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Preferential cytotoxicity of the type I ribosome inactivating protein alpha-momorcharin on human nasopharyngeal carcinoma cells under normoxia and hypoxia

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Abstract

All primary nasopharyngeal carcinoma (NPC) tumors contain hypoxic regions which are implicated in decreased local control and increased distant metastases, as well as resistance to chemotherapy in advanced NPC patients. One of the promising therapeutic approaches for NPC is to use drugs that can target hypoxic factors in tumors. In the present investigation, the type I ribosome inactivating protein α -momorcharin (α -MMC), isolated from seeds of the bitter gourd *Momordica charantia*, reduced cell viability and inhibited clonogenic formation of human NPC CNE2 and HONE1 cells under normoxia and cobalt chloride-induced hypoxia. By comparison, α -MMC exhibited only slight cytotoxicity on human nasopharyngeal epithelial NP69 cells under normoxia. Interestingly, α -MMC suppressed the expression levels of hypoxia-inducible factor 1- α (HIF1 α) and vascular endothelial growth factor (VEGF) in hypoxic NPC, as well as the growth of human umbilical vein endothelial cells. Further study disclosed that α -MMC targeted endoplasmic reticulum and down-regulated unfolded protein response (UPR) in NPC cells. Moreover, α -MMC induced apoptosis in NPC cells in a dose- and time-dependent manner. It initiated mitochondrial- and death receptor-mediated apoptotic signaling in CNE2 cells, but there was hardly any effect on HONE1 cells. In addition, α -MMC brought about G0/G1 phase cell cycle arrest in CNE2 cells and S phase arrest in HONE1 cells. Collectively, α -MMC preferentially exhibited inhibitory effect on normoxic and hypoxic NPC cells partly by blocking survival signaling (*e.g.* HIF1 α , VEGF and UPR), and triggering apoptotic pathways mediated by mitochondria or death receptor. These observations indicate the potential utility of α -MMC for prophylaxis and therapy of NPC.

Keywords

α -Momorcharin; Unfolded protein response; Ribosome inactivating protein; Nasopharyngeal carcinoma; Hypoxic tumors

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1. Introduction

Nasopharyngeal carcinoma (NPC) is an endemic disease. In western countries, NPC occurs occasionally and is correlated with alcohol and smoking. This type of NPC is classified into type I squamous cell carcinoma. Type II and Type III NPC, which are associated with an infection of Epstein–Barr virus (EBV) [1,2], prevail in Southern China and Southeast Asia, with an incidence rate of 15–50/100,000 [3,4]. Up to 70% of patients with NPC present with a locally advanced stage at the time of diagnosis [5,6]. Although concurrent cisplatin administration and radiotherapy for advanced NPC improved overall survival rates [7], posttreatment local relapse and particularly distant metastasis remain as the major problematic issues which eventually lead to the death of patients with advanced NPC [8].

Clinical studies showed that 100% of primary NPC and 58% of cervical nodal metastases of NPC were found to contain hypoxic regions [9], which was ascribed to an extremely vascularized structure of the nasopharynx [10]. Notably, tumor hypoxia is believed to contribute to decreased local control and increased distant metastases, as well as resistance to chemotherapy and radiotherapy [11]. In particular, among the proteins associated with tumor hypoxia, hypoxia-inducible factor 1- α (HIF1 α) and its downstream vascular endothelial growth factor (VEGF) are key hypoxia regulatory molecules that promote angiogenesis and metastasis [12,13]. Overexpression of HIF-1 α and VEGF was commonly found in NPC [14]. HIF-1 α has become a prognostic factor [15], as well as a potential therapeutic target of NPC [16].

Moreover, hypoxia constitutes a physically important endoplasmic reticulum (ER) stress. Thus tumor cells in hypoxia often rely on an activated unfolded protein response (UPR) for survival [17]. The UPR initiates mechanisms to deal with the accumulation of misfolded and/or unfolded proteins through selective expression of ER chaperone proteins, and an ER-associated degradation (ERAD) protein degradation process [18]. In response to UPR, three ER trans-membrane stress sensors including pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) release from the ER chaperone glucose-regulated protein of 78 kDa (GRP78) and subsequently launched three arms of signaling pathway, respectively [19]. The kinase PERK phosphorylates the serine 51 residue of eukaryotic initiation factor 2 α (eIF2 α) to inhibit mRNA translation, which in turn activates transcription factor 4 (ATF4) upon UPR [20]. IRE1, the initial sensor of the other branch of UPR, processes X-box binding protein 1 (XBP-1) mRNA, removing a 26-nucleotide intron to generate a spliced XBP-1 mRNA which encodes the functional XBP-1 transcription factor [21]. It has been reported that cells with a compromised PERK-eIF2 α pathway are more sensitive to hypoxic stress *in vitro* and grow into smaller tumors *in vivo* [22]. The IRE1–XBP-1 pathway has also been considered as a vital factor for surviving hypoxic stress *in vitro* and, more importantly, for optimal tumor growth *in vivo* [23]. There are two approaches for therapeutic targeting of ER stress pathways. The first is to target tumors which have activated the unfolded protein response for survival [24]. The second approach is to artificially induce the unfolded protein response in cells that already have a high demand on this system causing an overload which triggers the cell death program [25].

