Cancer Letters ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

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

Original Articles

Smac mimetic and oleanolic acid synergize to induce cell death in human hepatocellular carcinoma cells

Juliane Liese [a,b], Behnaz Ahangarian Abhari [a], Simone Fulda [a,c,d,*]

[a] Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Frankfurt, Germany
[b] General and Visceral Surgery, Goethe-University, Frankfurt, Germany
[c] German Cancer Consortium (DKTK), Heidelberg, Germany
[d] German Cancer Research Center (DKFZ), Heidelberg, Germany

ARTICLE INFO

Article history:
Received 16 December 2014
Received in revised form 17 April 2015
Accepted 17 April 2015

Keywords:
Cell death
IAP proteins
Smac
Cancer

ABSTRACT

Chemotherapy resistance of hepatocellular carcinoma (HCC) is still a major unsolved problem highlighting the need to develop novel therapeutic strategies. Here, we identify a novel synergistic induction of cell death by the combination of the Smac mimetic BV6, which antagonizes Inhibitor of apoptosis (IAP) proteins, and the triterpenoid Oleanolic acid (OA) in human HCC cells. Importantly, BV6 and OA also cooperate to suppress long-term clonogenic survival as well as tumor growth in a preclinical *in vivo* model of HCC underscoring the clinical relevance of our findings. In contrast, BV6/OA cotreatment does not exert cytotoxic effects against normal primary hepatocytes, pointing to some tumor selectivity. Mechanistic studies show that BV6/OA cotreatment leads to DNA fragmentation and caspase-3 cleavage, while supply of the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) revealed a cell type-dependent requirement of caspases for BV6/OA-induced cell death. The receptor interacting protein (RIP)1 kinase Inhibitor Necrostatin-1 (Nec-1) or genetic knockdown of RIP1 fails to rescue BV6/OA-mediated cell death, indicating that BV6/OA cotreatment does not primarily engage necroptotic cell death. Notably, the addition of several ROS scavengers significantly decreases BV6/OA-triggered cell death, indicating that ROS production contributes to BV6/OA-induced cell death. In conclusion, cotreatment of Smac mimetic and OA represents a novel approach for the induction of cell death in HCC and implicates further studies.

Introduction

Hepatocellular carcinoma (HCC) is the most common liver tumor and one of the leading worldwide causes of death [1]. Up to 80% of HCC cases are caused by chronic infection with the hepatitis B virus (HBV), hepatitis C virus (HCV) [2], alcoholic liver diseases and non-alcoholic fatty liver diseases (NASH) [3]. Only 30% of patients are eligible for curative treatment, including liver resection, transplantation and percutaneous ablation [4,5]. However, tumor recurrence up to 50% after three years could be observed [6,7]. The poor prognosis caused by the limited therapeutic options and resistance to chemotherapies highlights the high medical need to investigate novel treatment strategies. One promising approach is to identify drugs which target apoptotic signaling and reactivate cell death programs.

Inhibition of apoptosis is important for the survival of cancer cells [8]. Tumor resistance to apoptosis in HCC could be caused by IAP proteins e.g., x-linked Inhibitor of Apoptosis (XIAP), cellular Inhibitor of Apoptosis (cIAP)1 and cIAP2, a family of key proteins involved in the regulation of cell death [9,10]. IAP proteins are overexpressed in HCC [9,11,12]; especially XIAP is expressed at high levels in nearly 90% of clinical tumor samples and is associated with poor prognosis and tumor recurrence [13]. Inhibition of IAP proteins, for example with second mitochondria-derived activator of caspase (Smac) mimetics, represents a promising approach to re-sensitize HCC cells to cell death induction. While the efficacy of Smac mimetics as single agents is limited in most cancers, they have proven their

Abbreviations: AIF, apoptosis-inducing factor; CAM, chorioallantoic membrane; cIAP, cellular inhibitor of apoptosis; DR5, death receptor-5; ENDOG, endonuclease G; ER, endoplasmatic reticulum; FCS, fetal calf serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IAP, inhibitor of apoptosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; NASH, non-alcoholic fatty liver diseases; Nec-1, necrostatin-1; OA, oleanolic acid; RFU, relative fluorescence units; RIP1, receptor interacting protein 1; Smac, second mitochondria-derived activator of caspases; SOD, superoxide dismutase; XIAP, x-linked inhibitor of apoptosis; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

