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Additive effect of apicidin and doxorubicin in sulfatase 1 expressing hepatocellular carcinoma *in vitro* and *in vivo*☆

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***Background/Aims*: There are limited chemotherapy options for hepatocellular carcinoma (HCC). The heparin-degrading endosulfatase SULF1 functions as a liver tumor suppressor. We investigated the effects of the histone deacetylase inhibitor apicidin in combination with doxorubicin in SULF1-expressing HCC cells *in vitro* and in SULF1-expressing xenografts in nude mice.**

***Methods*: We evaluated the effects of apicidin alone or combined with doxorubicin on apoptosis, caspase activity, and phosphorylation of Erk and Akt in SULF1-transfected Huh7 and Hep3B cells *in vitro* and *in vivo*.**

***Results*: Apicidin induced HCC cell apoptosis and caspase activation in a dose- and time-dependent manner. Apicidin-induced caspase activation was significantly inhibited by the caspase inhibitor Z-Vad-fmk. Apicidin also decreased phosphorylation of both Erk and Akt. Expression of constitutively-active Mek1 and Akt significantly decreased apicidin-induced apoptosis. The combination of doxorubicin with apicidin significantly increased the anti-tumor effect in the SULF1-expressing Huh7 and Hep3B cells as compared to either apicidin or doxorubicin alone, both *in vitro* and *in vivo*.**

***Conclusions*: The combination of a histone deacetylase inhibitor with doxorubicin may be a novel and promising therapeutic modality for HCCs, particularly for SULF1-expressing HCCs.**

***Keywords*: Histone deacetylase inhibitor; Doxorubicin; Hepatocellular carcinoma; Sulfatase 1 (SULF1); Apoptosis; Akt kinase; MAP kinase**

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Abbreviations: Akt, protein kinase Akt; CHAPs, cyclic-hydroxamic-acid-containing peptides; DAPI, 4,6-diamidino-2-phenylidole dihydrochloride; Erk, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; MAPK, mitogen activated protein kinase; SAHA, suberoylanilide hydroxamic acid; SB, sodium butyrate; TSA, trichostatin A; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-(*O*-methyl ester) fluoromethylketone.

1. Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer death worldwide [1]. Survival of patients diagnosed with HCC is poor and only 10–20% of HCCs are detected at a sufficiently early stage for radical, potentially curative therapy to be feasible [2]. Local therapies for tumor control do not result in durable responses, and there are only limited options for chemotherapy for HCC [3]. Therefore, new chemotherapeutic agents or methods to improve the efficacy of existing agents are needed for effective treatment of

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the majority of HCCs. The recent demonstration of efficacy of sorafenib in advanced HCC provides a rationale for targeted therapies for HCC [4,5].

The cellular gene expression program is regulated by the level of acetylated histones associated with nuclear chromatin. Pharmacological manipulation of chromatin remodeling by histone deacetylase (HDAC) inhibitors has been proposed as a strategy for treatment of cancer. HDAC inhibitors suppress the activities of multiple HDACs, leading to an increase in histone acetylation. Histone acetylation enhances the expression of genes that elicit cellular growth arrest, differentiation and apoptosis [6]. Apicidin is a novel HDAC inhibitor derived from a fungal metabolite [7–9]. Its anti-tumor action in HCC remains to be elucidated. Sulfatase 1 (SULF1) is a recently characterized heparin-degrading endosulfatase which is downregulated in 9 out of 11 human HCC cell lines and in approximately 30% of primary HCC's [10]. We have shown that SULF1 desulfates cell surface proteoglycans and prevents the formation of the signaling complex between heparin-binding growth factor ligands, heparan sulfate proteoglycans, and receptor tyrosine kinases at the cell surface. Expression of SULF1, therefore, inhibits receptor tyrosine kinase signaling and abrogates cellular growth and survival signaling, thus sensitizing cells to chemotherapy-induced apoptosis [10–13]. We have found that SULF1 increases histone H4 acetylation in HCC cell lines and potentiates the therapeutic effect of the HDAC inhibitors apicidin and scriptaid in these cells [7]. SULF1 also functions as a tumor suppressor in cancers of the breast [14–16], pancreas [13,17], ovary [11,18,19], head and neck [12], and in multiple myeloma [20].