Ribosome inactivating proteins (RIPs) possess enzymatic activity that brings about the cleavage of a specific adenine base in ribosomal RNA, thus bringing protein synthesis to a halt [26]. An approximately 30-kDa glycoprotein designated as α -momorcharin (α -MMC), which was isolated from the bitter gourd *Momordica charantia*, is a type I RIP with a sugar chain attached to Asn-227 [27]. In contrast to type II RIPs with an extra binding chain, type I RIPs contain only an enzymatic chain [26]. Previous studies showed that natural α -MMC exhibited an inhibitory effect on DNA synthesis of mouse monocyte macrophage P388, Balb/c macrophage J774, human placental choriocarcinoma JAR and sarcoma S180 cell lines, as well as enhanced the tumoricidal effect of mouse macrophages on mouse mastocytoma P815 cells [28]. In order to undermine the immunogenicity of α -MMC, engineered homogeneous mono-, di- and, tri-PEGylated α -MMC had been produced. Both *in vitro* and *in vivo* investigations manifested that they retained moderate antitumor activity with reduced immunogenicity apart from their innate immunosuppressive activity [29,30]. These PEG-conjugates effectively exerted their cytotoxicity toward many tumor cells including melanoma, liver cancer, breast cancer, non-small cell lung cancer, epidermoid carcinoma, and colon cancer cells [30]. The strategy facilitates the application of α -MMC in cancer therapy. However, the antiproliferative activity of α -MMC on NPC and the underlying mechanism remain to be explored.

In this study, α -MMC exerted its inhibitory effect on cell viability and clonogenic formation of NPC CNE2 and HONE1 cells under normoxic and hypoxic conditions *in vitro*. To further ascertain its molecular mechanism, we found that α -MMC targeted endoplasmic reticulum and down-regulated unfolded protein response (UPR) in CNE2 (*e.g.* down-regulated expression level of PERK, IRE1 α and CHOP) and HONE1 (*e.g.* down-regulated expression level of PERK and CHOP) cells. Moreover, α -MMC induced dose- and time-dependent apoptosis in both CNE2 and HONE1 cells. Further study disclosed that α -MMC initiated mitochondrial- and death-receptor mediated apoptotic signaling cascades in CNE2 cells (as evidenced by activation of caspase-9, caspase-8 and caspase-3, and mitochondrial membrane potential depolarization), but elicited a weaker response from HONE1 cells (as witnessed by slight cleavage of caspase-8, and devoid of cleavage of caspase-9 and caspase-3, and less mitochondrial membrane potential depolarization). α -MMC caused G0/G1 phase cell cycle arrest in CNE2 cells involving inhibition of the activity of protein-serine-threonine kinases B (Akt) and activation of glycogen synthase kinase-3 α (GSK-3 α) and GSK-3 β , and S phase arrest in HONE1 cells possibly due to activation of GSK-3 α and GSK-3 β .

2. Materials and methods

2.1. Materials

The human nasopharyngeal carcinoma (NPC) cell line CNE-2 was purchased from the Sun Yat-sen University of Medicinal Sciences, Guangzhou, China. Human NPC cell line HONE1 and transformed human nasopharyngeal epithelial cell line NP 69 were generously provided by Prof. S.W. Tsao (Department of Anatomy, The University of Hong Kong). Human umbilical vein endothelial cells (HUVEC) were generously provided by Prof. Y. Huang (School of Biomedical Sciences, The Chinese University of Hong Kong). Primary antibodies against PERK (#3192), IRE1 α (#3294), CHOP (#2895), β -actin (#4970), caspase-9

(#9502), caspase-3 (#9662) and Phospho-Akt (Ser473) (#9271), and secondary antibodies against horseradish peroxidase (HRP)-linked anti-mouse immunoglobulin G (IgG) (#7076) and anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibody against phosphorylated GSK3 (G8170-47) was obtained from United States Biological (MA, USA). Primary antibody against caspase-8 (551243) was bought from BD Pharmingen (CA, USA). Primary antibody against HIF-1 α (NB100-105) was obtained from Novus Biologicals (CO, USA).

2.2. Preparation of α -momorcharin

Alpha-momorcharin (α -MMC) was isolated as previously described [31]. Briefly, bitter gourd seeds were extracted by homogenizing in distilled water. The aqueous supernatant obtained after centrifugation ($16,000 \times g$, 30 min, 4 °C) was loaded on an Affi-gel blue gel column (2.5 cm $\times$ 15 cm, Bio-Rad, UK) in 20 mM Tris-Cl buffer (pH 7.0). The bound fraction was then loaded on an SP Sepharose column (GE Healthcare, Hong Kong) in 20 mM NH_4OAc buffer (pH 4.6). Bound proteins were eluted sequentially with three concentrations of NaCl (0.2 M, 0.5 M, and 1 M) in 20 mM NH_4HCO_3 buffer (pH 9.4). The fraction eluted with 0.2 M NaCl was pooled, dialyzed, and loaded on a Superdex 75 HR 10/30 FPLC column (GE Healthcare) in 20 mM NH_4HCO_3 buffer (pH 9.4). Then the major eluted fraction was collected and lyophilized to yield pure α -MMC.

