curcumin not only sensitises cancer cells to cisplatin[20], paclitaxel[21] and taxane[22], but also lowers cisplatin-associated nephrotoxicity.[23] Moreover, administration of dietary curcumin significantly reduces the incidence (28.0%) of diethylstilbestrol (DES)-induced mammary tumours in rat mammary glands[24] and inhibits colon carcinogenesis with the lessened side-effects.[25] It is currently not known whether curcumin can also alleviate MMC-associated side-effects and thus allowing its safe use for cancer chemotherapy.

We had found that curcumin alone could inhibit the growth of MCF-7 and MDA-MB-231 cells (data not shown), agreeing with a previously reported study.[26] We further show that curcumin reduces the concentration required for MMC to suppress breast cancer cell growth both *in vitro* and *in vivo* (Figs. 1A and 4A). As curcumin can ease cisplatin-induced nephrotoxicity, we investigated whether curcumin could similarly alleviate MMC-associated severe renal toxicity.[27] In this study, we show that a clinical dosage of 2 mg/kg of MMC greatly decreased tumour mass in MCF-7 xenograft model (Fig. 4A). However, this dosage also caused severe toxicities including 50% of death rate (Fig. 5A) and significant animal weight loss (Fig. 5B). Results from our experiments further indicate that MMC-conferred toxicities are probably due to

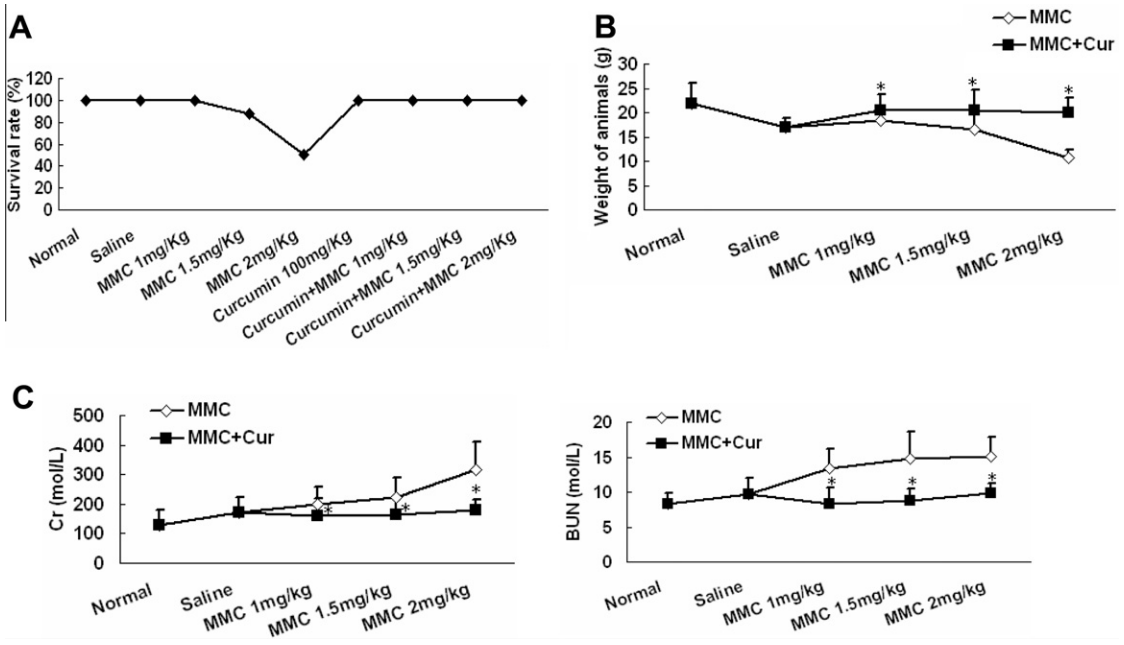

Fig. 5 – Side-effects of MMC or MMC plus curcumin together in MCF-7 xenografts. (A) Animal survival ratio (4-week treatment period). (B) Body weight of animals treated with MMC or MMC plus curcumin for 4 weeks. *$p < 0.05$ *versus* 2 mg/kg MMC alone. (C) Renal function of animals receiving MMC or MMC plus curcumin were assessed by measuring the levels of Cr and BUN in serum as described in Section . *$p < 0.05$ *versus* 2 mg/kg MMC alone.

failure in renal function (Fig. 5C). Interestingly, the inclusion of curcumin in MMC treatment prevented both animal death and weight loss (Fig. 5A and 5B). The protective role of curcumin is most likely achieved by its ability to improve renal function (Fig. 5C). Our studies strongly suggest that curcumin may be used to improve chemotherapy of various chemotherapeutic agents.

Reversing cancer cell resistance to apoptosis may be an effective way to suppress cancer malignancies. Both curcumin[28,29] and MMC[30,31] alone can induce apoptosis. In this study, we found that MMC/curcumin resulted in a 2- and 4-fold increase in the apoptotic rate in comparison with using curcumin and MMC alone. (Fig. 2B). Our results support the notion that a synergistic tumour-killing effect of MMC/curcumin treatment at least partially contributes to greater effect in inducing apoptosis than MMC or curcumin alone.

Moreover, we find that MMC/curcumin induced the cleavages of both initiator caspase-8/9 and executor caspase-3 much more effectively than either drug alone (Fig. 3). We also show that combined MMC and curcumin treatment simultaneously down-regulated the expression of anti-apoptotic factor bcl-2 and up-regulated the expression of pro-apoptotic protein bax (Fig. 3). Taken together, our results suggest that MMC/curcumin treatment triggers cancer cell death by both activating caspases and regulating the expression of bcl-2 family proteins.

In summary, our studies show that curcumin can improve MMC-based chemotherapy by both reducing MMC-associated side-effects and enhancing MMC tumoricidal effect. The combination treatment of MMC and curcumin may be an effective approach for breast cancer chemotherapy.

Conflict of interest statement

None declared.

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