*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 (IC$_{50}$ = 6.9) and CNE-2 (IC$_{50}$ = 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 phospho-retinoblastoma (Rb) protein expression contributed to arrest at G1-phase of the cell cycle. These events were associated with regulation of mitogen-activated protein kinases (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/day) led to an average of 45% remission of NPC xenograft tumors subcutaneously inoculated in nude mice. This is the first report that unveils the potential of a type II RIP, MCL, for prevention and therapy of NPC.

Keywords: nasopharyngeal carcinoma, therapy, *Momordica charantia* lectin, tumor, traditional Chinese medicine.
Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous, 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 three groups: typical keratinising squamous-cell carcinomas (type I), non-keratinising squamous carcinomas (type II), and undifferentiated carcinomas (type III) (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) (3, 5), other aetiological factors of NPC have been disclosed, including Epstein-Barr virus (EBV) 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/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 50-60%. 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 anti-diabetic, anti-HIV, and antitumor activities in both *in vitro* and *in vivo* investigations (for details see review (8)). Special attention goes to its applications on tumor therapy. Early in 1983, Jilka and colleagues found that an aqueous extract of BG fruits inhibited tumor formation in CBA/DI 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 anti-proliferative 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 (RIPs) are a class of RNA glycosylases that cleave an adenine-ribose glycosidic bond at position A-4324 within the conserved α-sarcin/ricin loop in the eukaryotic 28S ribosomal RNA bound by elongation factors or adenine-2660 in E. coli 23S rRNA (8). Based on their structure, RIPs are divided into three 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 since 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) (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 regarding these phenomena comprise regulation of MAPK signaling axis and activation of both extrinsic and intrinsic apoptotic cascades. Furthermore, the in vivo efficacy in CNE-2 bearing nude mice was demonstrated. This is the first report 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 (Santa Cruz, CA). Antibodies for caspase 8 (9746, monoclonal), caspase 9 (9508, monoclonal), caspase 3 (9665, polyclonal), p21 (2947, polyclonal), Bcl-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), phospho-Rb (Ser 780) (9307, polyclonal), Cytochrome c oxidase IV (COX-IV) (4844, polyclonal), and cytochrome c (4280, monoclonal) were provided by Cell Signaling (Danvers, MA). All reagents were from Sigma, USA, unless otherwise indicated. Reagents including N-Nitro-L-arginine methyl ester (L-NAME), SB-203580, and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO used was < 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 (16,000 g, 30 min, 4 °C), and the aqueous supernatant was loaded on a Blue Sepharose column (buffer: 0.02 M Tris-HCl). The unadsorbed fraction with hemagglutinating activity was loaded on a SP-Sepharose column (buffer: 0.02 M NH₄OAc). The unadsorbed fraction containing hemagglutinating activity was applied to a Q-Sepharose column (buffer: 0.02 M NH₄HCO₃). Bound proteins were eluted sequentially with three concentrations of NaCl (0.2, 0.5, and 1M) in 0.02 M NH₄HCO₃. The fraction eluted with 0.5 M 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% non-reducing 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) (14). Investigations of hemagglutinating activity, ribosome inactivating activity, and sugar-specificity were performed 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-D 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% fetal bovine serum, 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) (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 μM) of MCL for 24 to 48 h, and cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazo-lium bromide (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, Cowley, UK) using annexin-V-FITC/ propidium iodide (PI) (BD Phamingen, CA, USA) double staining as previously described (15). Furthermore,
observations of apoptotic bodies and chromatin condensation were made with Hoechst 33342 staining (1 µM), and visualized under ultraviolet illumination with a NIKON TE2000 microscope (Nikon, Japan) (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 TUNEL staining (green fluorescence), using an In Situ Cell Death Detection Kit (Roche, Indianapolis, IN). Briefly, tumor cells were treated with MCL (7.5 µM) for 24 h, followed by TUNEL staining according to the manufacturer's 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 µM, 24 h) 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) (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 µg/ml (15). The mechanism is that JC-1 exists in monomeric form in the cytosol (emitting green fluorescence) and also accumulates as aggregates in the mitochondria (emitting red 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) (17).

Western blot analysis

Effects of MCL treatment on (a) the expression and phosphorylation of mitogen-activated protein kinases (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 min in 6 x SDS loading buffer, followed by SDS-PAGE. The proteins were transferred to a PVDF membrane, which was then incubated with a primary antibody in 5% milk, followed by incubation with a horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody and visualized using the ECL detection system (Amersham Life Science, Piscataway, NJ).
Detection of Nitric oxide (NO) production

First, cells were incubated with (a) 3.75 μM MCL, (b) coincubated with 3.75 μM MCL and 100 μM L-NAME (a NO synthase inhibitor), and (c) coincubated with 3.75 μM MCL and 10 μM SB-203580 (a p38 MAPK inhibitor), respectively, for 24 h. L-NAME or SB-203580 was added 1 h 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 x 10^7 CNE-2 cells were trypsinized, washed with 1 x phosphate buffered saline (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/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 x PBS. Tumor volume was determined every two days with calipers using the following formula: tumor volume (mm^3) = length (mm) x width (mm)^2. Body weight was monitored every two days as an indicator of toxicity, and mice were euthanized when tumor size exceeded 2000 mm^3 (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, Indianapolis, IN) as per manufacturer’s instructions. The cells were visualized under a light microscope, and the percentage of apoptotic cells under 5 high–power fields (20 x) was calculated (7, 19).