2.3. Cell culture and assessment of cell viability

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) containing 10% fetal bovine serum (Hyclone, Gramlington, UK), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Millipore, Darmstadt, Germany) at 37 °C in a humidified 5% CO_2 incubator. After growing to 70–80% confluence, cells (2×10^3 – 4×10^3) were trypsinized and seeded into a 96-well plate. After incubation for 24 h, the cells were treated with α -MMC at specified concentrations and for different durations. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [32].

2.4. Clonogenic assay

CNE2 or HONE1 cells (0.5×10^3) were seeded on a 6-well culture plate for 24 h. Then the cells were treated with various concentrations of α -MMC for 48 h. After rinsing with fresh medium, cells were allowed to grow for 10 days to form colonies, and were then fixed with 4% formaldehyde and subsequently stained with crystal violet (0.4 g/L, Sigma, MO, USA). The images were captured by using the ChemDoc XRS system. The number of colonies was counted by using Quantity One Version 4.2. Plating efficiencies were determined for each treatment and normalized with reference to the control. The plating efficiencies of control CNE2 and HONE1 cells were $92.42 \pm 2.43\%$, and $90.21 \pm 4.15\%$, respectively.

2.5. Detection of apoptotic cells

The percentage of cells undergoing apoptosis was determined by flow cytometry (FACSCanto I, BD, New Jersey, USA) using signal or dual staining with Annexin V-FITC (BD Pharmingen, CA, USA) and propidium iodide (PI, Sigma, MO, USA) as previously

described [32]. Briefly, after seeding on a 6-well culture plate for 24 h, CNE2 and HONE1 cells (10^5) were respectively treated with the indicated concentration of α -MMC for different durations, respectively. Then the cells were trypsinized, centrifuged and washed thrice with phosphate buffered saline (PBS). The cell pellets were resuspended in binding buffer (0.01 M HEPES, pH 7.4, containing 140 mM NaCl and 25 mM CaCl_2) for subsequent Annexin V/PI staining and flow cytometry analysis. Furthermore, observations of chromatin condensation were made after staining with Hoechst 33342 (10 $\mu\text{g/mL}$, Invitrogen™, Oregon, USA) and by visualization under UV illumination with a Nikon TE2000U fluorescence inverted microscope.

2.6. Cell cycle analysis and measurement of mitochondrial membrane potential (Ψ_m) depolarization

CNE2 and HONE1 cells (10^5) were treated for different durations with 1 μM α -MMC. For cell cycle analysis, cells were trypsinized and stained with PI (50 $\mu\text{g/mL}$ PI in PBS, containing 0.05% Triton X-100 and 1 mg/mL RNase A) at 4 °C overnight with avoidance of exposure to light. Then the cell cycle was analyzed using flow cytometry. For Ψ_m detection, CNE2 and HONE1 cells (10^5) were respectively treated for different durations with 5 μM α -MMC. Then cells were stained with 2.5 $\mu\text{g/mL}$ 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma, MO, USA). In the absence of Ψ_m depolarization, JC-1 accumulates in the mitochondria as aggregates that emit red fluorescence. When Ψ_m depolarization occurs, JC-1 is localized in monomeric form in the cytosol and emits green fluorescence.

2.7. Labeling of α -MMC with fluorescent tag bimane amine

Pure α -MMC was dissolved in 0.1 M 2-(N-Morpholino) ethanesulfonic acid sodium salt (MES) buffer (pH 5.0) to a final concentration of 2 mM. The fluorescent tag bimane amine (Molecular Probes, Eugene, OR, USA) was added (final concentration 10 mM) together with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (final concentration 2 mM, Molecular Probes, Eugene, OR, USA). The reaction mixture was incubated overnight at 4 °C with gentle stirring before centrifugation (10 min, $18,000 \times g$) to remove precipitated peptide. An amicon tube (molecular mass cutoff 3000 Da) was used to remove salts, unbound bimane amine, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide from the labeled proteins. Then the powder of the labeled protein was obtained by freeze-drying. The fluorescence of bimane- α -MMC was examined in 12% of SDS-PAGE followed by UV illumination using ChemDoc XRS system. The purity of bimane- α -MMC was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (Bio-Rad laboratory, CA, USA).

