약학석사 학위논문

HSP90B1 세포표면 이동의 화학적 억제와 자가면역질환에 대한 효과

Chemical Inhibition of HSP90B1 Surface Translocation and Its Effect on Autoimmune Disease

2017 년 8 월

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이 논문을 약학석사 학위논문으로 제출함

2017 년 7 월

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임 종 하

임종하의 약학석사 학위논문을 인준함
2017 년 7 월

위 원 장 _____ (인)
부 위 원 장 _____ (인)
위 원 _____ (인)

ABSTRACT

Autoimmune disease is characterized by aberrant immunological activation. Heat shock protein beta 1 (HSPB1) or gp 96, HSP90 family member, is an endoplasmic reticulum (ER)-resident chaperone protein. It is involved in immunological activity and its cell surface localization induces autoimmune disease. In autoimmune disease, HSPB1 no longer retrogrades from golgi to the ER but translocates to cell surface instead. Cell surface expressed HSPB1 induces abnormal activation of dendritic cells (DCs) and DCs activate immune cells. Aberrantly activated immune cells attack normal tissues and consequently induce autoimmune disease. Aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1), found in multi-tRNA synthetase complex (MSC), supports HSPB1 in retrograde transport from golgi to the ER. In autoimmune disease, the interaction between HSPB1 and AIMP1 is disassociated, leading to translocation of HSPB1 to the cell surface. Therefore, the role of AIMP1 is essential in regulation of HSPB1 cell surface level and thereby in autoimmune disease.

Previously, chemical named GPM1 was introduced to control cell surface translocation of HSPB1 by substituting for the role of AIMP1 and to alleviate the autoimmune disease-associated symptoms *in vivo*. However, GPM1 had poor metabolic stability to be developed as a therapeutic drug. Metabolic biotransformation of a drug can alter the half-life of the drug and determine the drug response to the agent. Screening metabolic stability in the early stages of drug development is a critical step in drug discovery that it has significant effect on efficacy and safety. In this study, we identified GPM1 derivatives and found

'chemical KR-S-026S' dramatically reduced cell surface localization of HSPB1 and strikingly ameliorated autoimmune phenotype *in vivo*. KR-S-026S had enhanced effect and also had higher metabolic stability compared to GPM1. These results suggest that chemical KR-S-026S is a promising compound to alleviate autoimmune disease by substituting for the role of AIMP1 and support HSPB1 to retrograde to the ER.

Keywords : Autoimmune disease, HSPB1, AIMP1, GPM1, Therapeutic drug

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ABBREVIATION LIST

HSP90: Heat Shock protein 90

AIMP1: Aminoacyl-tRNA synthetase (ARS)-interacting multi-functional protein 1

Gp96: Glycoprotein 96

DC: Dendritic cells

ER: Endoplasmic reticulum

COPI: Coatomer protein complex

KDELR: Lys-Asp-Glu-Leu receptor

TLR: Toll-like receptor

MSC: Multisynthetase complex

TGF- β: Transforming growth factor beta

Smurf2: Smad ubiquitination regulatory factor 2

INTRODUCTION

Autoimmune disease is characterized by autoantibody production and immune complex stimulation (1). The mechanism that elicits this phenotype is aberrant activation of immune cells by abnormally matured dendritic cells (DCs); and those immune cells attack normal tissues, progressing to irreversible organ damage and consequently increasing the risk of premature death (2). At the interface of innate and adaptive immunity, DCs play crucial role in regulating immune responses. Matured DCs activate autoimmune T cells by presenting antigen for T cells; and T cells support the differentiation of autoreactive B cells, increasing the production of autoantibodies (3-5). The widespread deposition of immune complexes accumulates in the kidneys, leading to glomerulonephritis and renal failure (6). There are immunosuppressive therapy such as corticosteroids but have such side effects as infections, osteoporosis and atherogenesis (7).

