Research Article

Momordica Charantia Lectin, a Type II Ribosome Inactivating Protein, Exhibits Antitumor Activity toward Human Nasopharyngeal Carcinoma Cells In Vitro and In Vivo

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Abstract

The incidence of nasopharyngeal carcinoma (NPC) remains high in endemic regions, including southern China, northern Africa, and North America. One of the promising therapeutic approaches on NPC is drug screening from natural products, such as components from traditional Chinese medicine. In this study, the antitumor activity of Momordica charantia lectin (MCL), a type II ribosome inactivating protein from bitter gourd, on NPC was investigated. MCL evinced potent cytotoxicity toward NPC CNE-1 (IC50 = 6.9) and CNE-2 (IC50 = 7.4) cells but minimally affected normal NP 69 cells. Further investigation disclosed that MCL induced apoptosis, DNA fragmentation, G1-phase arrest, and mitochondrial injury in both types of NPC cells. The reduction of cyclin D1 and phosphoretinoblastoma (Rb) protein expression contributed to arrest at G1-phase of the cell cycle. These events were associated with regulation of mitogen-activated protein kinases (MAPK; including p38 MAPK, JNK, and ERK) phosphorylation and promoted downstream nitric oxide (NO) production. Concurrent administration of the p38 MAPK inhibitor SB-203580 significantly diminished NO production and lethality of MCL toward NPC cells. Further studies revealed that MCL increased cytochrome c release into the cytosol, activated caspases-8, -9, and -3, and enhanced production of cleaved PARP, subsequently leading to DNA fragmentation and apoptosis. Finally, an intraperitoneal injection of MCL (1.0 mg/kg/d) led to an average of 45% remission of NPC xenograft tumors subcutaneously inoculated in nude mice. This is the first article that unveils the potential of a type II RIP, MCL, for prevention and therapy of NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is a nonlymphomatous, squamous cell neoplasm that occurs in the epithelial lining of the nasopharynx and exhibits varying degrees of differentiation (1). It is characteristic with noticeable ethnic and geographic distribution for it is prevalent in South China, Southeast Asia, North Africa, and North America (2, 3). NPC is categorized into 3 groups: typical keratinizing squamous cell carcinomas (type I), non-keratinizing squamous carcinomas (type II), and undifferentiated carcinomas (type III; ref. 1). In southern China, about 2% of patients have type I histology, 3% belong to type II, and 95% belong to type III (1, 4). Besides genetically instinct defects such as disease susceptibility loci (such as TNFRSF19, MDS1-EVI1, CDKN2A-CDKN2B, and HLA molecules; refs. 3, 5), other etiologic factors of NPC have been disclosed, including Epstein–Barr virus infection, consumption of preserved foods, cigarette smoking, and occupational exposure to formaldehyde and wood dust, and other environmental factors (1, 3, 4, 6). The annual incidence of NPC in the endemic regions is about 250 cases per one million individuals (3, 6). The coadministration of radiotherapy and adjuvant chemotherapy with cisplatin is the standard treatment for NPC, but the 5-year survival rate is only about 50% to 60%. One obstacle ahead is the high risk of locoregional relapse and distant metastasis (1, 7).

One of the promising therapeutic approaches on NPC is drug screening from natural products, such as components from traditional Chinese medicine. In this study, we focus our attention on bitter gourd/BG (Momordica charantia, family Cucurbitaceae), a nutritious and healthy food, with a distinctive bitter taste. Multiple medicinal...
components in BG, including compounds and proteins, have been identified with antidiabetic, anti-HIV, and antitumor activities in both in vitro and in vivo investigations (for details see review; ref. 8). Special attention goes to its applications on tumor therapy. Early in 1983, Iljka and colleagues found that an aqueous extract of BG fruits inhibited tumor formation in CBA/D1 tumor cells, P388 tumor cells, and L1210 tumor cells bearing CBA/H mice, which was contributed partially by enhancement of immune functions (9). Both cell culture and animal experiments evinced the antiproliferative activity of BG fruit juice brought about by modulating cell-cycle regulatory genes and inducing apoptosis (10). The antitumor activities may at least partially be attributed to MAP30, α-momorcharin, and β-momorcharin and other medicinal proteins (8). For instance, coadministration of α- and β-momorcharins exhibited apoptotic inducing activity in prostate cancer cells, in both in vitro and in vivo experiments (11).