Statistical analysis

Results of all in vitro studies were collected from three independent experiments
performed 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. 1 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 M 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 non-reducing 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 one-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. 1F) 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 D-galactose and α-lactose at a concentration of 25 mM. These data were in accordance with previous reports (12, 13). The N-terminal amino acid sequence of one of the four subunits of MCL is NEQCSPQQRT, which coincides with the results of Tanaka and...
coworkers (23). Based on the total sequence reported by this research group (23), a bioinformatic investigation on MCL was carried out. As shown in Supplementary Fig. 2A, 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. 2B). 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 3-dimensional structure (ribbon diagram) of MCL was generated (Supplementary Fig. 2C).

**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 μM) of MCL for 24 and 48 h, respectively. As shown in Fig. 1A and Fig. 1B, 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 Fig. 1D). The IC_{50} values (24 h) for CNE-1 and CNE-2 cells were 6.9 ± 0.2 μM and 7.4 ± 0.4 μM, respectively. At 7.5 μM MCL, a concentration near IC_{50} 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 μM, 24 h). From 7.5 μM 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 Fig. 2C, 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 μM, 24 h) 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 μM 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). Since 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 3C 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- (3.75 and 7.5 μM) and time-dependent (24, 36, and 48 h) 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 μM, 24 h) 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 three major subgroups of MAPKs, including p-38 MAPK, Jun kinase (JNK/SAPK), and extra-cellular signal-regulated kinase (ERK) (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 μM MCL increased from 4 h, and 12 h, respectively, in a time-dependent way. Phosphorylation of JNK levels was not significantly altered in both cells of early exposure (before 8 h), and a great increase was appeared at 24 h 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 of phospho-ERK (p-ERK) levels in CNE-2 cells. A quantitative analysis of Fig. 4A is shown in Supplementary Fig. 3. 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 two types of NPC cells (Fig. 4B), whereas coadministration of 100 μM 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 (14)) contamination in MCL (data not shown). Furthermore, the NO production and cytotoxicity induced by MCL at 3.75 μM was partially blocked by cotreatment with the p38 MAPK inhibitor, SB-203580, at a dose of 10 μM (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 two distinct classes, the initiators (such as caspase 8, caspase 9) and the effectors (including caspase 3 and others) (33). We further elucidated the involvement of such caspases in MCL-induced cell death. As shown in Fig. 5A and Fig. 5B, MCL treatment resulted in a dose- (3.75 and 7.5 μM) and time- (24, 36 and 48 h)
dependent activation of the initiator caspases 8 and 9, and the executor caspase 3 in both
CNE-1 and CNE-2 cells. Accordingly, caspase activation leading to PARP cleavage was
observed. Since 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 μM) 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 μM (near IC<sub>50</sub> value) after
treatment for 48 h (data not shown).

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

After demonstrating 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 1mg MCL/kg body
weight for 12 consecutive days (i.p., 1.0 mg/kg/day), while the control group was treated
with PBS instead. As shown in Fig. 6A, MCL halted tumor growth on the 4<sup>th</sup> day (day 8) of
administration. This effect was consistent, and on the 12<sup>th</sup> 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. In order 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 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) (39), Chk1 inhibitor Gö6976 (40), lapatinib (also named as Tykerb, GW572016; the first dual kinase inhibitor of EGFR and HER-2) (41), and ApoG2 (the synthesized oxidation product of apogossypol from the cotton plant Gossypium) (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. 1). 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. 2). Though 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 two representative types of NPC cells by the regulation of MAPKs and caspase cascade.

The cytotoxicity and anti-proliferative 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 is responsible for the MCL effect. MCL increased the proportion of apoptotic cells, caused chromatin condensation and nuclear fragmentation (Fig. 2), which are
unique morphological 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 appears to be the cause rather than the effect of MCL-induced cell death since the phenomenon was detectable as early as 3 h 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, since 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 MAPKs and a variety of cellular programs like 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 down-stream 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 since 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 demonstrated with NO-inducing 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 is usually associated with the generation of reactive oxygen species (ROS) which could contribute to cell death (52), the effects of MCL in the expression levels of ROS and antioxidants (e.g., reduced glutathione) warrants further studies.

Based on 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 three types of Bcl-2 family members: multidomain antiapoptotic proteins (Bcl-2, Bcl-xL, Mcl-1L, 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) (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 coworkers (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 i.p. 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 LD_{50} of MCL is 3.16 mg/1kg 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 i.p. 0.5 mg/kg body weight, twice a week (11).