Glycoprotein 96 (gp96) or HSP90B1, member of heat shock protein (HSP) 90 family, is an endoplasmic reticulum (ER)-resident chaperone protein (8). Endoplasmic reticulum (ER) chaperones such as HSPB1, calreticulin, and isomerases recycle back to ER by retrograde transport from golgi through coatomer protein complex (COPI)-coated vesicles (9, 10). COPI is a protein complex that forms a coat around non-clathrin-coated vesicles to transport from the *cis* end of the golgi back to the rough ER (11). ER chaperones are equipped with C-terminal Lys-Asp-Glu-Leu (KDEL) motif which is recognized by the KDEL receptor, ERD2 (12, 13). Chaperones recognized by ERD2 induces oligomerization, leading to retrograde transport from golgi to the ER (14). However, many of these

chaperones can translocate to the cell surface under ER stress, and ER chaperones in the extracellular space can take on immunogenic characteristics (15). The association between autoimmune disease and HSPB1 has been studied that cell surface exposed HSPB1 directly interacts with and activates DCs via toll-like receptor (TLR) 2- and 4- signal transduction pathways (16). And abnormally matured DCs result in secretion of proinflammatory cytokines and upregulation of MHC class I and II molecules, consequently inducing autoimmune disease. In transgenic mouse that constitutively express cell surface HSPB1 induced by removing the C-terminal ER-retention signal KDEL of HSPB1, showed significantly increased level of activated DCs and spontaneously developed autoimmune disease phenotypes (17). Therefore, many studies understanding how these chaperone proteins translocate to the cell surface are in a process for the development of immune-based therapy.

Aminoacyl-tRNA synthetase (ARS)-interacting multi-functional protein 1 (AIMP1) or p43, a component of the multisynthetase complex (MSC), is involved in diverse physiological activities. AIMP1 works as a pleiotropic cytokine on endothelial cells, fibroblasts, and monocytes to regulate angiogenesis, wound healing, and (18). Moreover, AIMP1 downregulates transforming growth factor beta (TGF-β) signaling by stabilizing smad ubiquitination regulatory factor 2 (Smurf2), negative regulator of TGF-β signaling pathway (19). Furthermore, previous reports about AIMP1 in immune response elicited that AIMP1 can also regulate innate immune response by inducing maturation of DCs (20). AIMP1 is also associated with autoimmune disease that AIMP1 interacts with HSPB1 for ER

retention of HSPB1 and prevents extracellular translocation of HSPB1. The interaction between HSPB1 and KDEL receptor is enhanced by AIMP1, inducing dimerization of HSPB1 (21). AIMP1 has a crucial role in inhibition of extracellular translocation of HSPB1 and thereby in immunological implication. For instance, AIMP1-deficient mice developed autoimmune disease phenotypes that the mice had increased production of autoantibodies, high number of immune cells infiltrated through various organs, accumulated autoantibodies, and glomerularnephritis (18).

Previously, chemical named GPM1 was introduced to inhibit cell surface localization of HSPB1 by directly interacting with HSPB1. GPM1 binds at the vicinity of dimerization interface of HSPB1 and stabilizes HSPB1 dimer formation. GPM1 competes against AIMP1 to bind HSPB1 and functionally mimics AIMP1 that GPM1 inhibits extracellular translocation of HSPB1 by assisting the interaction between HSPB1 and KDEL receptor. Furthermore, autoimmune disease phenotypes of HSPB1tm transgenic mice which had chronically enhanced HSPB1 surface level, were ameliorated upon GPM1 treatment. Treatment of GPM1 in HSPB1tm transgenic mice decreased maturation of DCs, B cells and memory T cells, ameliorated glomerulonephritis phenotypes, and reduced autoantibodies level (22). In this study, we analyzed the chemical properties of GPM1 and observed that GPM1 had excessively low metabolic stability to become an ideal drug. Therefore, we synthesized GPM1 derivatives and suggest the most effective chemical with favorable chemical properties.

MATERIAL AND METHODS

Cell culture and chemical treatment

RAW 264.7 cells were purchased from Korean Cell Line Bank (KCLB) and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin at 37^{o}C in 5% CO_2.

Madin-Darby Canine Kidney (MDCK) epithelial cells were obtained from the American Type Culture Collection (ATCC) and were grown in Minimal Essential Medium containing 10% FBS and 2 mM fresh L-glutamine at 37^{o}C in 5% CO_2.

All GPM1 derivatives were synthesized by Syngene International Ltd Company. All chemicals were dissolved in dimethyl sulfoxide (DMSO).

Western blot Analysis

Whole cell protein extracts were isolated with cold lysis buffer (25 mM Tris-HCL pH 7.4, 150 mM NaCl, 10% glycerol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.1% SDS, and protease inhibitor) for 30 min at 4^{o}C. After collected lysates were centrifuged at 13,200 rpm for 15 min at 4^{o}C, supernatant proteins were quantified by Bradford assay (Biorad), followed by equalization of the sample concentration with sample buffer and lysis buffer.