* Corresponding author. Tel.: +49 69 67866557; fax: +49 69 6786659157.
E-mail address: simone.fulda@kgu.de (S. Fulda).

http://dx.doi.org/10.1016/j.canlet.2015.04.018
0304-3835/

Please cite this article in press as: Juliane Liese, Behnaz Ahangarian Abhari, Simone Fulda, Smac mimetic and oleanolic acid synergize to induce cell death in human hepatocellular carcinoma cells, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.04.018

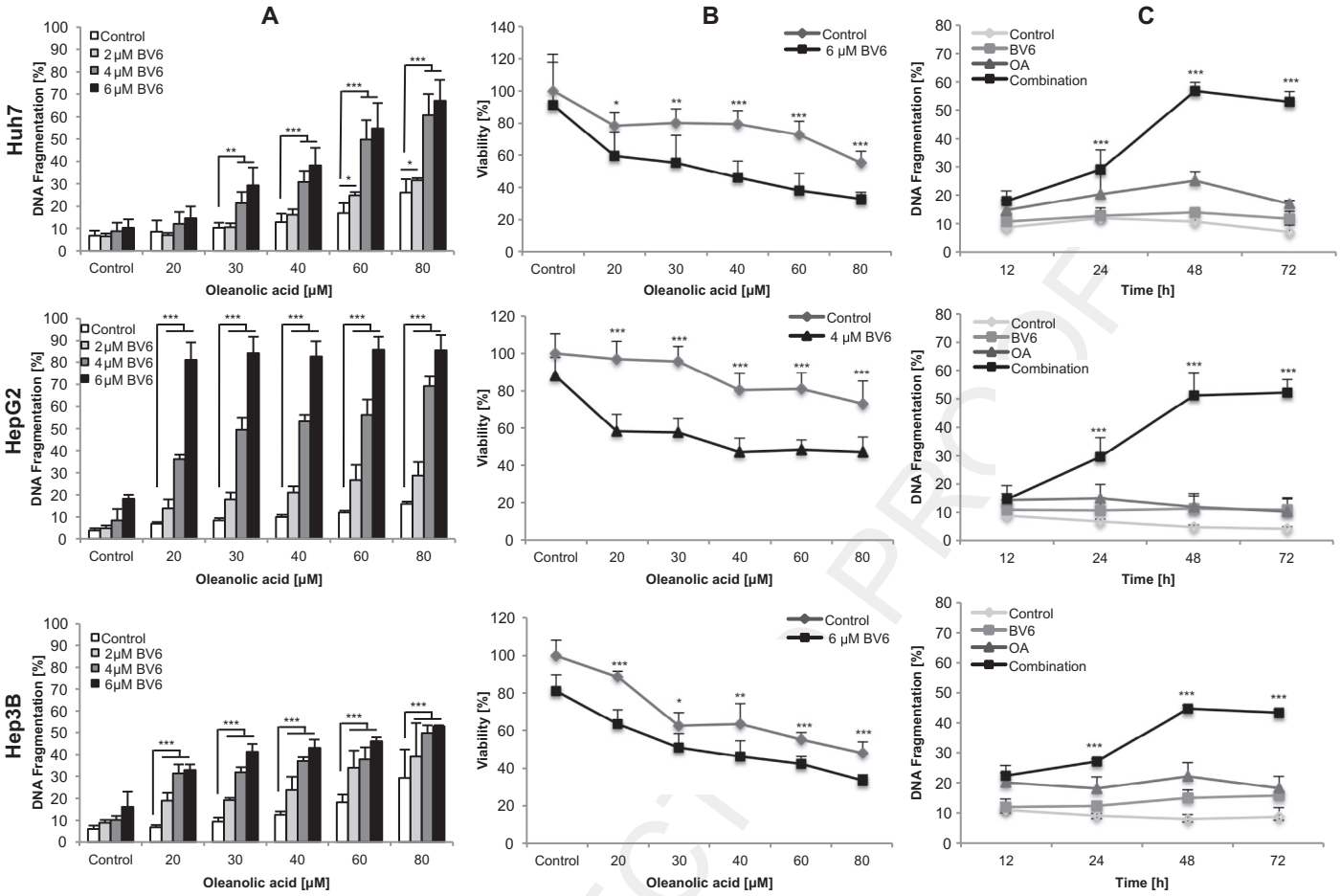

Fig. 1. BV6 and OA synergistically induce cell death in HCC cells. HCC cells (Huh7, HepG2, Hep3B) were treated for 72 hours with indicated concentrations of BV6 and OA (A, B) or for indicated times with BV6 (Huh7, Hep3B: 6 µM BV6, HepG2: 4 µM BV6) and/or 60 µM OA (C). Cell death was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (A, C), cell viability by MTT assay (B). Mean and SD of three independent experiments performed in triplicate are shown; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

effectiveness in combination with different cytotoxic stimuli, e.g. chemotherapeutics, in a number of preclinical studies [14–16]. In HCC, the anticancer activity of Smac mimetics could be improved by combined treatment with a Bcl-2 inhibitor [9]. Furthermore Smac mimetics could potentiate APO2/TRAIL- and Doxorubicin-mediated apoptosis in HCC cell lines [12]. Currently, Smac mimetics are tested in clinical trials as single agents and in combination therapies in different cancers [8].

Another approach to induce cell death in HCC cells is the use of Oleanolic acid (OA), a natural triterpenoid [17,18]. In Chinese medicine, OA has been used for many decades in the treatment of liver disorders, such as viral hepatitis [17]. Recently, an antitumor effect of OA *in vitro* and *in vivo* has been shown in HCC [17]. Natural OA as well as its synthetic derivatives display chemoprevention and antitumor effects in breast cancer, glioblastoma, prostate cancer and pancreatic cancer [18].

Searching for new strategies to overcome treatment resistance of HCC, in the present study we investigated the effects of Smac mimetic and OA.

Materials and methods

Cell culture and reagents

