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Targeting Liver Cancer and Associated Pathologies in Mice with a Mitochondrial VDAC1-Based Peptide

Srinivas Pittala, Yakov Krelin and Varda Shoshan-Barmatz

Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, 84105, Israel

Abstract

Hepatocellular carcinoma (HCC) is the third most lethal cancer worldwide. Despite progress in identifying risk factors, the incidence of HCC is increasing. Moreover, therapeutic options are limited and survival is poor. Therefore, alternative and innovative therapeutic strategies are urgently required. R-Tf-D-LP4, a cell-penetrating peptide derived from the mitochondrial multifunctional protein the voltage-dependent anion channel (VDAC1), is identified here as a highly effective liver cancer treatment. Recently, we demonstrated that R-Tf-D-LP4 induced apoptosis and inhibited tumor growth in mouse models. We now demonstrate that R-Tf-D-LP4 induced apoptosis in cancer liver-derived cell lines and inhibited tumor growth in three different liver cancer mouse models. These included diethylnitrosamine (DEN)-induced HCC, metabolically high-fat diet–induced HCC, and using a subcutaneous HepG2 cell xenograft model. Intravenous injection of the peptide into tumor-carrying DEN-treated mice resulted in dose-dependent inhibition of tumor growth up to complete tumor elimination. TUNEL staining of liver sections demonstrated peptide-induced apoptosis. Hematoxylin/eosin and Sirius red staining of liver sections showed decreased fibrotic formation. Immunohistochemical staining demonstrated reduced numbers of α-SMA–expressing cells in R-Tf-D-LP4–treated mouse livers. Additionally, macrophage presence in liver tissue was reduced in R-Tf-D-LP4–treated mice. Liver sections from DEN-treated mice showed steatohepatic pathology, reflected as fatty liver, inflammation, ballooning degeneration, and fibrosis; all were eliminated upon peptide treatment. Peptide treatment also inhibited tumor development in a nonalcoholic steatohepatitis–hepatocellular carcinoma mouse model induced by HFD. In HepG2 subcutaneous tumor xenografts, R-Tf-D-LP4 inhibited tumor growth. Conclusion: These results show that the VDAC1-based peptide R-Tf-D-LP4 has multiple effects on liver cancer cells, leading to impairment of cell energy and metabolism homeostasis, induction of apoptosis, and elimination of liver cancer-associated processes, and thus represents a promising therapeutic approach for liver cancer.

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Introduction

Hepatocellular carcinoma (HCC) is a major form of adult primary liver cancer, the third most common cause of cancer-related deaths worldwide, and the second leading cause of death from malignancy following lung cancer [1]. The development of HCC has been associated with various risk factors, including viral infection, alcoholic-induced liver disease, nonalcoholic fatty liver disease and toxins, such as aflatoxin-B found in contaminated food [2]. Other risk factors for HCC include hereditary hemochromatosis, alpha-1-anti-trypsin deficiency, autoimmune hepatitis, and Wilson's disease [3].

Abbreviations: VDAC1, voltage-dependent anion channel 1; HK, hexokinase; Cyto *c*, cytochrome c; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma; HFD, high fat diet; AIF, apoptosis-inducing factor; LDH, lactate dehydrogenase; NASH, nonalcoholic steatohepatitis; TfR, transferrin receptor.

Address all correspondence to: Varda Shoshan-Barmatz, Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.
E-mail: vardasb@bgu.ac.il

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Current therapeutic options are very limited, and survival after diagnosis is poor. Therefore, alternative and innovative therapeutic strategies are urgently required.

Mitochondria occupy a central position in cell life and death, playing crucial roles in cellular energy generation and metabolism, calcium homeostasis, cell signaling, proliferation, differentiation, and cell death, with mitochondria dysfunction having been implicated in many diseases, including cancer [4]. The voltage-dependent anion channel 1 (VDAC1) at the outer mitochondrial membrane serves as the mitochondrial gateway, mediating metabolic cross talk between the mitochondria and the rest of the cell [5–7]. VDAC1 is crucial for a range of cellular processes, including ATP rationing, Ca^{2+} homeostasis, apoptosis execution, and others [5,6,8,9]. These activities are regulated via the interaction of VDAC1 with many proteins central to the regulation of cell survival and cellular death pathways [4–6,8,9]. Thus, VDAC1, found at the crossroads of a variety of cell survival and cell death signals, serves as a controller of mitochondrial metabolism and apoptosis and thus can serve as a target for cancer therapy. VDAC1 is overexpressed in many other cancers [10,11], pointing to VDAC1 as playing a pivotal role in cancer cell survival [6,12].

Cancer cells utilize a variety of strategies to circumvent apoptosis, including quenching of the mitochondrial apoptotic pathway by overexpression of antiapoptotic proteins, such as Bcl-2 and hexokinase (HK-I, HK-II), thereby preventing the release of cytochrome *c* (Cyto *c*) from mitochondria [5,6,12–15]. VDAC1 functions in mitochondria-mediated apoptosis through its involvement in the release of apoptotic proteins to the cytosol and in the regulation of apoptosis via interaction with pro- and antiapoptotic proteins [16–21]. VDAC1 directly interacts with Bcl2 and Bcl-xL, leading to protection against apoptosis [19]. HK-I and HK-II interact with VDAC1 and, when overexpressed, prevented apoptosis in native but not mutated VDAC1-expressing cells [21–23]. Such proteins are highly expressed in many cancers and confer resistance to chemotherapy [24,25]. Thus, targeting cancer cell death evasion strategies by activating apoptosis and minimizing self-defense mechanisms of these cells are rational anticancer strategies. Accordingly, we engineered VDAC1-based peptides that interfere with the activity of the prosurvival proteins Bcl-2, Bcl-xl, and HK [16–21].

