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Original Articles

Pharmacologic inhibition of AKT leads to cell death in relapsed multiple myeloma

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ABSTRACT

Multiple myeloma (MM) is a neoplastic plasma cell disorder with high disease recurrence rates. Novel therapeutic approaches capable of improving outcomes in patients with MM are urgently required. The AKT signalling plays a critical regulatory role in MM pathophysiology, including survival, proliferation, metabolism, and has emerged as a key therapeutic target. Here, we identified a novel AKT inhibitor, HS1793, and defined its mechanism of action and clinical significance in MM. HS1793 disrupted the interaction between AKT and heat shock protein 90, resulting in protein phosphatase 2A-modulated phosphorylated-AKT (p-AKT) reduction. Moreover, we observed reductions in the kinase activity of the AKT downstream target, IκB kinase alpha, and the transcriptional activity of nuclear factor kappa B, which induced mitochondria-mediated cell death in MM cells exclusively. We confirmed the cytotoxicity and specificity of HS1793 via PET-CT imaging of a metastatic mouse model generated using human MM cells. We also evaluated the cytotoxic effects of HS1793 in primary and relapsed MM cells isolated from patients. Thus, HS1793 offers great promise in eliminating MM cells and improving therapeutic responses in primary and relapsed/refractory MM patients.

1. Introduction

Multiple myeloma (MM) is an incurable cancer where patients eventually develop drug resistance even with high-dose chemotherapy treatment. Current strategies for treating MM involve combination therapies that include proteasome inhibitors, immunomodulatory drugs, and targeted inhibitors of deregulated cell signalling. Nevertheless, many patients still develop drug resistance and experience disease relapse. Thus, more effective drugs targeting well-defined events in MM are needed.

AKT is involved in cell survival, proliferation, and metabolism [1]. Activated AKT promotes oncogenesis by activating several downstream pathways that mediate proliferative or survival responses [2]. Indeed, the constitutive activation of AKT has been reported in several cancers, including haematological malignancies and glioblastoma.

AKT is frequently activated in MM cells and the incidence of AKT activation correlates positively with disease activity [3]. In other human cancers, aberrant AKT signalling results from mutations in the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway, but this is not the case in MM [4,5]. Instead, cytokine stimulation, especially from interleukin-6 (IL-6), and upregulated AKT expression induce dysregulated AKT signalling in MM [6–8]. In numerous preclinical studies, loss of AKT function induced MM cell death [9], indicating that treatment with an AKT inhibitor, alone or in combination with other agents, may

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improve survival of MM patients. Several AKT inhibitors are currently known, such as afuresertib [6] (the ATP-competitive AKT inhibitor), MK-2206 [10] (the allosteric AKT inhibitor), and perifosine [11] (the alkylphospholipid). However, the development of additional AKT inhibitors and their clinical applications in treating MM remain elusive.

In this study, we screened an in-house library to identify compounds that inhibited phosphorylated-AKT (p-AKT), utilising the AKT ELISA. We identified a novel AKT inhibitor (HS1793) from the screening hits, and defined its mechanism of action and clinical significance in MM. HS1793 is a resveratrol derivative that exerts anti-tumour effects in breast, prostate, colon, and renal cancer [12–16]; however, its anti-tumour mechanisms are unclear. Here, we showed that HS1793 inhibits AKT-HSP90 binding, resulting in decreased AKT activity. Moreover, HS1793 inhibited the phosphorylation of IKKα (a downstream target of AKT), blocking the transcriptional activity of NF-κB, and ultimately inducing mitochondria-mediated MM cell death. Our data further revealed that HS1793 exhibits anti-tumour effects in a metastatic mouse model and in primary and relapsed MM cells isolated from patients. Hence, HS1793 acts as an AKT inhibitor and successfully induces therapeutic responses in relapsed or refractory MM patients.

2. Materials and methods

2.1. Cell culture and antibodies

The human KMS20 and KMS26 MM cell lines were established at the Kawasaki Medical School (Kawasaki, Japan) from 65- and 77-year old female patients, respectively [17–19]. RPMI8226 cells were obtained from the Korean Cell Line Bank, and human primary bone mononuclear cells were purchased from Lonza (Basel, Switzerland). The cell lines were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Antibodies against PARP, caspase-3, AKT, p-AKT (S473), IKKα, p65, and tubulin were purchased from Cell Signalling Technology (Danvers, MA). Antibody against Prx3 was purchased from Abclone (Seoul, Korea). Antibodies against HSP90 and GST were purchased from Santacruz (CA, USA).

