Toxicology and Applied Pharmacology 286 (2015) 168–177

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

α-Hispanolol sensitizes hepatocellular carcinoma cells to TRAIL-induced apoptosis via death receptor up-regulation

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ARTICLE INFO

Article history:
Received 17 December 2014
Revised 25 March 2015
Accepted 21 April 2015
Available online 28 April 2015

Keywords:
Hispanolone derivatives
Apoptosis
TRAIL
Death receptors
Caspase 8

ABSTRACT

Hispanolone derivatives have been previously described as anti-inflammatory and antitumoral agents. However, their effects on overcoming Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance remain to be elucidated. In this study, we analyzed the cytotoxic effects of the synthetic hispanolone derivative α-hispanolol (α-H) in several tumor cell lines, and we evaluated the induction of apoptosis, as well as the TRAIL-sensitizing potential of α-H in the hepatocellular carcinoma cell line HepG2. Our data show that α-H decreased cell viability in a dose-dependent manner in HeLa, MDA-MB231, U87 and HepG2 cell lines, with a more prominent effect in HepG2 cells. Interestingly, α-H had no effect on non-tumoral cells. α-H induced activation of caspase-8 and caspase-9 and also increased levels of the proapoptotic protein Bax, decreasing antiapoptotic proteins (Bcl-2, X-IAP and IAP-1) in HepG2 cells. Specific inhibition of caspase-8 abrogated the cascade of caspase activation, suggesting that the extrinsic pathway has a critical role in the apoptotic events induced by α-H. Furthermore, combined treatment of α-H with TRAIL enhanced apoptosis in HepG2 cells, activating caspase-8 and caspase-9. This correlated with up-regulation of both the TRAIL death receptor DR4 and DR5. DR4 or DR5 neutralizing antibodies abolished the effect of α-H on TRAIL-induced apoptosis, suggesting that sensitization was mediated through the death receptor pathway. Our results demonstrate that α-H induced apoptosis in the human hepatocellular carcinoma cell line HepG2 through activation of caspases and induction of the death receptor pathway. In addition, we describe a novel function of α-H as a sensitizer on TRAIL-induced apoptotic cell death in HepG2 cells.

Introduction

Apoptosis is a highly preserved mechanism that contributes to tissue homeostasis and also participates in the elimination of potentially dangerous cells, including the precursors of tumor cells (Song and Steller, 1999). Two main pathways: the extrinsic and intrinsic are involved in apoptosis. The extrinsic pathway is activated by external ligand binding to membrane receptors called death receptors, belonging to the TNF (Tumor necrosis factor) superfamily. Ligand-activated death receptors recruit the adaptor molecule Fas-associated death domain (FADD) protein which recruits and activates an initiator enzyme, usually caspase-8, in the death-inducing signaling complex (DISC) (Peter and Krammer, 1998). Then caspase-8 can activate downstream apoptotic effectors such as caspases 3, 6 and 7 (Enari et al., 1996). The intrinsic pathway involves the release of proapoptotic factors such as cytochrome *c* from the mitochondria to the cytoplasm. Cytochrome *c* forms a multi-protein complex with Apaf-1 and dATP called apoptosome, promoting the caspase cascade through autoactivation of procaspase-9 to caspase-9 (Slee et al., 1999). The translocation of cytochrome *c* to the cytosol can be mediated by Bcl-2 family proapoptotic proteins including Bax and Bid. Processed caspase-8 can cleave Bid, generating the activated form and supplying a link between the extrinsic and intrinsic pathways (Li et al., 1998).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an initiator of the extrinsic apoptotic pathway with potential in cancer therapy (Pavet et al., 2011; Yerbes et al., 2011). Binding to its cognate death receptors DR4 and DR5 (TRAIL-R1 and TRAIL-R2) on the cell surface (Almasan and Ashkenazi, 2003) induces rapid apoptosis of many types of cancer cells while sparing normal cells (LeBlanc and Ashkenazi, 2003). However, several studies have shown that some tumor cells are not sensitive to TRAIL based-therapies, possibly due to intrinsic or acquired TRAIL resistance (Dimberg et al., 2013; Shankar and Srivastava, 2004; Yamanaka et al., 2000). This is the case of human hepatocellular carcinoma (HCC), one of the most common types of human malignant

