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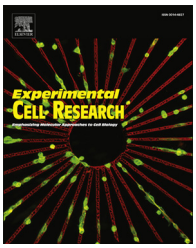

Research Article

Sorafenib enhances proteasome inhibitor-mediated cytotoxicity via inhibition of unfolded protein response and keratin phosphorylation

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ABSTRACT

Hepatocellular carcinoma (HCC) is highly resistant to conventional systemic therapies and prognosis for advanced HCC patients remains poor. Recent studies of the molecular mechanisms responsible for tumor initiation and progression have identified several potential molecular targets in HCC. Sorafenib is a multi-kinase inhibitor shown to have survival benefits in advanced HCC. It acts by inhibiting the serine/threonine kinases and the receptor type tyrosine kinases. In preclinical experiments sorafenib had anti-proliferative activity in hepatoma cells and it reduced tumor angiogenesis and increased apoptosis. Here, we demonstrate for the first time that the cytotoxic mechanisms of sorafenib include its inhibitory effects on protein ubiquitination, unfolded protein response (UPR) and keratin phosphorylation in response to endoplasmic reticulum (ER) stress. Moreover, we show that combined treatment with sorafenib and proteasome inhibitors (PIs) synergistically induced a marked increase in cell death in hepatoma- and hepatocyte-derived cells. These observations may open the way to potentially interesting treatment combinations that may augment the effect of sorafenib, possibly including drugs that promote ER stress. Because sorafenib blocked the cellular defense mechanisms against hepatotoxic injury not only in hepatoma cells but also in hepatocyte-derived cells, we must be careful to avoid severe liver injury.

Introduction

Hepatocellular carcinoma (HCC) is currently the sixth most common solid tumor worldwide and the third leading cause of cancer-related death [1]. Effective treatments for HCC include surgery, percutaneous ablation and liver transplantation and provide a high rate of complete responses, but are only feasible in a minority of patients [2]. For those patients who have

Abbreviations: Ab, antibody; ALLN, acetyl-leucyl-leucyl-norleucinal; ATF, activating transcription factor; CHOP, C/EBP homologous protein; DAPI, 4', 6-diamidino-2-phenylindole; eIF2, eukaryotic translation-initiation factor 2; ER, endoplasmic reticulum; GADD153, growth arrest DNA damage 153; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; IRE, inositol-requiring protein; JNK, c-Jun N-terminal kinase; K, keratin; LC3, light chain 3; MA, methyladenine; MAPK, mitogen-activated protein kinase; MDB, Mallory–Denk body; mTOR, mammalian target of rapamycin; PARP, poly-ADP-ribose-polymerase; PDGFR, platelet-derived growth factor receptor; PERK, PKR-like ER kinase; PI, proteasome inhibitor; TRAF2, tumor necrosis factor receptor-associated factor-2; UPR, unfolded protein response; VEGFR, vascular endothelial growth factor receptor; XBP1, X-box binding protein 1

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advanced stage disease, systemic pharmacologic treatment is the final and main therapy. Unfortunately, the response rate to traditional chemotherapies for HCC patients is quite low and outcomes are poor [3]. Therefore, new drugs with a higher therapeutic index are urgently needed for these patients.

Advances in treatment of HCC have recently been made with sorafenib, an oral multi-kinase inhibitor. Improvements in overall survival have been reported in separate phase III trials conducted in Western countries and in Asia [4,5]. Sorafenib targets the Raf serine/threonine kinases, including Raf-1 and B-Raf, and tyrosine kinases such as vascular endothelial growth factor receptor (VEGFR) 2, VEGFR 3, platelet-derived growth factor receptor (PDGFR) β, c-Kit, and Flt3 [6]. The direct effects of sorafenib on tumor cells include Raf kinase signaling-dependent and independent mechanisms [7]. Recent study demonstrates that sorafenib affects the organization of microfilaments [8]. However, the direct effects of sorafenib on tumor cell survival have not been fully elucidated.

As was the case with sorafenib, an increased understanding of the molecular alterations characteristic of HCC has led to the identification of other potential therapeutic targets. One candidate is the 26 S proteasome, an ATP-dependent multi-catalytic protease involved in the ubiquitin-degradation of key regulatory proteins. The ubiquitin–proteasome system is one of the major distinct proteolytic pathways and is involved in numerous biological processes including cell cycle and apoptosis [9]. Proteasome inhibitors (PIs) are a new class of chemotherapeutic drugs with great therapeutic potential [10]. Bortezomib (formally PS-341, Velcade) inhibits the 26S proteasome. It has been approved clinically for the treatment of refractory multiple myeloma and mantle cell lymphoma [11]. We previously reported that PIs induced the accumulation of ubiquitinated misfolded proteins and apoptosis [12–14]. The endoplasmic reticulum (ER) is the organelle responsible for proper folding of misfolded proteins. The unfolded protein response (UPR) is a compensatory cellular defense mechanism, which is activated by stresses stemming from the burden of misfolded proteins in the ER [15,16].