Samples were separated by SDS-PAGE and transferred to polyvinylidenefluoride (PVDF) membranes (Milipore) at 55 mA, 6 V for 1 h 30 min (BioRad). After the membranes were blocked with 5% skim milk in 0.5% TBS-T for 1 h, they were incubated with primary antibody diluted in 0.5% sodium azide, 1% skim milk in

TBS-T overnight at 4°C. Membranes were washed 3 times with 0.5% TBS-T buffer for 7 min respectively and incubated with secondary antibody (Thermo Scientific) diluted in 1% skim milk in TBS-T for 1 h at room temperature. ECL solution (Santacruz biotechnology) was applied to the membrane according to the manufacturer's protocols.

Antibodies were obtained from the following sources: rabbit polyclonal HSPB1 antibody (Santacruz Biotechnology), anti-sodium potassium ATPase antibody (Abcam), α-tubulin antibody (Santacruz Biotechnology).

Immunofluorescence

Cells were seeded onto 9 x 9 mm cover slips (Bellco Glass Inc.) at 2.5 x 10^4 cells/well overnight and were treated with chemical compounds for 16 h. Cells were fixed with 4% paraformaldehyde (PFA, Biosesang) for 10 min at room temperature and blocked with CAS-block (Life Techonology) without permeabilization step being followed. After blocking step, cells were subjected to immunofluorescene staining with rabbit polyclonal HSPB1 (1:100) antibody (Santacruz Biotechnology) diluted in PBS with 10% CAS-block for 2 h at 4°C. Cells were incubated with FITC-conjugated anti-rabbit (1:200) secondary antibody (Invitrogen) diluted in PBS with 10% CAS-block for 1 h at 4°C. Then, cells were stained with DAPI (1 μg/ml, Sigma-Aldrich) for 5 min and mounted with mounting medium (Biomeda) on Micro Slides (Leica). Cells were examined by confocal fluorescence microscopy (Nikon Instruments).

Flow Cytometry

Cell surface level of HSPB1 was measured with Beckman Coulter flow cytometer. RAW 264.7 cells (1 x 10^6 cells) were washed with cold PBS and were suspended in fluorescence-activated cell sorting (FACS) buffer (1% BSA in PBS). Cells were fixed with 2% PFA (Biosesang) diluted in PBS for 10 min and incubated with 1 μg of Fc blocker (BD Pharmingen) for 20 min at 4°C to block Fc receptors. After blocking non-specific bindings, cells were incubated with 0.8 μg of rabbit polyclonal HSPB1 antibody (Santacruz Biotechnology) for 1 h at 4°C and incubated with 1 μg of FITC-conjugated anti-rabbit secondary antibody (Invitrogen) for 20 min at 4°C. FACS data were analyzed with Navios software.

Fractionation

Cells were prepared in two 100 mm dishes at 2 x 10^6 cells/dish. On the next day, cells were washed with cold-PBS and scraped into 1.5 ml tube. Cell pellet was collected by centrifuge at 1,000 rpm for 3 min at 4°C. After resuspending the pellet with buffer A (25 mM Tris-HCL pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 10 mM NaCl), samples were homogenized 20-30 times. 1/10 volume of 2.5 M sucorse was added and centrifuged at 1,000 *g* for 10 min at 4°C. Supernatant was centrifuged at 15,000 *g* for 15 min at 4°C and plasma membrane was isolated after resuspending the pellet with buffer B (25 mM Tris-HCl pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.25 M sucrose). Additionally, Centrifuging the supernatant at 100,000 *g* for 1 h at 4°C separated cytosol (supernatant) and microsomal protein (pellet).

Microsomal Stability Assay

Compounds were diluted in DMSO to obtain 1 mM stock. Test compounds or controls were incubated for 5 min at 37^{o}C after addition of 0.1 M phosphate buffer pH 7.4 and 10 mM NADPH. Human and rat liver microsomes (purchased from Sigma-Aldrich) were added for final concentration to be 0.5 mg/ml and incubated in a shaking water bath at 37^{o}C. Samples were centrifuged at 10,000 *g* for 5 min at 4^{o}C and supernatants were collected.