Ribosome inactivating proteins (RIP) are a class of RNA glycosylases that cleave an adenine-ribose glycosidic bond at position A-4324 within the conserved σ-aracin/ricin loop in the eukaryotic 28S ribosomal RNA bound by elongation factors or adenine-2660 in E. coli 23S rRNA (8). On the basis of their structure, RIPs are divided into 3 classes, including type I RIPs (with only a RIP chain), type II RIPs (with a RIP chain and a lectin chain), and type III RIP (also named atypical type I RIP) (8). Type II RIPs are more toxic because the lectin chain can bind to the surface of target cells and facilitate the internalization of the RIP chain, resulting in manifestation of its RNA N-glycosidase activity and inactivation of ribosomes (8). A famous example is ricin, which is a heterodimeric toxin consisting of an RIP chain (~30 kDa), and a lectin chain (~30 kDa) produced by seeds of the castor oil plant (Ricinus communis ref. 8). RIPs exhibit tremendous promise for the therapy of tumors as exemplified by MAP30 (8), a type I RIP from BG, and ricin (8).

We have now established a new way for the purification of Momordica charantia lectin (MCL), a representative type II RIP, and investigated its in vitro as well as in vivo antitumor activities toward CNE-1 and CNE-2 NPC cells. We found that MCL could selectively decrease the viability of NPC cells, and at doses approximating the IC50 value, it manifested little effect on normal nasopharyngeal cells. The cytotoxicity of MCL was associated with (a) induction of cell apoptosis, characterized as increased levels of apoptotic bodies, nuclear condensation, and DNA damage; (b) G1 cell-cycle arrest, which was caused by decreased levels of cyclin D1, reduced phosphorylation of retinoblastoma (Rb); (c) and damage of mitochondrial potential. Molecular mechanisms with regard to these phenomena comprise regulation of mitogen-activated protein kinase (MAPK) signaling axis and intrinsic apoptotic cascades. Furthermore, the in vivo efficacy in CNE-2 bearing nude mice was shown. This is the first article that unveils the potential of type II RIP for application in NPC therapy.

Materials and Methods

Antibodies and reagents

The antibodies used in this study were as follows: primary antibodies for Bid (sc-11423, polyclonal), p53 (sc-126, monoclonal), tubulin (sc-9104, polyclonal), PARP (sc-25780, polyclonal), cleaved PARP (sc-23461-R, polyclonal), and Bak (sc-832, polyclonal) were purchased from Santa Cruz Biotechnology. Antibodies for caspase 8 (9746, monoclonal), caspase 9 (9508, monoclonal), caspase 3 (9665, polyclonal), p21 (2947, polyclonal), Bel-2 (2870, monoclonal), p-p38 (9215, monoclonal), p38 (9212, monoclonal), p-ERK (4376, monoclonal), ERK (4695, monoclonal), p-JNK (4671, monoclonal), JNK (9252, polyclonal), cyclin D1 (2922, polyclonal), phos-Rb (Ser 780) (9307, polyclonal), cytochrome c oxidase IV (COX-IV; 4844, polyclonal), and cytochrome c (4280, monoclonal) were provided by Cell Signaling. All reagents were from Sigma, unless otherwise indicated. Reagents including N-Nitro-o-arginine methyl ester (L-NAME), SB-203580, and N-benzylxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO used was less than 0.01% and had no adverse effect on cell viability.

Preparation of MCL

BG seeds were purchased from a local vendor and authenticated by Professor Shiu-Ying HU, Honorary Professor of Chinese Medicine, The Chinese University of Hong Kong. First, BG seeds were homogenized, centrifuged (16,000g, 30 minutes, 4°C), and the aqueous supernatant was loaded on a Blue Sepharose column (buffer: 0.02 mol/L NH4OAC). The unadsorbed fraction containing hemagglutinating activity was applied to a Q-Sepharose column (buffer: 0.02 mol/L NH4HCO3). Bound proteins were eluted sequentially with 3 concentrations of NaCl (0.2, 0.5, and 1 mol/L) in 0.02 mol/L NH4HCO3. The unadsorbed fraction containing hemagglutinating activity was loaded on a SP-Sepharose column (buffer: 0.02 mol/L NH4OAC). The unadsorbed fraction containing hemagglutinating activity was applied to a Q-Sepharose column (buffer: 0.02 mol/L NH4HCO3). Bound proteins were eluted sequentially with 3 concentrations of NaCl (0.2, 0.5, and 1 mol/L) in 0.02 mol/L NH4HCO3. The fraction eluted with 0.5 mol/L NaCl was pooled, dialyzed, and loaded on a Superdex 75 column. Pure MCL resided in the major peak.

