Cancer Letters ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

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

Original Articles

Disruption of the unfolded protein response (UPR) by lead compound selectively suppresses cancer cell growth

Hejing Huang [a,b], Huanan Liu [b], Changmei Liu [b], Lixia Fan [b], Xinwen Zhang [b], Anhui Gao [b], Xiaobei Hu [b], Kunzhi Zhang [b], Xianchao Cao [b], Kailong Jiang [b], Yubo Zhou [b,*,1], Jian Hou [a,1], Fajun Nan [b,1], Jia Li [b,1]

[a] *Department of Hematology, Changzheng Hospital, Second Military Medical University, Shanghai 201203, China*
[b] *National Center for Drug Screening, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*

ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form 10 February 2015
Accepted 12 February 2015

Keywords:
Selective cancer therapy
Unfolded protein response
2DG
UPR disrupter

ABSTRACT

Identifying chemotherapy candidates with high selectivity against cancer cells is a major challenge in cancer treatment. Tumor microenvironments cause chronic endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR) as an adaptive response. Here, one novel small-molecule compound, 17#, was discovered as a potent pan-UPR inhibitor. It exhibited good selection for growth inhibition when cancer cells were cultured in 2-deoxy-D-glucose (2DG), mimicking an *in vitro* glucose-deprived status. Additionally, 17# alone could mildly suppress the growth of HeLa tumor xenografts, and a synergistic anti-cancer effect was observed when 17# was combined with 2DG. A mechanistic study showed that 17#-induced selective anti-cancer effects were highly dependent on UPR inhibition, and overexpressing GRP78 or XBP1s reversed the 17#-induced growth inhibition and cell cycle arrest, partially by delaying the downregulation of the cell cycle regulator *cyclin B1*. Furthermore, 17# improved the sensitivity of anti-cancer drugs such as doxorubicin or etoposide. Our study presents evidence that disrupting the UPR has selective therapeutic potential and may enhance drug sensitivity.

Introduction

Successful cancer therapy depends on the selective killing of tumor cells. Identifying cytotoxic compounds with high selectivity against cancer cells is a major effort of current chemotherapy. The tumor microenvironment, which is characterized by hypoxia and nutrient deprivation, induces multiple adaptive cellular responses that play a crucial role in malignant progression and metastasis [1–3]. Various therapeutic strategies targeting the tumor microenvironment for selective cancer cell killing have been developed [4]. Accumulating evidence has demonstrated that the tumor microenvironment elicits an adaptive unfolded protein response (UPR) to maintain cancer cell homeostasis, which proves to be a cell-intrinsic mechanism crucial for tumorigenesis and chemotherapy resistance in various tumors [5]. Notably, the UPR pathways in most normal cells remain in a quiescent state but are activated in tumor cells. The UPR has been indicated as a biomarker for microenvironmental stressors that occur in tumors [6]. This discrepancy between cancer cells and normal cells offers an advantage for the drugs that target the UPR to achieve specificity in cancer therapy [7].

In mammals, the UPR is initiated by the activation of three main stress sensors, namely the ER transmembrane proteins inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which control the expression of downstream transcription factors ATF4, XBP1, and ATF6α-p50, respectively. Glucose-regulated protein 78 kDa (GRP78) interacts with each of the ER stress sensors and is considered the "master" regulator [8]. Because unfolded proteins accumulate in the ER lumina, they compete for binding with GRP78, leading to dissociation of GRP78 from the ER stress sensors, thus activating the UPR pathways.

All three branches of the UPR contribute to the development of cancer by driving tumor growth, cell transformation, invasion, and tumor dormancy [9]. Cancer cells that express dominant-negative IRE1 exhibit reduced proliferation, which could be attributed to its role in VEGF regulation. Additionally, XBP1s was shown to promote

Abbreviations: UPR, unfolded protein response; 2DG, 2-deoxy-D-glucose; IRE1, inositol-requiring enzyme 1; XBP1, X-box binding protein 1; GRP78, glucose-regulated protein 78 kDa; TM, tunicamycin.

* Corresponding author. Tel.: +0086 21 50801313.
E-mail address: ybzhou@simm.ac.cn (Y. Zhou).