Standard curve was prepared by diluting 1 mM stock solution to 10, 1, and 0.1 μM in 0.1 M phosphate buffer. Percent stability (percent of remaining compound after metabolism) was analyzed with LC-MS/MS using Nexera XR system (Shimadzu) and TSQ vantage (Thermo) and stability was calculated as follows:

$$\text{Stability (\%)} = \frac{100\text{xPeak are at 30min}}{\text{Peak area at 0min}}$$

Drug Permeability Assay

The transepithelial electrical resistance (TEER) value, which represents the degree of development of monolayer confluence and tight junction, of MDCK cells was calculated. MDCK cells were washed with transport medium (Hanks' Balanced Salt Solution, HBSS, Gibco) and equilibrated in transport medium for 30 min at 37^{o}C. Using STX-2 chopstick electrode and volt-ohm meter (World Precision Instruments), electrical resistance of the cell was measured. TEER (Ω*cm^2) value for each monolayer is the average of raw monolayer resistance value multiplied by the membrane area (0.33 cm^2) with background value subtracted. MDCK cells with

TEER values less than 90 $\Omega*cm^2$ (cutoff value for MDCK cells) were not used.

The apparent permeability (Papp) level is determined by amount of chemical compound crossing apical (donor)-to-basolateral (acceptor) direction through cell monolayer (MDCK) cultured on 96-well polycarbonate membrane filters (Corning). Compounds were added at 50 μM with final DMSO concentration of 1% on the apical side solution (Gibco BRL) and incubated for 4 h at 25°C. Both Apical and basolateral solutions (Gibco BRL) were analyzed by LC-MS/MS and Papp value was calculated based on the amount of compound on the basolateral medium.

Cell proliferation analysis using IncuCyteTM FLR

Cells were plated in 96-well plate at 10,000 cells/well in triplicate. Chemicals were treated on the next day and photomicrographs were taken every 2 h using an IncuCyteTM FLR (Essen BioScience). The confluence of the cultures was monitored with IncuCyte Kinetic Live Cell Imaging System.

Drug Administration

NZB/W F1 female mouse with age of 20 weeks expressing autoimmune disease were obtained from Jackson laboratory. Chemicals were treated from the age of 24 weeks for each experimental group. Groups were divided into three groups: vehicle group, GPM1 treated group and KR-S-026S treated group. GPM1 and KR-S-026S were treated at 30 mg/kg per injection. All chemicals were dissolved in 5% DMSO in PBS, pH 7.4; and administered intraperitoneally every 24 h for 2 months.

Proteinuria measurement

Urea from each groups of mouse were collected at the age of 23, 24, 26, 28, 30, 32 weeks. Collected urines were centrifuged at 3000 rpm for 5 min and pellets were examined by Accute TBA-40FR, Toshiba Medical System Co., Japan.

RESULTS

Screening strategy to select the most effective compound targeting HSPB1 in cell surface translocation

In the previous study, we screened 6,482 chemical compounds that can directly interact with HSPB1 and decrease cell surface level of HSPB1 with no *in vivo* toxicity. Among those, GPM1 was selected for its ability to reduce cell surface level of HSPB1. It ameliorated the phenotypes of autoimmune disease in HSPB1tm transgenic mice in which cell surface HSPB1 level is chronically enhanced. However, GPM1 had poor chemical property to be further developed as a therapeutic drug (Fig 1A); therefore we synthesized GPM1 derivatives having similar chemical structure, 4,6-dimethoxy-pyrimidine (Fig 1B). We selected the most effective derivative in decreasing the cell surface HSPB1 level with enhanced chemical property.

KR-S-026S was selected by flow cytometry and GPM1 was used as a positive control that GPM1 reduced 40% of HSPB1 surface level (Fig 1C). Compounds KR-S-020, KR-S-022, KR-S-025S and KR-S-026S showed the most decreased cell surface HSPB1 level compared to other derivatives. KR-S-020, KR-S-022, KR-S-025S and KR-S-026S reduced 56%, 57%, 59% and 63% of HSPB1 surface level, respectively (Fig 1C). However, considering patentability, KR-S-020 and KR-S-022 were omitted and KR-S-025S was also omitted due to its poor cell permeability level (data not shown). Therefore, KR-S-026S was selected as the final compound (Fig 1A).

Remarkable effect of KR-S-026S in regulating cell surface level of HSPB1

KR-S-026S was selected by flow cytometry because it diminished the surface level of HSPB1 most dramatically compared to other GPM1 derivatives. We confirmed the effect of KR-S-026S on HSPB1 surface level.