Identification and characterization of MCL

The purity of the acquired MCL fraction and its molecular weight (native conditions) were investigated by 15% nonreducing SDS-PAGE (12, 13). N-Terminal amino acid sequence was analyzed using an HP 1000A Edman degradation unit and an HP 1000 HPLC system (Hewlett Packard; ref. 14). Investigations of hemagglutinating activity, ribosome inactivating activity, and sugar specificity were done using methods previously described (13, 14). Bioinformatic works, including sequence alignment (using ClustalX 1.83 and boxshade server), construction of phylogenetic tree (using ClustalX 1.83 and TreeVier), and predictive 3-dimensional (3D) structure (using the
on line Phyre server) were conducted as mentioned previously (8).

**Cell culture and cell viability assay**

Human NPC cell lines CNE-1 (well differentiated) and CNE-2 (poorly differentiated) were purchased from the Sun Yat-sen University of Medicinal Sciences, Guangzhou, China. Transformed human nasopharyngeal epithelial cell line NP 69 was generously provided by Prof. S.W. Tsao (Department of Anatomy, The University of Hong Kong). Cell line characterization was done by monitoring cell morphology, karyotyping, and interspecies contamination. The cells were last tested in January 2011. The CNE-1 and CNE-2 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). The NP 69 cells were cultured in keratinocyte-SFM medium (Gibco) plus supplements for keratinocyte-SFM (Gibco; ref. 14). All cell lines were maintained at 37°C in a humidified incubator under an atmosphere of 95% air and 5% CO2. Logarithmically growing cells were incubated with increasing concentrations (0–60 μmol/L) of MCL for 24 to 48 hours, and cell viability was determined by MTT assay (14) and by counting the number of viable cells on the basis of trypan blue exclusion, respectively.

**Assessment of apoptosis, chromatin condensation, and DNA fragmentation**

Effects of MCL on apoptosis of tumor cell were examined with a FACSort flow cytometer (Becton Dickinson) using Annexin V–FITC/propidium iodide (PI; BD Phamingen) double staining as previously described (15). Furthermore, observations of apoptotic bodies and chromatin condensation were made with Hoechst 33342 staining (1 μmol/L) and visualized under UV illumination with a NIKON TE2000 microscope (Nikon; ref. 15). Moreover, apoptotic bodies were counted in 3 different fields of microscopic observation. One hundred cells were examined in one field. In addition, DNA fragmentation was determined with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (green fluorescence), using an In Situ Cell Death Detection Kit (Roche). Briefly, tumor cells were treated with MCL (7.5 μmol/L) for 24 hours, followed by TUNEL staining according to the manufacturer instructions, and monitored by flow cytometry.

**Assays of cell-cycle analysis and mitochondrial depolarization**

The cell-cycle distribution of cells after MCL treatment (0–15 μmol/L, 24 hours) was studied by using flow cytometry with PI staining (20 μg/mL PI in PBS, containing 1% Triton X-100 and 10 μg/mL RNase A; ref. 16). On the other hand, measurement of changes of mitochondrial transmembrane potential (ΔΨm) was made using the same procedure, but with JC-1 staining at a concentration of 2.5 μmol/mL (13). The mechanism is that JC-1 exists in monomeric form in the cytosol (emitting red fluorescence) and also accumulates as aggregates in the mitochondria (emitting green fluorescence) in normal cells. But in apoptotic/necrotic cells, the ΔΨm collapse halts the mitochondrial accumulation of JC-1 but maintains it in cytosol (emitting green fluorescence; ref. 17).

**Western blot analysis**

Effects of MCL treatment on (a) the expression and phosphorylation of MAPKs, (b) the activation of apoptotic cascades, and (c) the expression of Bcl-2 family proteins were determined by Western blot (18). Briefly, cellular lysates were heated at 99°C for 10 minutes in 6× SDS loading buffer, followed by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane, which was then incubated with a primary antibody in 5% milk, followed by incubation with a horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibody and visualized using the ECL detection system (Amersham Life Science).

**Detection of nitric oxide production**

First, cells were incubated with (a) 3.75 μmol/L MCL, (b) coincubated with 3.75 μmol/L MCL and 100 μmol/L L-NAME [a nitric oxide (NO) synthase inhibitor], and (c) coincubated with 3.75 μmol/L MCL and 10 μmol/L SB-203580 (a p38 MAPK inhibitor), respectively, for 24 hours. L-NAME or SB-203580 was added 1 hour prior to MCL. Second, the generation of nitrite and nitrate, as the surrogate markers for NO, in the cell culture supernatant was determined by using Griess reagent, as previously described (14, 15).

