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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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A B S T R A C T

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-alpha (HIF1α) 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.* HIF1α, 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.

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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

Abbreviations: CHOP, C/EBP homologous protein; ERAD, endoplasmic reticulum-associated degradation; HIF1 α, hypoxia inducible factor 1α; HUVEC, human umbilical vein endothelial cells; IRE1α, inositol-requiring protein 1α; 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; $\Delta\Psi_m$, mitochondrial membrane potential.

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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-alpha (HIF1α) and its downstream vascular endothelial growth factor (VEGF) are key hypoxia regulatory molecules that promote angiogenesis and metastasis [12,13]. Overexpression of HIF-1α and VEGF was commonly found in NPC [14]. HIF-1α 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 2α (eIF2α) 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-eIF2α 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 mastocytomal 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, IRE1α 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-3α (GSK-3α) and GSK-3β, and S phase arrest in HONE1 cells possibly due to activation of GSK-3α and GSK-3β.

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), IRE1α (#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 capase-8 (551243) was bought from BD Pharmingen (CA, USA). Primary antibody against HIF-1 alpha (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 × g, 30 min, 4 °C) was loaded on an Affi-gel blue gel column (2.5 cm × 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 Healthecare, 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

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