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Zinc protoporphyrin suppresses cancer cell viability through a heme oxygenase-1-independent mechanism: The involvement of the Wnt/β-catenin signaling pathway

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ABSTRACT

Zinc protoporphyrin (ZnPP), a known inhibitor of heme oxygenase-1 (HO-1), has been reported to have anticancer activity in both *in vitro* and *in vivo* model systems. While the mechanisms of ZnPP's anticancer activity remain to be elucidated, it is generally believed that ZnPP suppresses tumor growth through inhibition of HO-1 activity. We examined this hypothesis by altering cellular levels of HO-1 in human ovarian (A2780) and prostate cancer (DU145) cells and found that ZnPP inhibits cancer cell viability through an HO-1-independent mechanism. Neither over-expression nor knockdown of HO-1 significantly alters ZnPP's cytotoxicity in human cancer cells, indicating that HO-1 does not mediate ZnPP's inhibitory effect on cancer cell growth. Consistent with these observations, tin protoporphyrin (SnPP), a well-established HO-1 inhibitor, was found to be much less cytotoxic than ZnPP, and docosahexaenoic acid (DHA), an HO-1 inducer, enhanced ZnPP's cytotoxicity. In an effort to define the mechanisms of ZnPP-induced cytotoxicity, we found that ZnPP but not SnPP, diminished β-catenin expression through proteasome degradation and potently suppressed β-catenin-mediated signaling in our model systems. Thus, ZnPP-induced cytotoxicity is independent of HO-1 expression in cancer cells and the Wnt/β-catenin pathway is potentially involved in ZnPP's anticancer activity.

1. Introduction

HO-1 is an inducible and rate-limiting cellular enzyme that cleaves the α-mesocarbon bridge of heme, generating carbon monoxide (CO), iron ion and biliverdin. Biliverdin is further converted by biliverdin reductase to bilirubin, a molecule possessing antioxidant activity. The degradation of heme, a potent pro-oxidant, and the generation of CO and bilirubin, both being antioxidants, render HO-1 as a cytoprotective enzyme [1,2]. While HO-1 generally supports normal cell viability and growth, the cytoprotective activity of HO-1 is also believed to support tumor growth and render cancer cells resistant to chemotherapy [3,4]. Since cancer cells are considered to be more vulnerable to oxidative stress than normal cells [5–7], inhibiting HO-1 to elevate cellular oxidative stress is considered a viable approach to more effectively kill cancer cells or to enhance the cytotoxicity of chemotherapeutics [2–4]. To test this assumption, Sahoo and co-workers first reported that pegylated ZnPP, an established HO-1 inhibitor, suppressed solid tumor growth *in vivo* likely through inhibition of HO-1 [8]. Subsequent studies provided experimental evidence to further support the idea that inhibition of HO-1 by ZnPP inhibits cancer cell growth in both *in vitro* and *in vivo* model systems [9–11]. However, there has been no direct evidence to indicate that ZnPP suppresses tumor growth specifically through the inhibition of HO-1 activity. To further complicate the matter, HO-1 inhibitors, such as ZnPP, may also induce HO-1 gene transcription within hours of treatment [12]. Therefore, the cellular mechanisms of HO-1 inhibitor-induced growth inhibition of cancer cells remain to be further defined.

Several metalloporphyrins have been described as HO-1 inhibitors such as SnPP, ZnPP, and copper protoporphyrin (CuPP) [13]. These compounds act as substrates that compete with heme for HO-1, thereby reducing the levels of CO and bilirubin [14,15]. Among these, SnPP seems to be the most effective at inhibiting HO-1 activity and CuPP is the least effective [13,16]. However, studies have shown that among these inhibitors, ZnPP seems to be the

Abbreviations: CuPP, copper protoporphyrin; DHA, docosahexaenoic acid; GAPDH, glyceraldehydes-3-P-dehydrogenase; HO-1, heme oxygenase-1; SnPP, tin protoporphyrin; ZnPP, zinc protoporphyrin.

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most effective at inducing tumor cell apoptosis [17]. In addition, a recent study indicated that ZnPP suppresses cyclin D1 gene expression in cancer cells independent of HO-1, and SnPP did not [18]. Thus, it is likely that ZnPP-induced growth inhibition or cytotoxicity in cancer cells is HO-1 independent.

The present study was designed to determine whether ZnPP suppresses cancer cell growth through the inhibition of HO-1 in human cancer cells. Two human cancer cell lines were primarily used to answer this question: an ovarian cancer cell line (A2780) with barely detectable HO-1 expression and a prostate cancer line (DU145) with higher and constitutive HO-1 expression. Additional cancer cell lines such as A375 (melanoma) and MCF-7 (breast cancer) were also utilized to indicate that the effects of ZnPP are not cell line dependent. Our results show that neither over-expression of HO-1 in A2780 cells nor knockdown of HO-1 in DU145 alters ZnPP-induced suppression of cancer cell viability, strongly indicating an HO-1-independent mechanism that mediates ZnPP's cytotoxicity. Furthermore, we demonstrate that ZnPP, but not SnPP or CuPP, effectively suppresses the canonical Wnt/β-catenin signaling pathway, thus establishing ZnPP as a previously unrecognized metalloporphyrin inhibitor of Wnt signaling which may prove to be useful in suppressing cancer (and possibly cancer stem cell) growth [19].