The HCC cell lines HepG2, Huh7 and Hep3B were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM medium (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) and 1 mM Sodium Pyruvate (Invitrogen). Primary human hepatocytes were isolated by collagenase perfusion and cultured in William's Medium E (Sigma, Deisenhofen, Germany) with 10% FCS, 1% penicillin/streptomycin, 5 µg/ml Insulin (Sigma), 50 µM Hydrocortison (Sigma), 2% DMSO and 20 ng/ml EGF (Sigma). The study was approved by the local Ethical Committee and informed consent was obtained in accordance with the Declaration of Helsinki. Smac mimetic BV6 that neutralizes XIAP, cIAP1 and cIAP2 [19] was kindly provided by Genentech (South San Francisco, CA, USA). TRAIL receptor 2 agonistic antibody Lexatumumab was kindly provided by Human Genome Sciences, Inc. (Rockville, MD, USA). Pan-caspase inhibitor zVAD.fmk was purchased from Bachem (Heidelberg, Germany), and Nec-1 from Biomol (Hamburg, Germany). All other chemicals were purchased from Sigma unless indicated otherwise.

Determination of cell death and cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) or by crystal violet assay (0.75% crystal violet, 50% ethanol, 0.25% NaCl and 1.57% formaldehyde). Apoptosis was determined by analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei using flow cytometry (FACSCanto II, BD Biosciences, Heidelberg, Germany) as described previously [20].

Colony formation assay

To determine colony formation, 0.2×10^6 cells were seeded in a 6-well tissue culture plate, allowed to settle for 24 hours and treated with OA and/or BV6 for 72 hours. Then, cells were re-seeded with 200 cells (Huh7) or 400 cells (HepG2) in a 6-well tissue culture plate and were stained after culture for 12 days with crystal violet solution. Colonies were counted and the percentage of surviving colonies relative to the untreated controls was calculated.

Please cite this article in press as: Juliane Liese, Behnaz Ahangarian Abhari, Simone Fulda, Smac mimetic and oleanolic acid synergize to induce cell death in human hepatocellular carcinoma cells, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.04.018