[1] They contributed equally to the study and were listed as co-corresponding authors.

http://dx.doi.org/10.1016/j.canlet.2015.02.029
0304-3835/

blood vessel formation [10], and played a pivotal role in tumorigenicity of triple negative breast cancer [11]. The PERK-deficient tumors exhibit reduced viability and impaired angiogenic ability during hypoxia [12]. ATF6α can induce a cyto-protective response, which is essential for the adaptation of dormant cells to chemotherapy and the environmental conditions [13]. Moreover, the UPR is also a source of pro-inflammatory signaling whose downstream mediators may hamper antitumor immunity by remodeling the immune response in the tumor microenvironment [5].

Most tumors can escape from the inhibition of a single kinase, although kinase inhibitors are the largest class of new cancer drugs [14]. Additionally, drugs are frequently observed binding to more than one target; thus, polypharmacology approaches can be advantageous, complementing the "one drug-one target" strategy in cancer drug discovery [15]. Based on the UPR as a cancer adaptive response, we propose that disrupting the UPR would likely be a suitable polypharmacology model for a potential cancer-selective therapeutic option.

The ER stress indicator XBP1-venus has been used to monitor physiological and pathological ER stress *in vivo* [16], and XBP1–luciferase activity has been used to indicate the status of ER stress in primary tumors, with an inverse correlation with glucose avidity and direct correlation with hypoxia and tumor growth [6]. Increased XBP1–luc activity reflected the contribution of hypoxia as well as glucose and nutrient deprivation during stress. The UPR is a dynamic signaling network in which all three UPR signaling pathways activate a gene transcription program to balance both pro-apoptosis and pro-adaptation signaling in response to cellular stress, in which *GRP78* and *CHOP* are the classical pro-adaptation and pro-apoptosis genes regulated by all three UPR pathways [17]. Here, we have developed a cellular UPR inhibitor screen assay using stably transfected HEK293 cells with XBP1–luciferase. The screen assay was combined with real-time PCR of classical UPR transcription program genes (*GRP78*, *CHOP* and *ATF4*) to discover potential UPR inhibitors with the ability to inhibit XBP1 splicing, as well as the UPR transcription program.

In our study, we identified 17#, a novel small-molecule compound that abrogates the UPR in cancer cells stressed by divergent ER stress stimuli. As expected, 17# showed good selectivity for cell growth inhibition, cell cycle arrest and apoptosis when cancer cells were cultured under glucose-deprived conditions and displayed good anti-tumor effects on tumor xenografts. A mechanistic study showed that 17#-induced selective anti-cancer effects were indeed dependent on UPR inhibition and that overexpressing GRP78 or XBP1s could reverse 17#-induced growth inhibition and cell cycle arrest, partially by delaying the decrease in the cell cycle regulator *cyclin B1* gene expression.

Materials and methods

Cell lines and culture conditions

The HeLa, A549, H1299, HCT116 and HEK293 cell lines were obtained from the Cell Bank (Chinese Academy of Sciences, Shanghai, China). HeLa cells are epithelial cells from cervical adenocarcinoma, which contain human papilloma virus (HPV-18). A549 cells are epithelial cells from human lung carcinoma. H1299 cells are from human non-small cell lung cancer, and derived from metastatic lymph node. HCT116 cells are epithelial cells from colorectal carcinoma. HeLa, A549, H1299 and HCT116 were used to see whether the inhibitory effect of 17# could be applied to different tumor cell lines. HEK293 cells are epithelial cells from human embryonic kidney. They were used for establishing the HEK293–XBP1-splicing–Luc cell line. HEK293–XBP1-splicing–Luc cells were HEK293 cells stably transfected with the XBP1-splicing–Luc vector, which was subcloned from the pCAX-F-XBP1-ΔDBD-venus plasmid, a kind gift from Dr. Masayuki Miura (The University of Tokyo) [16], where XBP1 splicing activity could be indicated with the luciferase activity.

The MEF cells are fibroblast cells from mouse embryo, a kind gift from Dr. Yingming Zhao (Shanghai Institute of Materia Medica). Peripheral blood mononuclear cells (PBMCs) were obtained from healthy people who underwent physical examination, with informed consent. PBMCs were separated by Ficoll density gradient centrifugation from peripheral blood, using lymphocyte separation medium. Both MEF cells and PBMCs are non-malignant cells. In our study, we used them as representatives of cells from normal tissues, aiming at testing whether 17# was toxic to normal cells.

HeLa, HEK293, MEF and PBMCs cells were maintained in HG-DMEM. A549 cells were maintained in F12 medium. H1299 and HCT116 cells were maintained in RPMI-1640 medium and 5A medium, respectively. All culture media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Utah, USA) and 100 units/ml each of penicillin and streptomycin in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 °C.