Though 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 Onconase from Rana pipiens) (55), there are still some bottlenecks concerning 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, MCL 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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References


Figure Legends

Fig. 1. MCL-mediated selective cytotoxic activity toward NPC cells. MCL induced cell death in NPC cells in a dose- and time-dependent manner, but not in NP69 cells which are transformed from normal human nasopharyngeal epithelial cells. Cells were incubated with a series of concentrations of MCL (0 to 60 μM) for 24 h or 48 h. Cell viability was determined by MTT assay (A and B), and by cell counting on the basis of trypan blue exclusion (C and D). Data represent mean ± SD of three independent experiments. *, #, &, p < 0.05 vs. control of CNE-1, CNE-2, and NP69, respectively.

Fig. 2. MCL induces apoptosis in NPC cells. (A) MCL treatment in CNE-1 and CNE-2 cells increased the percentage of cells exhibiting early apoptosis and late apoptosis/necrosis. NPC cells were cultured for 24 h either alone or with a series of concentrations of MCL, and the cell fate was monitored by flow cytometry after double staining with FITC-Annexin V and propidium iodide. The data were analyzed by WinMDI 2.9 software. (B) and (C) Treatment of CNE-1 and CNE-2 cells with MCL induced the production of apoptotic bodies. After treatment with 7.5 μM MCL for 24 h, NPC cells were stained with Hoechst 33342 dye, and the formation of apoptotic bodies (indicated by asterisks) and chromatin condensation (denoted by arrows) was revealed under a fluorescence microscope. Contemporarily, the percentage of cells showing apoptotic bodies was counted (C). (D) MCL-induced DNA fragmentation in CNE-1 and CNE-2 cells. NPC cells were cultured in the presence of 7.5 μM MCL for 24 h, and the percentage of cells exhibiting DNA fragmentation was calculated by flow cytometry using a TUNEL kit. Numbers in percentage of M1 region indicated the percentage of cells undergoing DNA fragmentation. a, control; b, 7.5 μM MCL. *, p < 0.05 vs. control.

Fig. 3. MCL induces G1 cell cycle arrest and ΔΨm collapse in NPC cells. (A) After
treatment with MCL (0 ~ 15 μM) for 24 h, cells were stained with propidium iodide, and DNA content was measured by flow cytometry followed by assessment using WinMDI 2.9. Results were presented as percentage of cells in G1, S, and G2-M phases of the cell cycle, and showed that MCL induced a marked G1 arrest in both CNE-1 (left panel) and CNE-2 (right panel) cells. (B) MCL treatment reduced the protein expression of cyclin D1 and phospho-Rb. Protein levels were measured by Western blot and β-tubulin was used as a control. (C) Quantitative data of the expressed levels of cyclin D1 and phospho-Rb. The quantification of two different Western blots is shown, performed with ImageJ software. (D) After treatment with MCL (0 ~ 15 μM) for 24 h, ΔΨm collapse was measured by flow cytometry using JC-1 staining. Percentage of cells undergoing ΔΨm collapse was recorded. *, p < 0.05 vs. control.

Fig. 4. Activation of p38 MAPK and induction of downstream NO in MCL-treated NPC cells. (A) MCL stimulates the p-38, JNK, and ERK pathways. After treatment of NPC cell lines CNE-1 and CNE-2 with 3.75 μM MCL for the indicated durations (0, 1, 2, 4, 8, 12, 24 h), cell lysates were prepared, and separated by SDS-PAGE prior to immunoblot detection of phosphorylated p38 MAPK, JNK, and ERK. (B) Induction of NO by MCL. Cells (mouse peritoneal macrophages, CNE-1 and CNE-2) were treated with 3.75 μM MCL or a combination of 3.75 μM MCL and 100 μM L-NAME for 24 h. L-NAME was added 1 h prior to MCL. The release of nitrite and nitrate, as the surrogate markers for NO, into the cell culture supernatant was determined using Griess reagent. (C) Coadministration of 10 μM SB-203580 (SB, 1 h pretreatment before adding MCL), a p38 MAPK inhibitor, partially blocked MCL-induced NO (left panel) and cytotoxicity (right panel). For (B) and (C), data represent mean ± S.D. of three independent experiments conducted in quadruplicate wells. *, p < 0.05 vs. control.
Fig. 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 vs. control.

Fig. 6. The antitumor effect of MCL in nude mice bearing CNE-2 xenograft. (A) Two groups of nude mice were inoculated with CNE-2 xenograft. On day 4 tumor was palpable. The MCL-treated group received a daily intraperitoneal injection of 1.0 mg MCL/kg body weight. The control group was treated with PBS buffer. The tumor volumes were calculated every two days. Eight xenografts were performed in each group, and data show the average tumor volume ± S.D. expressed in relation to the initial tumor volume. (B) On day 13, mice were sacrificed, and the tumor weight was measured. (C) CNE-2 xenografts were performed with TUNEL staining, and visualized under a light microscope. Left panel: representative figures of TUNEL staining. Right panel: Quantification of percentage of apoptotic cells was calculated under 5 high–power fields. *, p < 0.05 vs. control.
A

B

C

D

Relative expression of active caspase 3

Relative expression of cleaved PARP

Cytoplasm

Mitochondria

Annexin V

Cancer Research.


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