Doxorubicin (DOX) has been widely used for the treatment of HCC [21–23]. The anti-tumor effects of DOX are mainly through DNA-damage leading to apoptosis of tumor cells. However, DOX also increases phosphorylation of MAP kinase, which may decrease its anti-tumor efficacy [24]. Therefore, it is not surprising that reports of its efficacy are not consistent, with response rates varying up to 20%. Interestingly, early phase studies of the combination of doxorubicin with the multi-kinase inhibitor, sorafenib, suggest that the combination may be more efficacious than either agent alone. Combining DOX with a targeted therapeutic approach may, therefore, provide additional treatment options [23,25].

In the present study, we investigated the anti-tumor efficacy of apicidin in HCC *in vitro* and *in vivo*, and evaluated the efficacy of the combination of apicidin with DOX in HCC cells with or without expression of SULF1. We addressed the following questions: (1) Is apicidin-induced apoptosis caspase dependent? (2) Does apicidin decrease phosphorylation of Erk and Akt, which are among the most important pathways in development of HCCs? (3) Does apicidin induce apoptosis and inhibit tumor growth of HCC xenografts? (4) Does the combination of apicidin with DOX increase caspase activation and anti-tumor effects in SULF1-expressing HCC cells *in vitro*? and (5) Does the combination of apicidin with DOX show increased anti-tumor effects in SULF1-expressing HCC xenografts *in vivo*?

2. Materials and methods

2.1. Chemicals and antibodies

Apicidin was purchased from Calbiochem (Cambridge, MA); SB, TSA, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Sigma (St. Louis, MO); DOXIL (doxorubicin HCL liposome) was purchased from Ortho Biotech, Raritan, NJ; polyclonal antibodies against human acetylated histone H3, acetylated histone H4, phospho-ERK44/42, total ERK44/42, phospho-AKT ser 473 and total AKT were purchased from Cell Signaling (Beverly, MA); monoclonal human anti-procaspase 9 was purchased from Pharmingen (Boston, MA); anti-actin antibody and horseradish peroxidase-conjugated mouse IgG and rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); ECL reagents were purchased from Pierce (Rockford, IL). Apo-One Homogeneous Caspase 3/7 Assay kits were purchased from Promega (Madison, WI).

2.2. Cell lines

The three HCC cell lines, Hep3B, SNU449 and SNU182 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by ATCC. Huh7 was from Dr. Gregory Gores' lab. Cells were maintained in a 37 °C, 5% CO_2 incubator, and in logarithmic growth.

2.3. Detection of apoptosis

Fluorescence microscopy: Apoptosis of Huh7, Hep3B, SNU449 or SNU182 *in vitro* was measured by identification of apoptotic nuclear changes (chromatin condensation and nuclear fragmentation) using fluorescence microscopy after staining with the DNA binding dye, DAPI [7]. Three hundred cells were randomly counted, and each treatment was repeated at least three times, in two wells each time.

The identification of apoptotic cells by morphological criteria using light-microscopic examination of haematoxylin–eosin (H & E)-stained sections is generally considered to be the reference standard in paraffin embedded tissues [26–30]. Morphological characteristics including the presence of apoptotic bodies, nuclear condensation and cytoplasmic shrinkage were assessed in H & E-stained tissue sections of the xenografts. In all cases, at least 500 tumor cells in 6 randomly-selected high power fields/slide were counted and morphologically apoptotic cells were expressed as a percentage of the total number of cells counted (apoptotic index) [29,31,32].

2.4. Caspase 3 and 7 activity assay

To determine whether the combination of apicidin with DOX enhances activation of caspases 3 and 7, the activity of caspases 3 and 7 in 2 μg of protein from whole cell lysates of HCC cells was measured [7].

2.5. Western blotting

Twenty micrograms of total cell protein were separated by electrophoresis [7,10]. Blots were probed with rabbit polyclonal anti-acetylated histone H3 and H4, mouse monoclonal anti-procaspase 9, rabbit polyclonal anti-phospho-Erk and anti-total Erk, and rabbit polyclonal anti-phospho-Akt ser473 and anti-total Akt. The level of Actin was also measured to control for equal loading.