2.8. Confocal imaging microscopy

CNE2 or HONE1 cells were seeded on the Coverglass-Bottom Dish (35 mm $\times$ 10 mm, SPL life Sciences, Gyeonggi-Do, Korea) for 24 h. After cells had been treated with bimane- α -MMC for 24 h, the culture medium in the dishes was discarded, and the cells were stained with 1 μM ER-Tracker Red (Molecular Probes, Eugene, OR, USA) in DMEM at 37 °C in a humidified 5% CO_2 incubator for 30 min. Then the cells were rinsed with PBS thrice and confocal imaging was performed on the Olympus FV1000-ZCD Laser Scanning Confocal System (Tokyo, Japan) connected to an IX81 inverted microscope with 63 $\times$ oil immersion

objective lens. LD405 laser was used to excite bimane amine and the emission was acquired by a Band-pass Filter (425–475 nm). LD559 laser was applied for ER-Tracker Red excitation, and the images were captured through the Band-pass Filter (570–670 nm). All images were analyzed by Olympus Fluoview Version 2.0.

2.9. Western blot analysis

After cells had been treated with α -MMC for different durations, cell lysates were prepared by using RIPA lysis buffer (50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, MO, USA), protease cocktail inhibitor (Sigma, MO, USA), and phosphatase cocktail inhibitor II (Sigma, MO, USA). Then cell lysates were subjected to Western blot analysis using various antibodies mentioned in Section 2.1, as previously described [32]. Each experiment was repeated thrice and the representative pictures are shown in the figures. The results were quantified by plotting the density intensity of the band using Quantity One Version 4.6.2. β -Actin was used as an internal control.

2.10. Hypoxic pretreatment

For investigation employing hypoxic cells, NPC cells were pretreated with 150 μ M cobalt chloride (CoCl_2 , Sigma, MO, USA) for 24 h. Then the hypoxic cells were examined for cell viability and colony formation and using Western blot.

2.11. Statistical analysis

Results were collected from 3 independent experiments conducted in triplicate, and data are expressed as mean $\pm$ SD. The histograms were drawn using Origin Version 6.0. Students' *t*-test and two-way ANOVA were used for between-group comparisons. Difference with a *P* value < 0.05 was considered as statistically significant.

3. Results

3.1. Cytotoxicity of α -MMC on human nasopharyngeal carcinoma (NPC) cells

Cell viability was assessed by using the MTT assay. Human nasopharyngeal carcinoma (NPC) CNE2 and HONE1 cells, and transformed human nasopharyngeal epithelial NP69 cells were treated with increasing dosages of α -MMC (0–10 μ M) for 24 h and 48 h, respectively. α -MMC evinced a concentration- and time-dependent inhibitory effect on proliferation of CNE2 and HONE1 cells (Fig. 1a and b), but exhibited only slight cytotoxicity toward NP69 cells (Fig. 1c). Furthermore, the clonogenic assay was used to investigate the long-term inhibitory effect of α -MMC on NPC. As shown in Fig. 1, α -MMC reduced the efficiency of colony formation in both CNE2 (Fig. 1d) and HONE1 cells (Fig. 1e).

3.2. α -MMC retained cytotoxicity on hypoxic NPC cells

Tumor hypoxia, which has been implicated in tumor metastasis and chemotherapy resistance [11], usually occurred in NPC *in vivo* [9]. To further investigate the anti-tumor effect of α -MMC under hypoxic conditions, NPC cells pretreated with cobalt chloride (CoCl_2), a

chemical inducer of HIF1 α , were exposed to various concentrations of α -MMC for 48 h. As shown in Fig. 2a, the level of HIF1 α , a key regulator in hypoxia, was augmented after pretreatment with 150 μ M CoCl₂ for 24 h in CNE2 cells, as well as in HONE1 cells. This augmentation did not attenuate the cytotoxicity of α -MMC on CNE2 cells or HONE1 cells (Fig. 2b). Moreover, α -MMC also maintained its inhibitory effect on colony formation in both hypoxic CNE2 and HONE1 cells (Fig. 2c).

To further explore the molecular mechanism of α -MMC on hypoxic signaling in NPC cells, the factors responsible for hypoxia were investigated after the treatment of hypoxic CNE2 and HONE1 cells with 0 μ M, 0.5 μ M and 1 μ M α -MMC for 24 h and 48 h, respectively. Western blot analysis disclosed that α -MMC down-regulated the expression level of HIF1 α and the downstream VEGF which were up-regulated in both CoCl₂-pretreated CNE2 and HONE1 cells, respectively (Fig. 2d). Moreover, α -MMC impeded, in a dose- and time-dependent fashion, the growth of human umbilical vein endothelial cells (HUVEC) which is critical to angiogenesis (Fig. 2e). These results suggested that α -MMC retained its antiproliferative effect on hypoxic CNE2 and HONE1 cells which was associated with down-regulation of HIF1 α and VEGF expression.

3.3. α -MMC targeted endoplasmic reticulum and down-regulated unfolded protein response in NPC cells

In order to determine whether α -MMC can gain entry into cells and ascertain its subcellular localization, the fluorescent tag bimane amine was used to label α -MMC as described in Section 2. As depicted in Fig. 3a, bimane amine labeled α -MMC (bimane- α -MMC) of high purity was obtained. The fluorescent dye ER-Tracker Red was exploited for endoplasmic reticulum labeling in CNE2 and HONE1 cells after treatment with 1 μ M bimane- α -MMC for 24 h, respectively. Confocal imaging analysis unveiled that bimane- α -MMC was localized in endoplasmic reticulum in both CNE2 and HONE1 cells (Fig. 3b). Furthermore, massive swelling-like endoplasmic reticulum occurred in both CNE2 and HONE1 cells after treatment with bimane- α -MMC (Fig. 3b). These results suggested that endoplasmic reticulum was a subcellular target of α -MMC in NPC cells.