**Animal studies**

The *in vivo* antitumor activity of MCL was studied in athymic nude (nu/nu) mice following the procedure reported elsewhere with modifications (7, 16). First, a total of 1 × 10⁷ CNE-2 cells were trypsinized, washed with 1× PBS, and injected subcutaneously into the right flank of each mouse. Mice were checked each day for xenograft/tumor development. Once the tumors were palpable, mice were randomly divided into 2 groups (8 mice per group). The MCL group received a daily intraperitoneal injection of 1.0 mg MCL/kg body weight (1.0 mg/kg/d). The control group was treated with 1× PBS. Tumor volume was determined every 2 days with calipers using the following formula: tumor volume (mm³) = length (mm) × width (mm)². Body weight was monitored every 2 days as an indicator of toxicity, and mice were euthanized when tumor size exceeded 2,000 mm³ (19).

**TUNEL staining assay**

On day 16, all (remaining) mice were sacrificed, and sections of tumor tissue in both groups were prepared. As mentioned above, TUNEL assay was carried out using an In Situ Cell Death Detection Kit (Roche) as per manufacturer’s instructions. The cells were visualized under a light microscope, and the percentage of apoptotic cells under 5 hpf (20×) was calculated (7, 19).
Statistical analysis
Results of all in vitro studies were collected from 3 independent experiments conducted in triplicate, and data are expressed as mean ± SD. The 2-tailed Student t test was used for between-group comparisons, and differences were considered significant at P < 0.05.

Results
A new effective method established for the isolation of MCL
The common way for the purification of MCL is to make use of galactopyranosyl columns (such as cross-linked arabinogalactan and galactosyl Sepharose 4B columns) based on its galactose-specific activity (12, 13, 20–22). As Supplementary Fig. S1 illustrates, here a new method has been established for the purification of MCL. MCL was unadsorbed on Blue and SP Sepharose columns, but adsorbed on a Q Sepharose column, which could subsequently be eluted with 0.5 mol/L NaCl. The eluate was finally loaded on a Superdex 75 column, and purified MCL was acquired in fraction Sup1. MCL appeared as a single band with a molecular weight near 130 kDa in SDS-PAGE under nonreducing conditions (without β-mercaptoethanol). These findings are commensurate with previous reports (12, 13). About 24 mg homogeneous MCL were harvested from 250 g dried seeds. Though the current method is not as simple as the commonly used 1-step purification procedure, it owns a specific advantage. This method can be applied to the simultaneous isolation of different medicinal proteins, such as MCL, α- and β-momorcharins, and a new ribonuclease RNase MC2 (8), from BG seeds (Supplementary Fig. S1F) and may facilitate the commercial exploitation of BG.

Characterization and bioinformatic analysis of MCL
MCL exhibited hemagglutinating activity toward rabbit erythrocytes (640 units/mg), and it also inhibited protein synthesis in a cell-free rabbit reticulocyte lysate system. Among a variety of sugars used for the testing of sugar specificity of a Phaseolus vulgaris lectin (15), the hemagglutinating activity (16 units) of MCL was specifically inhibited only by α-galactose and α-L-lactose at a concentration of 25 mmol/L. These data were in accordance with previous reports (12, 13). The N-terminal amino acid sequence of 1 of the 4 subunits of MCL is NEQCSPOQRT, which coincides with the results of Tanaka and coworkers (23). On the basis of the total sequence reported by this research group (23), a bioinformatic investigation on MCL was carried out. As shown in Supplementary Fig. S2A, sequence alignment between MCL and other type II RIPs revealed marked sequence similarity among them. Their genetic relationships are established in the form of a phylogenetic tree (Supplementary Fig. S2B). Among the 6 RIPs, ricin and CS-RIP (a type II RIP from Camellia sinensis) exhibited a relatively close evolutionary relationship with MCL. Currently, only preliminary X-ray studies of MCL have been reported (12), and there is a lack of detailed NMR spectroscopic data. By using the on-line Phyre server (24), a predictive 3D structure (ribbon diagram) of MCL was generated (Supplementary Fig. S2C).

MCL induces cytotoxicity in NPC cells in a time- and dose-dependent manner
To investigate the in vitro antitumor activity of MCL, CNE-1 and CNE-2 cells were exposed to increasing concentrations (0–60 μmol/L) of MCL for 24 and 48 hours, respectively. As shown in Fig. 1A and B, after culture with MCL, the viability of both CNE-1 and CNE-2 cells underwent a decline in a time- and dose-dependent fashion. Similarly, MCL caused a time- and dose-dependent inhibition of cell proliferation in both types of NPC tumor cells (Fig. 1C and D). The IC50 values (24 hours) for CNE-1 and CNE-2 cells were 6.9 ± 0.2 and 7.4 ± 0.4 μmol/L, respectively. At 7.5 μmol/L MCL, a concentration near IC50 of both types of NPC tumor cells, there was only slight lethality toward normal human nasopharyngeal epithelial cell line NP 69.