We have developed VDAC1-based peptides that effectively induced cancer cell death in a panel of genetically characterized cancer cell lines, regardless of cancer type or mutational status, with perceived specificity toward cancerous cells [16–21,26]. The peptides possess a triple mode of action, namely, energy and metabolism homeostasis impairment, interference with the action of antiapoptotic proteins, and triggering apoptosis [27]. VDAC1-based peptides represent new anticancer therapies, allowing for an overcoming of the chemoresistance of cancer cells.

Here, we used the VDAC1-based peptide R-Tf-D-LP4 to treat HCC. R-Tf-D-LP4 is a cell-penetrating peptide comprising a VDAC1-derived sequence termed LP4 containing amino acids in the D-conformation, fused to the human transferrin receptor (hTfR)-recognition sequence HAIYPRH (Tf). TfR is highly expressed in many different cancers, including liver cancers [28]. In three HCC mouse models, namely, chemically induced using diethylnitrosamine (DEN), metabolically induced using the high-fat diet (HFD)-32, and using subcutaneous Hep-G2 cell xenografts, the peptide dramatically reduced liver cancer. R-Tf-D-LP4 treatment of liver cancer is mediated via alteration of cancer cell metabolic activity, apoptosis induction, cell proliferation arrest, and reduced inflammatory and fibrotic activities. In addition, in a DEN-induced model, R-Tf-D-LP4–treated mice no longer showed steatohepatitis pathology. Thus, the R-Tf-D-LP4 peptide can serve as novel liver cancer therapy.

Materials and Methods

Materials

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, DEN, leupeptine, phenylmethylsulfonyl fluoride, propidium iodide (PI), 4′,6-diamidino-2-phenylindole, trypan blue, glucose, beta-mercaptoethanol, dimethyl sulfoxide (DMSO), cytochalasin B, poly-(D, L-lactic-co-glycolide), and streptozotocin were purchased from Sigma (St. Louis, MO). Dithiothretol was purchased from Thermo Fisher Scientific (Waltham, MA). The cell transfection agent JetPRIME was from PolyPlus (Illkirch, France). Annexin-V was obtained from Alexis Biochemicals (Lausen, Switzerland). Dulbecco's modified Eagle's medium, 7.5% BSA solution, and the supplements fetal calf serum and penicillin-streptomycin were purchased from Biological Industries (Beit-Haemek, Israel). Tissue array sections (US Biomax) were purchased from (US Biomax Inc., Derwood, MD). Primary antibodies used in immunoblotting, immunohistochemistry (IHC) and immunoflouresence (IF), as well as their dilutions, are listed in Table 1. Horseradish peroxidase (HRP)–conjugated anti-mouse and anti-rabbit antibodies, TUNEL, and CellTiter-Glo Luminescent Cell Viability assay kits were obtained from Promega (Madison, WI). 3,3-Diaminobenzidine was obtained from (ImmPact-DAB, Burlingame, CA). The R-Tf-D-LP4 peptide (KWTWK-216-NSNGATWALNVATELKK-199-EWTWS**HRPYIAH)** was synthesized by GL Biochem (Shanghai, China) with >95% purity. The peptide was dissolved in DMSO and diluted to a stock solution of 5 mM in 5% DMSO, and its concentration was determined by following absorbance at 280 nm and using the specific molar extinction coefficient, as calculated based on amino acid composition (http://www.biomol.net/en/tools/proteinextinction.htm).

Cell Culture, R-Tf-D-LP4 Treatment, and Cell Death Analysis

Human HepG2, HuH7, and mouse BNL1MEA.7R.1 liver cancer cell lines were maintained at 37°C in a 5% CO_2 atmosphere in the recommended culture medium and supplements added with 10% fetal calf serum, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

For peptide treatment, cells ($1.5\text{-}2 \times 10^5$ cells/well) were incubated for 6 hours in serum-free medium with various concentrations of the peptide at 37°C. Cell death was analyzed by PI and Annexin V-FITC staining by flow cytometery.

Mitochondria-Bound HK Detachment and Cytochrome C Release Assays

Peptide-induced HK detachment from mitochondria to the cytosol was assessed using HepG2 cells expressing GFP-HK-I. HK-I/II mitochondrial detachment and Cyto *c* release to the cytosol were analyzed by subcellular fractionation into cytosolic and mitochondria fractions and immunoblotting using anti-HK-I or anti–Cyto *c* antibodies. Images were captured using a confocal microscope (Olympus 1X81).

HepG2 cells (3×10^5) were incubated for 3 hours with R-Tf-D-LP4 (3, 5, 10 μM). The cells were harvested, washed with PBS, and gently resuspended in ice-cold buffer (100 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose, 20 mM HEPES/KOH, pH 7.5, 0.2 mM EDTA, 1 μg/ml leupeptin, 5 μg/ml cytochalasin B, and 0.1 mM phenylmethylsulfonyl fluoride) containing 0.02% digitonin and incubated for 10 minutes on ice. Aliquots were centrifuged at