2. Materials and methods

2.1. Materials

The β-catenin luciferase reporter (M50 Super 8× topFlash, Addgene plasmid 12456) was obtained from Dr. Randall T. Moon (Department of Pharmacology and Center for Developmental Biology, Howard Hughes Medical Institute, Seattle, WA). The GFP-tagged HO-1 expression vector was kindly provided by Dr. Fumio Kishi (Department of Molecular Genetics, Kawasaki Medical School, Okayama, Japan [20]). The human HO-1 promoter construct (pHOGL3/4.5, [21]) was obtained from Dr. Anupam Agarwal (Department of Medicine, University of Alabama at Birmingham, Birmingham, AL). The HO-1 antibody was from Stressgen (Ann Arbor, MI). Antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-catenin were from Cell Signaling Technology, Inc. (Danvers, MA). The procaspase 3 antibody was from Santa Cruz Biotechnology, Inc. (Dallas, TX). The dual luciferase reporter assay kit and the cell viability CellTiter 96 Aqueous Solution (MTS assay) were from Promega (Madison, WI). The fluorescent probe for the detection of zinc [22] was purchased from Invitrogen (Carlsbad, CA). MG132 was from Cayman Chemical (Ann Arbor, MI). Bortezomib was from Selleck Chemicals (Houston, TX). The β-actin antibody and other chemical reagents were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

The A2780 cell line (human ovarian cancer) was a kind gift from Dr. Stephen Howell (University of California, San Diego). The DU145 cell line (human prostate cancer), MCF-7 cell line (human breast cancer) and A375 cell line (human melanoma) were purchased from American Type Culture Collection (ATCC, Manassas, VA). A2780 and MCF-7 cells were cultivated in RPMI 1640 medium, and DU145 and A375 cells were cultivated in DMEM medium. Both RPMI 1640 and DMEM mediums were supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely grown in a 75-mm flask at 37 °C in a humidified environment containing 5% CO_2. All cells were sub-cultivated twice a week and applied to the various experiments as described in the results section.

2.3. Preparation and application of ZnPP, SnPP and CuPP

ZnPP, SnPP and CuPP were purchased from Frontier Scientific, Inc (Logan, UT). The manufacturer's advice and a previous report [12] were followed for proper handling of these compounds. A working stock of ZnPP, SnPP, and CuPP was freshly prepared for each individual experiment. All tubes used to prepare the stock solution were covered by aluminum foil to avoid light reaction with the compounds. The compounds were initially dissolved in complete DMSO, and further diluted with 50% DMSO in 1X PBS buffer prior to addition to the cell culture medium. The final DMSO concentration in the cell culture medium was below 0.5% in all experiments conducted. Vehicles were included as controls. Cells were treated with the compounds in indirect low-light conditions and incubated in the dark for various lengths of time prior to individual assays, similar to previous reports [12,18].

2.4. Cell viability assay

Cell viability was analyzed *via* reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), as we have previously described [23,24]. In brief, around 5000 cells of each cell line were plated per well in a 96-well tissue culture plate containing 100 μl of medium. This resulted in 40–50% cell confluence after 24 h of plating. The medium was then replaced with fresh medium containing ZnPP, SnPP, or CuPP at the indicated concentrations and the cells were grown for the indicated periods of time. At the end of the incubation, the growth medium was replaced with fresh medium and 20 μl of the MTS solution was added. Cells were incubated at 37 °C for 1 h, and the absorbance of each well was recorded at 490 nm. Data are presented as a percentage of the values detected in untreated control cells.

2.5. siRNA knockdown and over-expression of HO-1

DU145 cells were used for the knockdown experiments. The HO-1 siRNA was purchased from Dharmacon, Inc. (Lafayette, CO). Cells grown in DMEM with 10% FBS were plated at a density of 4×10^5 cells per well in a 6-well plate. Twenty-four hours after plating, the medium was removed, and cells were transfected with 25 nM HO-1 siRNA using the DharmaFECT transfection reagent (Dharmacon, Inc., Lafayette, CO) following the manufacturer's protocol. Twenty-four hours after transfection, the cells were plated and treated with ZnPP for 48 h. Cell viability was analyzed using MTS assay. HO-1 knockdown was confirmed at protein level by Western blot analysis.

An A2780 cell line stably over-expressing HO-1 was generated. In brief, cells were grown in RPMI 1640 with supplements, and plated at a density of 3×10^6 cells per 100-mm dish. The following day, the medium was removed, and a transfection complex containing 2 μg of the GFP-HO-1 construct and 3 μl of FuGENE HD transfection reagent (Promega, Madison, WI) in 2 ml of serum-free medium was added. After 5 h of incubation, the medium was replaced with 8 ml of RPMI containing 10% fetal bovine serum, and cells were incubated overnight. The cells were then lifted and plated into a 75-mm cell culture flask. G418 (Invivogen, San Diego, CA) was added into the media with a final concentration of 500 μg/ml. After four weeks of selection with G418, cells were sorted with an Influx Cell Sorter (BD Biosciences, San Jose, California) Top 10% of brighter GFP expressing cells were selected and cultured in a flask with complete RPMI medium containing 500 μg/ml of G418 for another month. Cells were then sorted again with the Influx Cell Sorter and the top 20% of brighter GFP expressing cells were collected and used for cell viability experiments. Over-expression of GFP-HO-1 was confirmed by Western blot analysis, and by a