MCL induces apoptosis and DNA fragmentation in vitro
To unveil the possible mechanism involved in the cytotoxicity of MCL, the event of apoptosis was evaluated in MCL exposed CNE-1 and CNE-2 cells. In this study, Annexin V/PI staining was used to monitor early ( Annexin V positive) and late apoptosis/necrosis (both dyes positive). Compared with control, the percentage of cells in early apoptosis increased in a dose-dependent manner after MCL exposure (0–15 μmol/L, 24 hours). From 7.5 μmol/L onward, sharp increases in the percentage of late apoptotic/necrotic cells were noticed in both tumor cell lines (Fig. 2A). Furthermore, characteristic features of apoptosis, including chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies were detected (15). As shown in Fig. 2B and C, both NPC tumor cells displayed features consistent with apoptosis including condensation of nucleus (arrows) and formation of apoptotic bodies (asterisks; and C) after exposure to MCL (7.5 μmol/L, 24 hours) compared with control. Consistently, the number of TUNEL-positive cells significantly increased from a value near baseline to almost 40% in both cell lines after exposure to 7.5 μmol/L MCL (Fig. 2D).

Exposure of NPC cells to MCL induces G1 arrest and mitochondrial membrane potential depolarization
To further investigate the mechanism of MCL-mediated apoptosis, cell-cycle arrest and mitochondrial membrane depolarization were analyzed by flow cytometry. As Fig. 3A shows, MCL treatment resulted in a highly significant increase in the G1 population in a dose-dependent fashion (P < 0.05 in each case). Because cyclin D1 and phospho-Rb reflect G1 cell-cycle progression (25, 26), changes of their protein levels after MCL exposure were investigated. As Fig. 3B and C indicate, treatment with MCL produced a time-related decrease of the protein levels of both cyclin D1 and phospho-Rb. In view of the high frequency that G1 cell-cycle arrest takes place in a p53- and/or p21-dependent way (27), changes of protein levels of p53 and p21 were also investigated. Interestingly, MCL caused a dose-dependent
(3.75 and 7.5 μmol/L) and time-dependent (24, 36, and 48 hours) inhibition of protein expression of both p53 and p21 (data not shown). On the other hand, the percentage of cells with depolarized mitochondria increased in accordance with elevated MCL concentration (0–15 μmol/L, 24 hours) in both CNE-1 and CNE-2 cells (Fig. 3D).

**Regulation of MAPKs and the production of downstream NO play a role in MCL toxicity in NPC cells**

To determine the involvement of the regulation of MAPKs and the downstream NO production in MCL lethality, an on-time monitoring of the phosphorylation levels of 3 major subgroups of MAPKs, including p-38 MAPK, Jun kinase (JNK/SAPK), and extracellular signal-regulated kinase (ERK; refs. 28, 29) was undertaken. In Fig. 4A, immunoblot analysis shows that the amount of phospho-p38 (p-p38) in both CNE-1 and CNE-2 cells treated with 3.75 μmol/L MCL increased from 4 and 12 hours, respectively, in a time-dependent way. Phosphorylation of JNK levels was not significantly altered in both cells of early exposure (before 8 hours), and a great increase was seen at 24 hours in both cells. Interestingly, the fates of ERK phosphorylation in both cells were opposite: MCL caused an increased activation of ERK in CNE-1 cells. However, it inhibited phospho-ERK (p-ERK) levels in CNE-2 cells. A quantitative analysis of Fig. 4A is shown in Supplementary Fig. S3. In light of the previous reports that (a) activation of MAPKs signal cascade leads to an increase of the production of NO, a major antitumor molecule (14, 15), and (b) proteins such as lectins (14, 15), type I and type II RIPs (30, 31) may stimulate NO production, the NO-inducing activity of MCL as well as the relationship with one MAPK, p-38, were investigated. MCL caused profound changes of NO formation, as indexed by the production of nitrite and nitrate, in both mouse peritoneal macrophages.
and the 2 types of NPC cells (Fig. 4B), whereas coadministration of 100 μmol/L L-NAME, a NO synthase inhibitor (32), attenuated MCL-induced NO production. At the same time, the positive control, blue tiger king lectin, also induced NO production (15). There was no detectable lipopolysaccharide (an environmentally available NO inducer; ref. 14) contamination in MCL (data not shown). Furthermore, the NO production and cytotoxicity induced by MCL at 3.75 μmol/L was partially blocked by cotreatment with the p38 MAPK inhibitor, SB-203580, at a dose of 10 μmol/L (Fig. 4C). These results suggested that MCL treatment activated the p38 cascade and increased the downstream production of NO, resulting in cell toxicity.