Autophagy is another protein degradation system and involves the engulfment of a portion of cytoplasm by a double-membrane structure, the autophagosome. The autophagosome fuses with the lysosome, becoming the autolysosome, which undergoes autodigestion. Autophagy maintains cellular homeostasis and participates in processes including differentiation, remodeling, growth control, cell defense, and adaptation to adverse environments [17], and is involved in eliminating abnormal proteins [18]. Despite the pro-death function of autophagy via autophagic cell death, autophagy has been better known as one important pro-survival mechanism, especially for cells under stress conditions. Recently, sorafenib has been reported to induce autophagy [19–21]. In addition, sorafenib affected tumor cell viability via induction of autophagy [22].

Keratins are intermediate filament-forming proteins that are preferentially expressed in epithelial cells [23]. Keratins provide mechanical support and fulfill a variety of additional functions [24]. Keratin 8 (K8) and K18 are the cytoskeletal intermediate filament proteins of hepatocytes and behave as stress proteins with injury-inducible expression. Our previous studies demonstrated that keratins and their phosphorylation are very important for protection of hepatocytes from various types of stresses [24–29]. Changes in keratin phosphorylation are important for protection of hepatocytes against cell injury. Mallory–Denk bodies (MDB) are a hyaline inclusion and are found in hepatocytes of patients with various liver diseases [14,30]. Phosphorylation of K8 and K18 plays an essential role in regulating keratin filament organization and MDB formation [29,31].

As the clinical application of sorafenib evolves, there is increasing interest in clarifying the mechanisms underlying its antiproliferative activity as well as in examining the effects of this agent in combination with other anticancer drugs. In the present study, we used human hepatoma and immortalized human hepatocyte cell lines to examine how sorafenib induces its cytotoxic effect and how proteasome inhibition modulates the anti-tumor effect of sorafenib.

Materials and methods

Cells culture and reagents

We used human hepatoma cell lines established from hepatocellular carcinoma cell lines (Huh7 and Hep3B) and a highly differentiated immortalized human hepatocyte cell line (OUMS29) [32]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Cells were maintained in a 37 °C incubator with 5% CO_2. The following materials were used: sorafenib (LKT Laboratories, St. Paul, MN, USA); acetyl-leucyl-leucyl-norleucinal (ALLN) and epoxomicin as PIs (Calbiochem, La Jolla, CA, USA); and 3MA (Sigma-Aldrich, St. Louis, MO, USA). Sorafenib and PIs were dissolved in dimethyl sulfoxide (DMSO) for preparation. 3MA was stored as powder and dissolved in DMEM immediately before use. The all reagents were added to the cells at the same time. Then, cells were treated with the reagents for indicated times before fixation or harvest.

Antibodies

The antibodies to the following antigens were used: K18, p62, ubiquitin (for immunoblotting), GADD153 (CHOP), XBP1 (Santa Cruz Biotechnology; Santa Cruz, CA, USA); beclin 1 (Novus Biological; Littleton, CO, USA); poly-ADP-ribose-polymerase (PARP), cleaved caspase 3, JNK, phospho JNK (Thr183/Tyr185), phospho c-Jun (Ser73), phospho eIF2α (Cell Signaling Technology, Danvers, MA, USA); light chain 3 (LC3) (Medical and Biological Laboratories, Nagoya, Japan); K8 and actin (Sigma-Aldrich, St. Louis, MO, USA); ubiquitin (for immunocytochemical staining) (Dako, Glostrup, Denmark). The anti-phospho keratin antibody used included mouse anti-phospho K8 (Ab LJ4 directed to K8 pS73) and rabbit anti-phospho K18 (Ab 8250 directed to K18 pS33 and Ab 3055 directed to K18 pS52) were kind gifts from Dr. Omary (Michigan university) [33].

GFP-LC3 transfection and beclin1 knock down

Microtubule-associated protein 1 light chain 3 fused to green fluorescent protein (GFP-LC3) was a kind gift from Dr. Yoshimori (Osaka University, Japan). Transfection was performed using Effecten Transfection Reagent (Qiagen GmbH; Hilden, Germany) 24 h after cell plating, followed by fixation at 48 h after transfection. For beclin1 knockdown, siRNA duplex oligoribonucleotides were purchased from Invitrogen (BECN1 Stealth RNAi siRNA;