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http://dx.doi.org/10.1016/j.taap.2015.04.012
0041-008X/

tumors, which presents a poor response to conventional chemotherapies due to a marked resistance to the induction of cell death by TRAIL (Dimberg et al., 2013; Shankar and Srivastava, 2004; Yamanaka et al., 2000). Identification of sensitizing agents capable of overcoming such resistance, may offer an improved treatment for TRAIL-resistant cancer. In this sense, several compounds including histone deacetylase inhibitors (Schuchmann et al., 2006), proteasome inhibitors as bortezomib (Ganten et al., 2005), kinase inhibitors, such as sorafenib (Chen et al., 2010) and natural products as andrographolide, quercetin, or dihydroflavonols (Kim et al., 2008; Yang et al., 2012; Zhou et al., 2008) have been reported to enhance TRAIL-induced apoptosis in HCC cells.

The labdane diterpenoid hispanolone was first isolated from *Ballota hispanica*, a Labiatae species growing in Spain (Savona et al., 1978). Hispanolone exhibited very low cytotoxicity and anti-inflammatory activity in the in vivo model of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema assay (Nieto-Mendoza et al., 2005). Our group has previously reported that hispanolone derivatives exert anti-inflammatory effects through inhibition of the activation of nuclear factor-κB (NF-κB) in LPS-activated RAW 264.7 macrophages, and suppressing mouse ear edema induced by (TPA) and inhibiting myeloperoxidase activity, an index of neutrophil infiltration (Giron et al., 2008). Furthermore, some of these labdane diterpenes also had antitumoral effects by activating apoptotic cell death machinery (Traves et al., 2013). In this study, we evaluated the cytotoxic effects of the synthetic hispanolone derivative α-hispanolol (α-H) in several tumor cell lines, as well as the TRAIL-sensitizing potential of this diterpene in the hepatocarcinoma cell line HepG2. Our results showed that α-H decreased cell viability in a dose-dependent manner in all cell lines we tested, except in MDA-MB231 cells (human breast cancer cell), without affecting non-tumor cells. Analysis of the signaling pathways involved in the cytotoxic effects of α-H in HepG2 cells showed that α-H induced caspase activation (both extrinsic and intrinsic pathways) and modified the levels of proapoptotic and antiapoptotic factors. Interestingly, inhibition of caspase-8 prevented the cascade of caspase activation, suggesting that the extrinsic pathway has a critical role in the apoptotic events induced by α-H. Moreover, α-H sensitized HepG2 cells to TRAIL-mediated apoptosis through up-regulation of the death receptors DR4 and DR5. Thus, α-H might represent a novel therapeutic option for the treatment of hepatocarcinoma tumors with acquired TRAIL resistance.

Material and methods

Materials. α-Hispanolol (α-H) was obtained as previously described (Giron et al., 2008; Savona et al., 1978). Stocks were dissolved in DMSO and subsequently diluted in PBS before use (maximum concentration 0.01%). Western blot reagents were purchased from GE Healthcare (Pittsburgh, PA, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescent probes for caspase activity were purchased from BD Biosciences (San José, CA, USA) and Bachem (Bubendorf, Switzerland). Caspase inhibitors were purchased from BD Biosciences (San José, CA, USA). TRAIL was purchased from Sigma (St Louis, MO, USA). Culture media were purchased from Lonza (Basel, Switzerland). Neutralizing antibodies against TRAIL-R1 (DR4) and TRAIL-R2 (DR5) were purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture conditions. HeLa (human cervical cancer cell line), MDA-MB231 (human breast cancer cell), U87 (human primary glioblastoma cell line), HepG2 (human hepatocellular liver carcinoma cell line) and CHL (human hepatocyte derived cell line) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml streptomycin. All cell lines were mycoplasma tested and kept frozen under liquid nitrogen until resuscitated for use.