MCL activates caspase-mediated apoptosis but has no effect on the expression of classic Bcl-2 family proteins in NPC cells

Apoptosis is executed by a group of cysteine-dependent aspartate-specific proteases termed caspases which comprise 2 distinct classes, the initiators (such as caspase 8, caspase 9) and the effectors (including caspase 3 and others; ref. 33). We further elucidated the involvement of such caspases in MCL-induced cell death. As shown in Fig. 5A and B, MCL treatment resulted in a dose-dependent (3.75 and 7.5 μmol/L) and time-dependent (24, 36, and 48 hours) activation of the initiator caspases 8 and 9, and the executor caspase 3 in both CNE-1 and CNE-2 cells. According to caspase activation leading to PARP cleavage was observed. Because caspase-9 activation is initiated by cytochrome c in cytoplasm (34, 35), we further confirmed that MCL enhanced cytochrome c translocation into the cytoplasm and, in turn, activated caspase 9 (Fig. 5C). Furthermore, the caspase inhibitor Z-VAD-FMK (20 μmol/L) decreased MCL-induced apoptosis in both NPC cells (Fig. 5D). On the other hand, in view of the reports that some Bcl-2 family proteins are involved in caspase-mediated apoptosis and cell-cycle arrest (27), the protein levels of some representative Bcl-2 family members were investigated.
Unexpectedly, MCL did not significantly affect the protein expression of Bid, Bak, and Bcl-2, even at the dose of 7.5 μmol/L (near IC50 value) after treatment for 48 hours (data not shown).

**MCL halts the growth of CNE-2 cell xenograft tumors**

After showing the antitumor potential of MCL in vitro, we went on to assess its effect in nude mice. After subcutaneous inoculation of nude mice with CNE-2 cells for 4 days, the tumor xenograft was palpable and treatment was initiated. Two groups were involved in the experiments, including the MCL group treated with 1 mg MCL/kg body weight for 12 consecutive days (i.p., 1.0 mg/kg/d), whereas the control group was treated with PBS instead. As shown in Fig. 6A, MCL halted tumor growth on the fourth day (day 8) of administration. This effect was consistent, and on the 12th day (day 16), nearly 45% reduction of tumor volume was observed. On day 16, all mice were sacrificed and the mean tumor weights of both groups were compared. As Fig. 6B shows, MCL significantly decreased the mean tumor weight.
compared with control group. The dose of MCL used did not exhibit detectable toxicity to nude mice. To correlate the in vivo antitumor activity of MCL with its apoptosis-inducing activity, tumor sections of both groups were stained with TUNEL to compare the numerical difference in apoptotic cells. As shown in Fig. 6C, compared with control (6.4%), the proportion of apoptotic cells in the MCL-treated group increased to 14.8% ($P < 0.05$). Together, these findings indicate that MCL also manifested antitumor activity against CNE-2 xenograft, which was at least partially attributed to the induction of apoptosis of tumor cells.

**Discussion**

As one of the most important epithelial neoplasms worldwide, patients with NPC always present at diagnosis advanced stage disease, or relapse, invasion, and dissemination after first-line therapy, thus resulting in a poor
Figure 5. Effects of MCL on activation of apoptotic cascades in NPC cells. A and B, MCL activated the apoptotic caspase cascade in NPC cells. After different treatments, cells were harvested and their protein extracts were analyzed by Western blot (A). The results of active caspase 3 and cleaved PARP were quantified with ImageJ software (B). C, MCL induced relocalization of cytochrome c (cyto c) to the cytoplasm. After the indicated treatment, cytoplasmic isolates and mitochondrial isolates were prepared for Western blot analysis. β-Actin and cytochrome c oxidase (cox-IV) were internal controls for cytoplasmic isolates and mitochondrial isolates, respectively. D, administration of the caspase inhibitor Z-VAD-FMK repressed MCL-induced apoptosis. After different treatments, as indicated at the bottom of the histogram, cells were harvested, stained with Annexin V–FITC/PI, and processed for flow cytometry. *, P < 0.05 versus control.
prognosis (1, 36). Hence, there is an urgent need for novel therapies with increased safety and efficacy. For example, researchers have investigated the potential/effectiveness of (a) allogeneic Epstein–Barr virus–specific cytotoxic T cells (37), (b) molecular therapies targeting epithelial–mesenchymal transition and cancer stem cells (38), and (c) different natural/synthesized components, including resveratrol (a natural polyphenolic compound present in different plants; ref. 39), Chk1 inhibitor G06976 (40), lapatinib (also named as Tykerb, GW572016; the first dual kinase inhibitor of epidermal growth factor receptor and HER-2; ref. 41), and ApoG2 (the synthesized oxidation product of apogossypol from the cotton plant Gossypium; ref. 7, 16).