The homeostasis of endoplasmic reticulum was regularly manipulated by unfolded protein response (UPR) which was reported to counter stress for survival in hypoxic tumor [17]. In view of morphological changes of endoplasmic reticulum elicited by α -MMC, the factors contributing to UPR were considered after treatment of CNE2 and HONE1 cells with specified concentrations (0, 0.5, 1 and 5 μ M) of α -MMC for different durations (24 and 48 h), respectively. Immunoblotting analysis showed that the expression levels of PERK, IRE1 α and CHOP in CNE2 cells were attenuated in response to α -MMC in a time- and dose-dependent manner (Fig. 3c). The expressions levels of PERK and CHOP, rather than IRE1 α , were down-regulated in HONE1 cells after treatment with α -MMC (Fig. 2c). These data signified that down-regulation of UPR was involved in the cytotoxicity of α -MMC on NPC cells.

3.4. α -MMC induced dose- and time-dependent apoptosis in NPC cells

To illustrate the form of cell death induced by α -MMC, Annexin V/PI (single or dual) staining was applied for evaluation of apoptotic cells. As shown in Fig. 4a, the percentage of Annexin V-positive cells rose, in a concentration-dependent fashion, after exposure of CNE2 and HONE1 cells respectively to escalating dosages (0, 0.1, 0.5, 1 and 5 μ M) of α -MMC for 48 h. Moreover, CNE2 and HONE1 cells underwent time-dependent apoptosis (Fig. 4b) after treatment with 5 μ M α -MMC for different durations (0, 12, 24, 36 and 48 h), respectively. In addition, Hoechst staining demonstrated that characteristic features of apoptosis, including chromatin condensation and DNA fragmentation, were detected in CNE2 and HONE1 cells after treatment with 5 μ M α -MMC for 48 h (Fig. 4c).

3.5. α -MMC induced mitochondrial- and death receptor-mediated apoptotic signaling cascades in CNE2 cells, but ablation in HONE1 cells

It is reported that various apoptotic pathways induced by RIPs in cancer cells converged in mitochondria [33]. The factors responsible for these pathways hereby were investigated herein after the treatment of CNE2 and HONE1 cells with indicated concentrations of α -MMC for different durations, respectively. Western blot analysis manifested that the activation of caspase-9 and caspase-8, respectively accounting for mitochondrial- and death receptor pathways, occurred, in a time- and dose-dependent manner, in CNE2 cells (Fig. 5a). Furthermore, activated caspase-3, which served as downstream of both caspase-9 and caspase-8, was also detected in CNE2 cells (Fig. 5a). However, slight cleavage of caspase-8 without caspase-9 and caspase-3 activation was identified in HONE1 cells (Fig. 5a). Flow cytometry analysis of mitochondrial membrane potential (Ψ_m) revealed that α -MMC treatment produced a marked time-dependent Ψ_m depolarization in CNE2 cells (Fig. 5b), but not in HONE1 cells (Fig. 5b). Overall, α -MMC triggered mitochondrial- and death receptor-mediated apoptotic signaling cascades in CNE2 cells, but not in HONE1 cells.

3.6. α -MMC induced differential cell cycle arrest in CNE2 and HONE1 cells

To further investigate the mechanism of apoptosis induced by α -MMC in NPC cells, flow cytometry analysis of cell cycle was conducted after the treatment of CNE2 and HONE1 cells respectively with 1 μ M α -MMC for different durations. As shown in Fig. 6a, α -MMC induced time-dependent G0/G1 phase arrest in CNE2 cells, and S phase arrest in HONE1 cells. These data suggested that α -MMC exerted its cytotoxicity on NPC cells *via* induction of cell cycle arrest.

Furthermore, glycogen synthase kinase-3 (GSK-3), which can be inactivated through dependence or independence on Akt-mediated phosphorylation at Ser21 of GSK-3 α and Ser9 of GSK-3 β [34,35], was thought to stall cell cycle progression by regulating cyclin D1 phosphorylation and proteolysis [36]. These factors were herein investigated after CNE2 and HONE1 cells had been respectively exposed to indicated concentrations of α -MMC for different durations. Western blot analysis showed that α -MMC treatment down-regulated the phosphorylation level of Akt, GSK-3 α and GSK-3 β in CNE2 cells (Fig. 6b). Surprisingly, up-regulation of phosphorylation level of Akt was found in HONE1 cells (Fig. 6b). In addition, α -MMC treatment induced down-regulation of phosphorylation level of GSK-3 α and GSK-3 β in HONE1 cells (Fig. 6b). Collectively, α -MMC caused G0/G1 phase cell

cycle arrest in CNE2 cells involving inhibition of activity of Akt and activation of GSK-3 α and GSK-3 β , and S phase arrest in HONE1 cells possibly due to activation of GSK-3 α and GSK-3 β .