In order to confirm that KR-S-026S is effective for HSPB1 only on the cell membrane instead of whole HSPB1 level in the cell, we fractionated RAW 264.7 cell into whole cell lysate, cytosol, and plasma membrane. We confirmed that the whole level of HSPB1 was not reduced under the treatment of GPM1 or KR-S-026S. Additionally, each samples were equally quantified as seen by equal levels of α-tubulin (cytosolic marker) and Na^+/K^+ ATPase (plasma membrane marker) (Fig 2A). Moreover, HSPB1 in the cytosol portion remained unchanged under the treatment of GPM1 or KR-S-026S. However, treatment of GPM1 or KR-S-026S decreased level of HSPB1 in the plasma membrane portion. And KR-S-026S dramatically reduced level of HSPB1 in the plasma membrane (Fig 2A). These data show that the treatment of GPM1 or KR-S-026S do not affect the whole HSPB1 level, but reduce level of HSPB1 only in the plasma membrane fraction.

Furthermore, chemical effect on surface HSPB1 level was visualized under fluorescence microscopy. Permeabilization step was omitted to visualize HSPB1 only on the cell membrane. About eight hundred RAW 264.7 cells were counted in five different areas and calculated for the mean intensity. With the background value being subtracted, GPM1 decreased 60% and KR-S-026S reduced 90% surface HSPB1 intensity (Fig 2B). Taken together, KR-S-026S showed improved result than GPM1 in surface level reduction of HSPB1.

Chemical properties of KR-S-026S in chemical stability, permeability, and its effect on cell proliferation

After selection of KR-S-026S as the most promising compound, chemical properties of KR-S-026S were examined. In order to investigate the toxicity of the compound, RAW 264.7 cells were treated with DMSO, KR-S-026S, and doxorubicin. Doxorubicin was used as a negative control because it hinders cell proliferation by inhibiting progress of DNA replication. And KR-S-026S was added with different concentrations: 2.5, 5, 10, 20 μM. Cell proliferation was not affected by the treatment of DMSO or KR-S-026S; however, treatment of doxorubicin diminished cell proliferation over time (Fig 3A). There was no significant difference in different concentrations of KR-S-026S. This data implies that KR-S-026S is not toxic on the proliferation of RAW 264.7 cell.

Next, human and rat liver microsomal stabilities of GPM1 and KR-S-026S were examined in order to compare the chemical stabilities of GPM1 and KR-S-026S. Chemicals were incubated with human or rat liver microsome for 30 min, and amount of chemicals remaining were measured. In human liver microsome, more than 99% of KR-S-026S was remained whereas only 0.1% of GPM1 was remained. In rat liver microsome, more than 89% of KR-S-026S was remained but GPM1 was not detected after 30 min (Fig 3B). This data implies that stability of KR-S-026S was dramatically enhanced compared to GPM1. Microsome stability indicates metabolic biotransformation of a drug. Low microsome stability indicates short half-life and thereby low response to the agent. In contrast, KR-S-026S has long half-life so that it has longer time to circulate in the body.

We then investigated cell permeability of KR-S-026S on MDCK cell to see its effectiveness in absorption level. Metoprolol was used as a positive control that it has high absorption level whereas atenolol was used as a negative control because it has low absorption level. 50 μM of KR-S-026S was treated and showed exceptionally high cell permeability with papp value of 24 x 10^{-6} cm/sec. Papp value of metroprolol and atenolol were 13 x 10^{-6} cm/sec and 0.2 x 10^{-6} cm/sec, respectively (Fig 3C). This data indicates that KR-S-026S has high cell permeability on MDCK cell and thereby high absorption level.

Effect of KR-S-026S on autoimmune disease in NZB/W F1 mice

We prepared NZB/W F1 mice, chronically expressing autoimmune disease, to examine the chemical effect on autoimmune disease. NZB/W F1 mice starts to develop autoimmune disease typically from the age of 18 weeks. Therefore, in order to assume and assure that all groups of mouse are induced with severe autoimmune disease, we started daily chemical treatment at the age of 24 weeks for 2 months. NZB/W F1 mouse were divided into 3 groups: vehicle, GPM1 treated group, and KR-S-026S treated group.