In this study, we investigated the in vitro and in vivo antitumor activity of MCL toward NPC cells. We established a new and effective liquid chromatographic technique for purifying MCL (Supplementary Fig. S1). MCL used in this study exhibits previously reported activities (12, 20, 22, 23), including hemagglutinating, ribosome inactivating, and galactose-specific activities. Bioinformatic studies reveal a close relationship between MCL and the classic type II RIP, ricin (Supplementary Fig. S2). Although very toxic, ricin and its engineered products have been reported with potent antitumor activity characterized by specificity, high efficacy, and stability (42). The results of the present study indicate that MCL exhibited selective cytotoxicity on 2 representative types of NPC cells by the regulation of MAPKs and caspase cascade.

The cytotoxicity and antiproliferative activity of MCL on NPC cells were higher than those in the normal NP69 cells (Fig. 1). This may be caused by the numerical difference of molecules recognizable by MCL lectin chain on the surface of the cells, for some investigations found that changes in cell surface sugars are associated with the development of cancer (43). Subsequent studies showed that induction of tumor cell apoptosis was responsible for the MCL effect. MCL increased the proportion of apoptotic cells, caused chromatin condensation, and nuclear fragmentation (Fig. 2), which are unique morphologic nuclear changes of apoptosis (44). At the same time, G1 phase cell-cycle arrest and mitochondrial damage were detected. It seems that MCL-induced mitochondrial damage seems to be the cause rather than the effect of MCL-induced cell death because the phenomenon was detectable as early as 3 hours after treatment (data not shown). These results agree with the prevailing response of most cancer cells exposed to chemotherapeutic components (7, 16, 39).

We then tried to reveal the molecular basis involved in MCL-induced G1 cell-cycle arrest. MCL caused a G1 cell-cycle arrest which was (partially) contributed by decreased levels of cyclin D1 and phospho-Rb, which are key members involved in cell proliferation and G1 cell-cycle progression (45). The results are commensurate with those brought about by the administration of other chemotherapeutic reagents on NPC cells, such as Grifolin (26). Furthermore, previous reports indicate that the G1 cell-cycle arrest is regulated in (a) a p53+, and p-21–dependent manner.
(27), or (b) a p53-independent transcriptional induction of p21 (46), or (c) a p21-independent manner (47). Paradoxically, we found that MCL perturbs G1 signaling distal to p53 and p21 expression. Western blot analysis indicated that MCL dose and time dependently decreased the expression of both proteins in CNE-1 and CNE-2 cells (data not shown). These data are in keeping with results from other workers using the same cell lines (16, 48). This is explainable because mounting evidence indicates that p53 mutation is among the most common genetic events in the development of human cancer (46), and both types of NPC cells have identical AGA (arginine) to ACA (threonine) changes at codon 280 of p53 (48).

Molecular linkages between MAP kinases and a variety of cellular programs such as proliferation, differentiation, development, transformation, and apoptosis have made the signaling cascade an object of intense research in recent years (28). The activation of p38 MAPK and JNK has been reported to play a significant functional role in cell death induction (19). And curiously, ERK phosphorylation has been linked with both antitumor (29) and protumor (49) activities. Our results show that MCL induced the phosphorylation of p38 MAPK in both NPC tumor cells and also increased the activation of JNK from different time points. It is important to note that MCL caused an increase of p-ERK in CNE-1 cells, as well as attenuated ERK phosphorylation in CNE-2 cells (Fig. 4). We further found that MCL could induce NO production in a NO synthase–dependent way (Fig. 4B). Pharmacologic interruption of p38 MAPK signaling, by the specific inhibitor SB-203580, weakened MCL lethality and the downstream NO production, indicating that this pathway plays an important part in cell death induction. The NO-inducing activity of MCL is of more than trivial interest because unphysiologically high levels of extracellular NO can induce apoptosis or necrosis (15). Both type I and type II RIPs (30, 31) and lectins (14, 15) have been shown to induce NO activity in mouse macrophages and/or tumor cells, and which chain(s) of MCL contributes to the production of NO awaits elucidation.