4. Discussion

Previous investigations demonstrated that the type I RIP α -momorcharin (α -MMC) is able to inhibit the proliferation of a variety of tumor cells [28,30]. It is revealed herein that α -MMC-induced suppression of human nasopharyngeal carcinoma (NPC) growth associated with down-regulation of unfolded protein response (UPR), cell cycle arrest, and mitochondrial- and death receptor-mediated apoptotic signaling pathway. Importantly, α -MMC retains its cytotoxicity on hypoxic NPC cells which entails restraint of hypoxic signaling such as down-regulation of HIF1 α and VEGF expression. The results advocate that α -MMC is a promising agent for NPC therapy and provide a new insight to treatment of hypoxic tumors using RIPs.

By adopting the fluorescent labeling technique, we reveal here that α -MMC is capable of entry into NPC cells and reach the endoplasmic reticulum. RIP uptake by cells is very important for their cytotoxicity. It has been reported that type II RIPs could be taken up by cells through different endocytic pathways either dependent on or independent of clathrin-coated pits [37,38]. Recently, the type I RIP trichosanthin (TCS) was demonstrated to be internalized by means of low density lipoprotein (LDL) receptor-related protein 1 (LRP1) expressed in JAR and BeWo choriocarcinoma cell lines [39]. Here, we present evidence that the type I RIP α -MMC can enter into NPC cells and reside in endoplasmic reticulum (Fig. 3b), a destination where RIPs regularly arrive at during intracellular traffic associated with endocytic process, and retrograde transport *via* the trans-Golgi network [37,38,40]. Furthermore, the observation that α -MMC induced down-regulation of the expression levels of PERK, IRE1 α and CHOP which are engaged in the unfolded protein response (UPR) in NPC cells (Fig. 3c), may contribute to the *N*-glycosidase activity of α -MMC in cleavage of adenine residue of 28S RNA in the 60S subunit of endoplasmic reticulum membrane-bound ribosome [41]. This ensures inhibition of protein synthesis and subsequent apoptosis [42]. As expected, inhibition of cell proliferation and colony formation (Fig. 1), and increase of the percentage of apoptotic cells (Fig. 4) were detected after treatment of NPC cells with α -MMC.

The notable finding in this study is that α -MMC can manipulate hypoxic signaling and sustain its cytotoxicity in hypoxic NPC cells. This proposes that α -MMC is a promising candidate for clinical therapeutic intervention of NPC. To date, concurrent administration of chemotherapy-radiotherapy for advanced NPC usually did not meet with success owing to posttreatment local relapse and particularly distant metastasis [8]. It is believed that tumor hypoxia, which is encountered in most of primary NPC [9], leads to distant metastases as well as resistance to chemotherapy and radiotherapy [11]. The hypoxia-inducible factor (HIF) is a central component of hypoxia [12]. The α -subunits of the HIF transcription factors are degraded by proteasomal pathways during normoxia [43] but are stabilized under hypoxic conditions [44]. Cobalt prevented von Hippel-Lindau protein (pVHL) mediated-ubiquitination and rapid degradation of HIF- α through binding directly to the oxygen-

dependent degradation (ODD) domain of HIF- α under normoxia [45]. Therefore cobalt chloride mimics hypoxia by stabilizing HIF- α . In the present study, cobalt chloride was used for hypoxic pretreatment of cells, and the data indicate that treatment of hypoxic NPC cells with α -MMC ensues in a decrement of the expression level of HIF1 α (Fig. 2d), a key regulator accounting for hypoxia tolerance and therapy resistance [46]. Thus this action of α -MMC may explain the observation that α -MMC adequately exerts an inhibitory effect on NPC cells and colony formation in hypoxia as well as in normoxia (Fig. 2b and c). In addition, consistent with the results that down-regulation of VEGF expression occurs in hypoxic NPC cells (Fig. 2d), α -MMC suppresses the proliferation of HUVEC (Fig. 2e) which is critical for angiogenesis. This implies that α -MMC is of considerable interest as a potential *in vivo* anti-tumor agent for its cytotoxic action on tumors and inhibitory effect on angiogenesis. Moreover, accumulating clinical evidence suggest that hypoxic tumors also exist in patients with cervical cancers, carcinomas of the head and neck, or soft-tissue sarcomas [47]. Therefore, the clinical usage of α -MMC could be extended to these hypoxic tumors due to its conspicuous action on the regulation of hypoxic signaling.