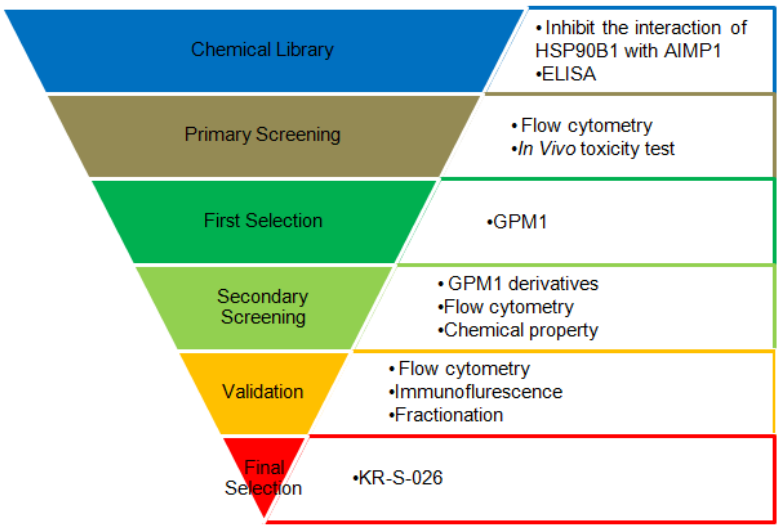
High level of proteinuria is an indication of an autoimmune disease that we measured proteinuria level to examine the severity of autoimmune disease in each groups. Because NZB/W F1 mouse model develops severe autoimmune disease, proteinuria level was increased in every group after treating chemicals for 8 weeks. However, proteinuria level of KR-S-026S treated group did not increase as much as vehicle or GPM1 treated group. Level of proteinuria increased 111%

and 71% for vehicle and GPM1 treated group respectively whereas it increased only 55% for KR-S-026S treated group (Fig 4A). Therefore, KR-S-026S alleviated one of the symptoms of autoimmune disease effectively.

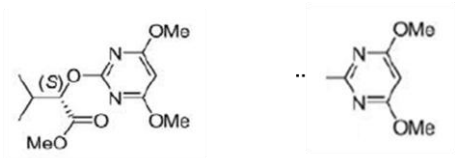
Additionally, each group contained 9 mouse at the beginning of the chemical treatment. However, 2 mouse in vehicle group died in third and fourth week respectively presumably due to severe autoimmune disease whereas no mouse were died in GPM1 or KR-S-026S treated groups (Fig 4B). Therefore, we can conclude that GPM1 and KR-S-026S have beneficial effect on survival of autoimmune disease induced NZB/W F1 mouse model.

This study highlights the critical role of cell surface expressed HSPB1 and suggests that HSPB1 can be a therapeutic target for autoimmune disease. Moreover, these results demonstrate that the treatment of KR-S-026S ameliorated symptoms of autoimmune disease in NZB/W F1 model by decreasing the cell surface level of HSPB1 (Fig 5A, B).

A.


Chemical Library
• Inhibit the interaction of HSP90B1 with AIMP1
•ELISA
Primary Screening
• Flow cytometry
•*In Vivo* toxicity test
First Selection
•GPM1
Secondary Screening
• GPM1 derivatives
•Flow cytometry
•Chemical property
Validation
• Flow cytometry
•Immunoflurescence
•Fractionation
Final Selection
•KR-S-026

B.


OMe
N
(S)
O
N
OMe
O
MeO
GPM1
14 GPM1 derivatives

C.