Chemicals

17# was first screened from the NCDS library and then synthesized by Professor Fajun Nan (The National Center for Drug Screening, Shanghai, China). 2-Deoxy-D-glucose (2DG) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile distilled water for use in cell lines. Tunicamycin (TM) was purchased from Millipore (Billerica, MA, USA) and dissolved in DMSO. Kolliphor was purchased from Sigma-Aldrich, and methyl cellulose was purchased from MP Biomedical. Lymphocyte separation medium was purchased from Haoyang Co. Ltd, Tianjin, China. The final DMSO concentration in the cell culture was maintained below 1% of the total medium volume.

Adenovirus system

The GRP78 recombinant adenovirus vector (Ad-GRP78) was a kind gift from Dr. Allen Volchuk (University of Toronto, Canada) [18]. The XBP1s recombinant adenovirus vector (Ad-XBP1s) was a kind gift from Professor Yong Liu (Shanghai Institutes for Biological Sciences) [19]. The CHOP recombinant adenovirus vector was subcloned from the mCHOP-WT-9E10-pcDNA1 plasmid (https://www.addgene.org/21913/), a kind gift from Dr. David Ron (University of Cambridge, UK), and expressed according to the protocol of AdEasy system.

RNA isolation and reverse-transcription

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration and integrity were tested using a UV/VIS spectrophotometer NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). Two and one-half micrograms of total RNA was reverse transcribed using PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China).

Semi-quantitative RT-PCR

The cDNA product was subjected to 35 cycles of PCR using the forward primer 5-TTACGAGAGAAAACTCATGGGC-3 and reverse primer 5-GGGTCCAAGTTGTCCAGAATGC-3 specific for XBP1. PCR products were analyzed on a 12% polyacrylamide gel. The size difference between the spliced and the unspliced XBP1 is 26 nucleotides [20].

Real-time PCR

Total RNA and the complementary DNA were prepared following instructions in 2.4 RNA isolation and reverse-transcription. Real-time PCR was carried out at 95 °C for an initial 5 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s using SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) and a 7300 Fast Real-Time PCR System (Applied Biosystems). The samples were analyzed in duplicate and normalized to actin expression using the $2^{-\Delta\Delta CT}$ method [21]. The primer sequences could be found in Supplementary Table S1.

Immunoblotting analysis and antibodies

Cell extracts were prepared in sodium dodecyl sulfate (SDS) loading buffer, subjected to electrophoresis through Tricine–SDS-PAGE gels and blotted with antibodies for β-actin (Upstate, Billerica, MA, USA); p-IRE1α (rabbit polyclonal) eIF2α (rabbit polyclonal), and p-eIF2α (rabbit polyclonal) from Epitomics Company; GRP78 (rabbit polyclonal), cyclin B1 (mouse monoclonal), p-cdc2 (Thr161) (rabbit polyclonal), p-cdc2 (Tyr 15) (rabbit polyclonal), cdc2 (rabbit polyclonal), PARP (rabbit polyclonal), p-chk1 (rabbit polyclonal), p-cdc25c (rabbit polyclonal), cdc25c (rabbit polyclonal), caspase 3 (rabbit polyclonal), cleaved caspase 3 (rabbit polyclonal), caspase 6 (rabbit polyclonal), PERK (rabbit polyclonal) and p-PERK (rabbit polyclonal) from Cell Signaling Technology; CHOP (rabbit polyclonal), ATF6 (rabbit polyclonal), XBP1s (rabbit polyclonal) and PCNA (mouse polyclonal) from Santa Cruz biotechnology. The working solutions of GRP78, cyclin B1, p-cdc2 (Thr 161), p-cdc2 (Tyr 15), cdc2, p-chk1, p-cdc25c, cdc25c, p-IRE1α, p-PERK, PERK, p-eIF2α, eIF2α, CHOP, ATF6α, PCNA, caspase 6, caspase 3, cleaved caspase 3 and XBP1s antibodies were 1:1000, while the working solutions of PARP and β-actin antibodies were 1:2000 and 1:10000 respectively. The immunoblots were visualized using the Enhanced Chemiluminescence (ECL) western blotting System (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Please cite this article in press as: Hejing Huang, et al., Disruption of the unfolded protein response (UPR) by lead compound selectively suppresses cancer cell growth, Cancer Letters (2015), doi: 10.1016/j.canlet.2015.02.029