Apart from modulation of hypoxia *via* inhibition of HIF1 α , the finding of this study indicates that α -MMC down-regulates the expression level of the markers responsible for UPR in NPC cells. UPR usually serves as a modulator dealing with stress in cancer cells, thereby preventing tumors from cell death under stressful conditions [48]. There are two alternative strategies to target UPR for cancer therapy: prolonging UPR that leads to programmed cell death [25], or inhibiting components of UPR so that tumors were unarmed of the weapons against stresses [24]. Here, α -MMC seems to exert the latter action on NPC cells as suggested by the observation that expression level of PERK, IRE1 α and CHOP is diminished in response to α -MMC (Fig. 3c). It has been reported that inhibition of UPR by ricin A-chain enhances its cytotoxicity [49]. Our data disclose that α -MMC undermines cell viability as well as manifests a long-term inhibitory effect on the clonogenic colony formation in NPC cells. Interestingly, there was only an approximately 10% increment of the proportion of Annexin V-positive cells in CNE2 and HONE1 cells subsequent to treatment with 1 μ M α -MMC (Fig. 4a), in spite of a *circa* 50% lowering of cell viability (Fig. 2b). A substantial percentage of apoptotic cells is not observed until the dosage of α -MMC is raised to 5 μ M (Fig. 4a). This signifies that the cytotoxicity of α -MMC at low concentrations probably contributes to stunting the rise of cell generation *via* down-regulation of UPR (Fig. 3c) or stalling cell cycle progression involving GSK3 β (Fig. 6). Besides, hypoxia as a physically important endoplasmic reticulum stress could lead to rapid initiation of UPR [50]. Thus the observation that hypoxic stress does not compromise the deleterious effect of α -MMC on cell viability and colony formation of NPC (Fig. 2b and c) contributes not only to the aforementioned attenuation of HIF1 α , but also to the reduction of UPR (Fig. 3c).

Our findings furnish evidence that α -MMC initiates differential mitochondrial- and death receptor-mediated apoptotic signaling pathways in CNE2 and HONE1 cells, although induction of apoptosis is observed in both of these two cell lines. Apoptotic pathways are traditionally mediated by death receptor and mitochondria [51]. Regarding the mitochondrial-mediated signaling pathway, the binding and insertion of pro-apoptotic proteins on the mitochondrial membrane enhance the mitochondrial membrane permeability, resulting in the release of cytochrome *c* that can activate caspase-9 and downstream

caspase-3, eventually leading to cell apoptosis [51]. These phenomena are expected in CNE2 cells (*e.g.* caspase-9 and caspase-3 activation, and Ψ_m depolarization, Fig. 5). In contrast, the cleavage of caspase-9 and caspase-3, and Ψ_m depolarization are not observed in HONE1 cells (Fig. 5). *CASP9* gene produces two spliced variants, the pro-apoptotic Casp9a and the pro-survival Casp9b, *via* the inclusion or exclusion of an exon 3, 4, 5, 6 cassette [52]. Thus the discrepancy may contribute to inhibition of phosphorylation of Akt in CNE2 cells (Fig. 6b), whereas enhancement of phosphorylation of Akt occurs in HONE1 cells (Fig. 6b), since activated Akt has been found to mediate exclusion of the exon 3, 4, 5, 6 cassette of caspase-9 [53]. Caspase-8 activation is a prominent feature of the death receptor-mediated apoptotic pathway [51]. Caspase-8 amplifies the mitochondrial apoptotic loop through cleavage of the BH3 protein Bid that creates holes in the mitochondria bring about mitochondrial collapse, or directly cleaves downstream caspase-3 [42]. Consistent with the paradigm, substantial activation of caspase-8 and caspase-3, and mitochondrial collapse have been noticed in CNE2 cells (Fig. 5). However, in HONE1 cells, the slight cleavage of caspase-8 observed seems insufficient to activate its two downstream caspase-3 and Ψ_m depolarization (Fig. 5). Alternatively, DNA damage stress signaling is implicated in RIP-induced apoptosis [33]. As anticipated, chromatin condensation is observed in both CNE2 and HONE1 cells (Fig. 4c).

In conclusion, α -MMC evinces cytotoxicity and colony formation of NPC CNE2 and HONE1 cells *in vitro*. α -MMC exerts its inhibitory effect through targeting at endoplasmic reticulum, down-regulation of UPR, and induction of cell cycle arrest and apoptosis in both CNE2 and HONE1 cells. Moreover, α -MMC modulates the hypoxic signaling and sustains its anti-tumor activity on hypoxic NPC cells, suggestive of its potential *in vivo* application. These observations indicate the possible utility of α -MMC for NPC therapy.

Acknowledgments

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Abbreviations

CHOP	C/EBP homologous protein
ERAD	endoplasmic reticulum-associated degradation
HIF1 α	hypoxia inducible factor 1 α
HUVEC	human umbilical vein endothelial cells
IRE1α	inositol-requiring protein 1 α
α-MMC	α -momorcharin
NPC	nasopharyngeal carcinoma
PERK	(protein kinase RNA-activated)-like endoplasmic reticulum kinase
RIPs	ribosome inactivating proteins

UPR	unfolded protein response
VEGF	vascular endothelial growth factor
Ψ_m	mitochondrial membrane potential.

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Fig. 1.