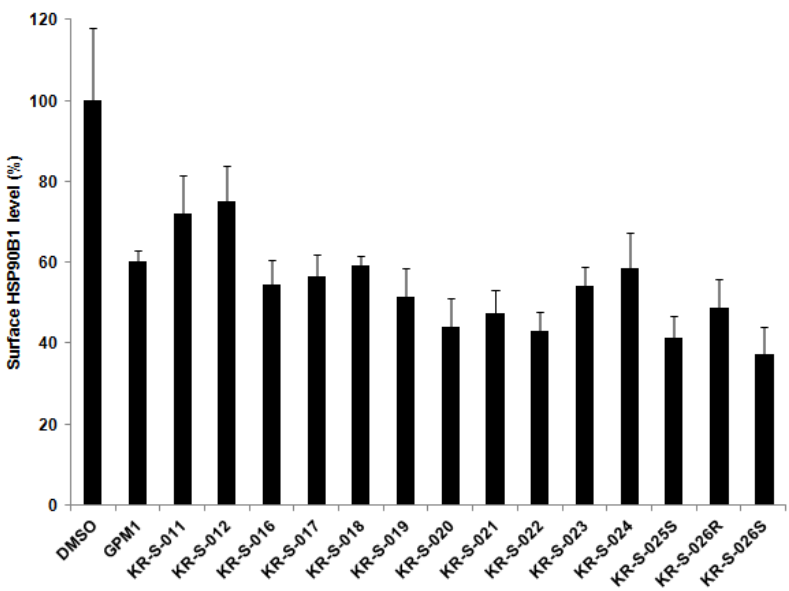

Surface HSP90B1 level (%)
120
100
80
60
40
20
0
DMSO
GPM1
KR-S-011
KR-S-012
KR-S-016
KR-S-017
KR-S-018
KR-S-019
KR-S-020
KR-S-021
KR-S-022
KR-S-023
KR-S-024
KR-S-025S
KR-S-026R
KR-S-026S

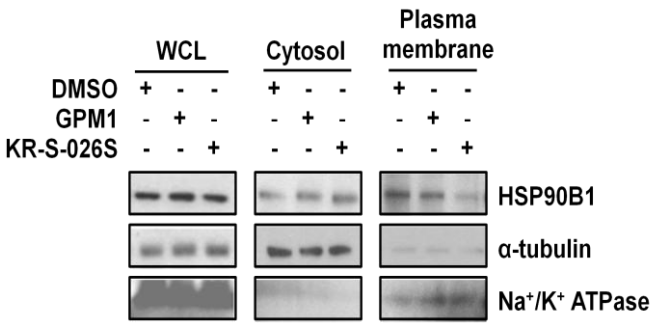
Figure 1. Screening strategy and selection of the most effective GPM1 derivative.

(A) Screening flow to identify the lead compound which functionally mimics AIMP1. Primary selection was performed to select the compound that can inhibit HSPB1 surface translocation and directly bind to HSPB1 with no *in vivo* toxicity. GPM1 was selected from the primary selection and GPM1 derivatives were synthesized for the secondary selection. Secondary selection was performed to select the compound with enhanced effect in reducing cell surface HSPB1 level and enhanced chemical property; and as a result, KR-S-026S was selected as a final compound.

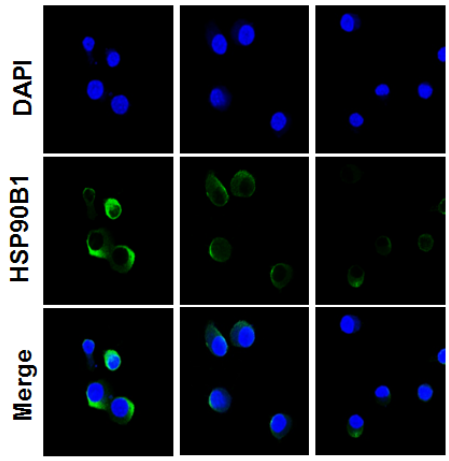
(B) GPM1 derivatives share similar chemical structure that they all have 4,6-dimethoxypyrimidine.

(C) RAW 264.7 cells were treated with DMSO or 10 μM of indicated chemicals dissolved in DMSO for 24 h. HSPB1 expressed on the cell surface was stained with HSPB1 antibody and FITC-conjugated anti-rabbit secondary antibody. Y-axis represents amount of HSPB1 remaining on the cell surface after chemical treatment in percentage.

A.


WCL
Cytosol
Plasma membrane
DMSO + - - + - - + - -
GPM1 - + - - + - - + -
KR-S-026S - - + - - + - - +
HSP90B1
α-tubulin
Na+/K+ ATPase

B.


DAPI
HSP90B1
Merge
DMSO
GPM1
KR-S-026S

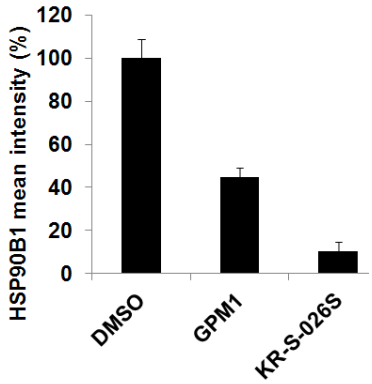
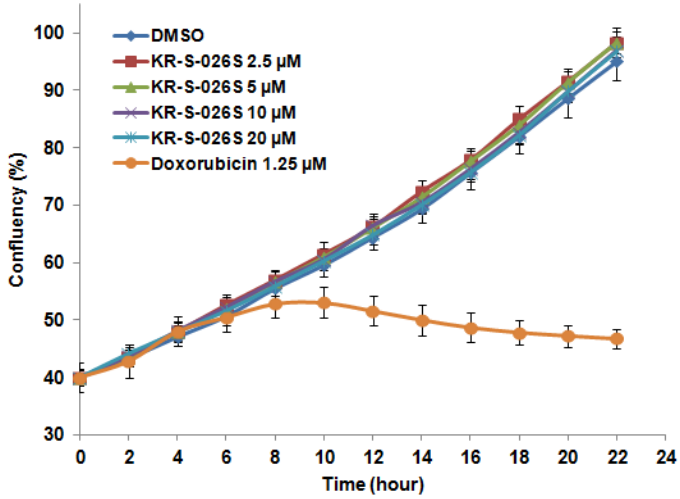