2.2. Plasmids

The full-length cDNA of human AKT1 (NM_005163), HSP90 (NM_005348), and PP2Ac (NM_002715) were received from the 21C HUMAN GENE BANK, Genome Research Center of Korea Research Institute of Bioscience and Biotechnology (KRIBB), South Korea. AKT was subcloned into the pCGN-HA (W. Herr, Cold Spring Habor Laboratory), pEBG-GST (kindly provided by Y. Liu, NIA, National Institutes of Health) and pM (Clonetech) vectors in order to construct the HA-tagged, GST-tagged and DNA binding-domain fused plasmids, respectively. PP2Ac was subcloned into the pEBG vector. HSP90 was subcloned into the pcDNA6.1-mycHis (Invitrogen) and pVP16 (Clontech) to construct the His-tagged and activation-domain fused plasmids, respectively. Constitutive active (CA)-AKT (Myristoylated AKT1) were generous gifts from Dr. William Sellers (Dana-Farber Cancer Institute and Havard Medical School, Boston, MA). All constructed plasmids were validated using a Sanger DNA sequencing analysis (Macrogen Inc., Korea), and the sequence were analysed by alignment with the reference sequence of each gene using a SanpGene program.

2.3. Screening for AKT inhibitors

AKT In-Cell ELISA kit (Thermo Scientific) was used to screen for the inhibitors of AKT. As given as the manufacturer's instructions, KMS20 MM cells were seeded at 3×10^4 cells/well in 96-well plates. Cells were treated separately with 400 different types of small molecules for 24 h, followed by a 4% formaldehyde fixation, and were then subjected to total p-AKT/AKT ELISA. The plates were washed thrice with TBST buffer and subjected to 1 × permeabilization buffer for 15 min and a blocking buffer for 30 min at room temperature. p-AKT or total AKT antibody in blocking buffer (1:2000) was added to the wells for 24 h at 4 °C. The plates were washed thrice with 1 × washing buffer and TMB substrate was added for 15 min. Finally, the absorbance of the plates was measured at 450 nm within 30 min. To compensate for changes in total AKT protein levels with treatment, total AKT protein levels were measured using the same experimental conditions, following which the p-AKT values were normalized against total AKT values. We cut-off at 0.6 of the pAKT vs. AKT ratio to identify the effective compounds, and identified the 7 hit compounds including 4-resveratrol analogues. We next confirmed the AKT phosphorylation and cell death with the 4-resveratrol analogues using a western blotting. From these data, we identified the final hit compound, HS1793.

2.4. Cell isolation from human MM patients

Plasma cells were isolated from the blood of primary and relapsed MM patients at Chonnam National University Hwasun Hospital, Hwasun, South Korea. All patients provided informed consent prior to the collection of blood and the samples were obtained according to the ethical standards of the Institutional Review Board for Human Research at Chonnam National University Hwasun Hospital. Mononuclear cells were isolated by density gradient centrifugation. The $CD38^{high}CD138^{+}CD45^{-}$ or $CD38^{low}CD138^{-}CD45^{+}$ subsets were isolated from the mononuclear cells of MM patients by fluorescence-activated cell sorting (FACS) using a FACSAria instrument (BD Biosciences, Franklin Lakes, NJ, USA) with the help of anti-CD38, anti-CD138, and anti-CD45 antibodies (Miltenyi Biotec, Auburn, CA, USA).

2.5. Immunoprecipitation (IP)

For co-immunoprecipitation, KMS20 cells were co-transfected with expression vectors encoding for pCGN-HA-AKT and pEBG-GST-PP2Ac using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, followed by treatment with HS1793 or vehicle for 24 h. Cells to Co-IP or endogenous IP were lysed in lysis buffer A (20 mmol/L Tris, 137 mmol/L NaCl, 10% glycerol, 1% NP-40, 2 mmol/L EDTA) with freshly added protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The lysate was immunoprecipitated with antibodies to detect AKT, HSP90, HA-AKT, or normal immunoglobulin G. Protein A/G Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were then applied and the final precipitated proteins were analysed via immunoblot with the corresponding antibodies.

2.6. Glutathione S-transferase (GST) precipitation and in vitro pull-down assay

HEK293T cells were co-transfected with expression vectors encoding for pEBG-GST-AKT or pEBG-GST-AKT deletion mutants (as indicated in each figure), and pcDNA6.1-HSP90-His plasmid using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. GST fusion proteins were precipitated from cell lysates and analysed by immunoblotting with anti-His-tag and anti-GST-tag antibodies. Recombinant His-HSP90 and GST-AKT proteins were purchased from Sigma Aldrich. The His-tagged HSP90 protein was bound to Ni-NTA-agarose beads (QIAGEN), and the bead-bound HSP90 protein was used to test for binding to GST-AKT1. Resin (50 μL) bearing equal amounts of either His lysate or His-HSP90 protein (5 μg) was incubated with 5 μg of GST-fusion AKT1 proteins in 1 mL of PBS for 3 h at 4 °C on a rotating wheel after 1 h pretreatment with HS1793. The resin was washed in 1 mL of lysis buffer A and then resuspended in SDS-polyacrylamide gel electrophoresis buffer. Samples were boiled for

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