Cytotoxicity of α -momorcharin (α -MMC) on human nasopharyngeal carcinoma (NPC). NPC CNE2 (a) and HONE1 cells (b), and transformed human nasopharyngeal epithelial NP 69 cells (c) were respectively exposed to increasing concentrations (0–10 μ M) of α -MMC for 24 and 48 h. MTT assay was used to assess cell viability. For colony formation assay, CNE2 or HONE1 cells (0.5×10^3) were treated with 0, 0.5 or 1 μ M α -MMC for 48 h. After rinsing with fresh medium, cells were allowed to grow for 10 days to form colonies. α -MMC undermined the clonogenic survival of CNE2 (d) and HONE1 cells (e), respectively.

* $P < 0.01$, when compared with control.

Fig. 2.

α-MMC suppressed the growth of hypoxic NPC cells. (a) Cobalt chloride (CoCl₂) up-regulated HIF1α expression level in both CNE2 and HONE1 cells. Western blot analysis of HIF1α expression after treatment of CNE2 or HONE1 cells with 150 μM CoCl₂ for 24 h. β-Actin was used as an internal control. (b) and (c) α-MMC maintained its inhibitory effect on cell viability (b) and colony formation (c) in both hypoxic CNE2 and HONE1 cells. After pretreatment with 150 μM CoCl₂ for 24 h, CNE2 and HONE1 cells were respectively exposed to indicated concentrations of α-MMC for 48 h. (d) α-MMC undermined the expression level of HIF1α and VEGF in both hypoxic CNE2 and HONE1 cells. After

pretreatment with 150 μM CoCl_2 for 24 h, CNE2 and HONE1 cells were respectively exposed to indicated concentrations of α -MMC for different durations. (e) α -MMC thwarted, in a dose- and time-dependent fashion, the growth of human umbilical vein endothelial cells (HUVEC). HUVEC were treated with increasing concentrations of α -MMC for 24 or 48 h. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, when compared with control.

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Fig. 3.

α -MMC targeted endoplasmic reticulum and down-regulated unfolded protein response (UPR) in NPC cells. (a) Labeling of α -MMC with fluorescent tag bimane amine. Pure α -MMC and bimane amine labeled α -MMC (Bimane- α -MMC) were respectively subjected to SDS-PAGE and stained with coomassie brilliant blue (CBB) (left panel). To detect the fluorescence of Bimane- α -MMC, the gel present in left panel was imaged by UV illumination using ChemDoc XRS system (right panel). (b) Confocal imaging analysis of co-localization of Bimane- α -MMC and endoplasmic reticulum. CNE2 or HONE1 cells were treated with bimane- α -MMC for 24 h, the culture medium in the dishes were discarded, and

then the cells were stained with 1 μ M ER-Tracker Red in DMEM for 30 min at 37 °C in a humidified 5% CO₂ incubator. Bright field: BF. Scale bar: 10 μ m. (c) Down-regulation of UPR induced by α -MMC in both CNE2 and HONE1 cells. CNE2 and HONE1 cells were respectively treated with indicated concentrations of α -MMC for different durations. β -Actin was used as an internal control. * P < 0.05, when compared with control.

Fig. 4.

α -MMC induced apoptosis in NPC cells. (a) α -MMC induced concentration-dependent apoptosis in NPC cells. CNE2 and HONE1 cells were respectively exposed to increasing dosages of α -MMC for 48 h. (b) α -MMC induced time-dependent apoptosis in NPC cells. CNE2 and HONE1 cells were treated with 5 μ M α -MMC for different durations, respectively. (c) α -MMC induced chromatin condensation in NPC cells. Hoechst 33342 staining was carried out after CNE2 and HONE1 cells had respectively been treated with 5 μ M α -MMC for 48 h. Magnification: 200 $\times$.

Fig. 5. α -MMC triggered mitochondrial- and death receptor-mediated apoptotic signaling pathway in CNE2 cells, but not in HONE1 cells. (a) Western blot analysis of the factors that contributed to mitochondrial- and death receptor-mediated pathways. CNE2 and HONE1 cells were respectively treated with indicated concentrations of α -MMC for different durations. β -Actin was used as an internal control. "&" Non-specific band. * $P < 0.05$, when compared with control. (b) Flow cytometry analysis of mitochondrial membrane potential (Ψ_m) using JC-1 staining. CNE2 and HONE1 cells were respectively treated with 5 μ M α -

MMC for different durations. The cells undergoing Ψ_m depolarization are shown (polygonal area).

Fig. 6.

α-MMC induced differential cell cycle arrest in CNE2 and HONE1 cells. (a) α-MMC caused time-dependent G0/G1 phase arrest in CNE2 cells (left panel), but S phase arrest in HONE1 cells (right panel). CNE2 and HONE1 cells were respectively treated with 1 μM α-MMC for indicated durations. (b) Western blot analysis of phosphorylation level of Akt, GSK-3α and GSK-3β. CNE2 and HONE1 cells were respectively treated with indicated concentration of α-MMC for different durations. β-Actin was used as an internal control. * $P < 0.05$, when compared with control.