Apoptosis is executed by caspases and caspase-3 is a key protease associated with DNA fragmentation and apoptosis (39). Two main pathways have been involved in activation of caspase-3, including the caspase-8–regulated plasma membrane extrinsic pathway and the caspase-9–regulated cell damage intrinsic pathway (39). We determined that MCL exposure caused direct activation of both caspase-8 and caspase-9, followed by activation of caspase-3 and cleavage of PARP (one of the early DNA damage responses; Fig. 5). These results again corroborate our observation that MCL caused the production of DNA fragmentation and apoptotic bodies (Fig. 2). Moreover, Δψm collapse is a conspicuous feature of apoptosis and always coincides with activation of Bcl-2 family members, the release of cytochrome c into the cytoplasm, and caspase activation (50, 51). As expected, MCL treatment in NPC cells caused Δψm collapse (Fig. 3D), cytochrome c translocation into the cytoplasm (Fig. 5C), and the activation of caspase 9 (Fig. 5A). In light of mitochondrial damage, it is usually associated with the generation of reactive oxygen species (ROS) which could contribute to cell death (32), the effects of MCL in the expression levels of ROS and antioxidants (e.g., reduced glutathione) warrants further studies.

On the basis of the knowledge that (a) MCL could induce the damage of mitochondria and cell-cycle transition, and the activation of caspase cascades, (b) Bcl-2 family proteins play multiple regulatory functions in signal transduction involved in these activities (7, 27). We further investigated changes in protein levels of some Bcl-2 family members in MCL-treated NPC cells. There are 3 types of Bcl-2 family members: multidomain antiapoptotic proteins (Bcl-2, Bcl-xL, Mcl-1, and Bfl-1/A1), multidomain proapoptotic proteins (Bax, Bok/Mtd, Bcl-Xs, and Bak), and BH3-only proapoptotic proteins (Bad, Bid, BimEL, Bmf, Mcl-1S, PUMA, and NOXA; ref. 27). Three representative members from each subfamily were included in this study, including Bcl-2, Bak, and Bid. Bcl2 phosphorylation has been shown to inhibit cell-cycle progression by delaying the G1–S transition (27, 53). Furthermore, the proapoptotic proteins Bak and Bid can inhibit cell-cycle progression, inhibit cell survival, and increase apoptosis (7, 27, 39). Strikingly, MCL had no significant effects on the production of these proteins. Hu and colleagues (7) studied the apoptotic activity of ApoG2 toward NPC cells (including CNE-1 and CNE-2) and observed that ApoG2 totally blocked the functions of Bcl-2 family proteins without affecting their expression levels. Whether the same phenomenon is true of MCL remains to be investigated.

Consistent with the significant apoptosis-inducing activity in vitro, administration of MCL at a physiologically safe dose resulted in a pronounced reduction in the growth of CNE-2 xenograft tumor in nude mice. Daily intraperitoneal injection of 1.0 mg MCL/kg body weight (1.0 mg/kg/d) abated both tumor volume and weight in mice by induction of tumor cell apoptosis (Fig. 6). The dose used had no toxicity on mice in the control group. This finding is reminiscent of other reports (11, 13). The LD50 of MCL is 3.16 mg/kg body weight in Swiss mice (13). Two RIP proteins from BG exhibit profound apoptosis-inducing activity in premalignant and malignant prostate cancer cells in vitro and in vivo. The dose used for the in vivo assay in male mice was intraperitoneal 0.5 mg/kg body weight, twice a week (11).

Although some proteins manifest significant antitumor activities both in vitro and in vivo (11, 42, 54), and some are under phase III clinical trial (such as a ribonuclease Oncospin from Rana pipiens ref. 55), there are still some bottlenecks with regard to the development of protein therapeutics in humans. For example, proteins would be digested after oral intake and whether the fragments produced still remain biologically active needs investigation. A gratifying report is that MAP30, a 30-kDa RIP from BG, could yield, after digestion, biologically active fragments, which retained full activities against tumor cells (56). Furthermore, we would like to consider other routes of drug administration,
such as intravenous injection and direct injection, into the tumor. In sum, MCI is a potential candidate for prophylaxis and therapy of NPC in endemic regions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Momordica Charantia Lectin, a Type II Ribosome Inactivating Protein, Exhibits Antitumor Activity toward Human Nasopharyngeal Carcinoma Cells In Vitro and In Vivo

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