Total extracts and Western blot analysis. Cells were lysed at 4 °C with ice-cold buffer of total extract (0.5% Chaps, 10 mM Tris pH 7.5, 1 mM Cl_2Mg, 1 mM EGTA, 10% Glycerol, 5 mM β-Mercaptoethanol and 1 μl/ml of Proteinase Cocktail Inhibitor), scraped off the plate and maintained for 15 min under continuous shaking. After centrifugation of the cell lysate the supernatant was stored at −80 °C. Protein content was estimated by the Bradford assay (Bradford, 1976). Protein extracts were subjected to SDS-PAGE (10–15% gels) and blotted onto polyvinylidenedifluoride membranes, which were incubated with the following antibodies: Bid (sc-11423), Bax (sc-526), Bcl-2 (sc-783), X-IAP (sc-11426), IAP-1 (sc-7943), DR4 (sc-53687), DR5 (sc-166624) and tubulin (sc-53646). After incubation with HRP-conjugated secondary antibody, protein bands were revealed with an enhanced chemiluminescence kit (Immobilon Westerns, Millipore, Merck, Darmstadt, Germany). Tubulin was used as a loading control. Band intensity of Western blots was analyzed by densitometry using Quantity One software (Bio-Rad, CA, USA). For all experiments, representative Western blots displaying results obtained from three independent experiments are shown.

MTT assay for cell viability. Cells were incubated in the presence of different concentrations of α-hispanolol for 24, 48 or 72 h, before they reacted with MTT (2 mg/ml) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) at 37 °C for 3 h. The reaction product, formazan, was extracted with dimethyl sulfoxide (DMSO) and the absorbance was read at 540 nm as previously described (Oramas-Royo et al., 2013). Assays were performed in triplicate, and results are expressed as the percent reduction in cell viability compared to untreated control cultures for at least three independent experiments. IC_{50} values refer to the concentration needed to inhibit 50% of cell viability in the presence of the compounds.

Caspase assays. The activities of caspase-3, 8 and 9 were determined fluorometrically in protein extracts, using the substrates Ac-DEVD-AMC, Ac-IETD-AFC and Ac-LEHD-AMC, respectively, according to the supplier's instructions (BD Biosciences, San José, CA, USA).

Flow cytometry analysis of apoptosis and necrosis. After treatment with the appropriate stimuli, cells were stained with 0.005% (w/v) propidium iodide (PI) and annexin V-FITC as recommended by the manufacturer (BD Biosciences, San José, CA, USA) and immediately analyzed in a FACSCanto II flow cytometer (BD Biosciences, San José, CA, USA). 10,000 events were counted by flow cytometry and an increase in the percentage of annexin-V-positive cells indicates increased apoptosis compared with cells without treatment.

Treatment of cells with neutralizing antibodies to death receptors. HepG2 cells were pretreated (1 h) with neutralizing antibodies raised against the extracellular domains of TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (R&D Systems, Minneapolis, MN, USA), either alone or in combination in a final concentration of 20 μg/ml in the medium. Isotype control IgG1 (final, 20 μg/ml) was added to the control group. Then HepG2 cells were incubated with a subtoxic dose of α-H (10 μM) and TRAIL (250 ng/ml) for 24 h.

Statistical analysis. All numerical data were presented as means ± S.D. for at least three independent experiments. Statistical significance was estimated by Student's *t* test for comparison between two groups. For comparison between two or more groups, one-way ANOVA, followed by Bonferroni's post hoc comparisons was used. Differences were considered significant at $^*P < 0.05$. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, CA, USA).