HSP90B1 mean intensity (%)
120
100
80
60
40
20
0
DMSO
GPM1
KR-S-026S

Figure 2. Dramatically decreased surface levels of HSPB1 upon KR-S-026S treatment.

(A) RAW 264.7 cells treated with indicated compounds were fractionated into cytosol and plasma membrane. Proteins from each segment were separated by SDS-PAGE and immunoblotted with anti-HSPB1, Na^+/K^+ ATpase, and α-tubulin antibodies. Na^+/K^+ ATpase and α-tubulin were used as markers for plasma membrane and cytosol, respectively.

(B) DMSO, 10uM of GPM1 and KR-S-026S were treated in RAW 264.7 cell for 24 h. Cells were fixed with PFA and permeabilization step was omitted in order to analyze HSPB1 only on the cell surface. Cell surface HSPB1 was detected using anti-HSPB1 antibody with secondary FITC-conjugated antibody and the green signal represents HSPB1. About eight hundred RAW 264.7 cells in five different area were calculated for the mean intensity of HSPB1 on the plasma membrane.

A.



B.

Human and rat liver microsome stability (% remaining during 30 mins)

	Human (%)	Rat (%)
KR-S-026S	>99	89.3 ± 11.4
GPM1	0.1 ± 0.1	~0

C.

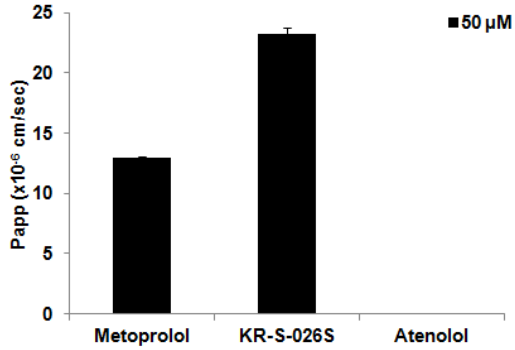


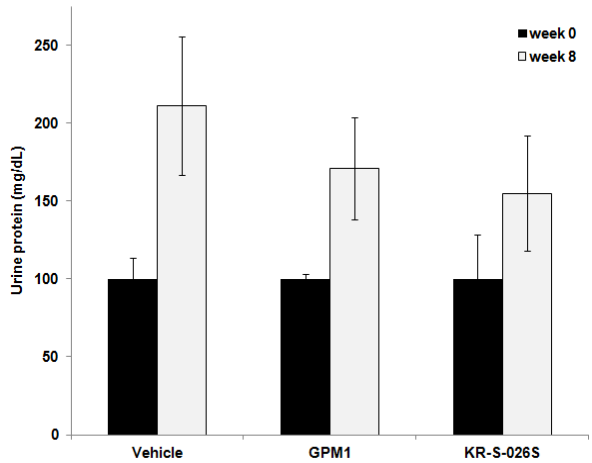
Figure 3. Chemical properties of KR-S-026S in stability, permeability, and cell proliferation level.

(A) RAW 264.7 cells were treated with KR-S-026S with indicated concentration and the real time cell proliferation was measured by Incucyte. Doxorubicin (1.25 μM) was used as a negative control.

(B) Chemical stability of KR-S-026S analyzed with human and rat liver microsomes was compared with GPM1. Human or rat liver microsome (0.5 mg/ml) was incubated with 10 μM of compounds for 30 min. Numerical value indicates amount of chemicals remaining after 30 min incubation.

(C) Permeability of KR-S-026S was measured by permeability assay with MDCK cell. Metoprolol and atenolol were used as positive and negative control, respectively. All chemicals were treated in the concentration of 50 μM and incubated for 4 h at 25^{o}C. Y-axis indicates amount of compounds pass through MDCK cell.

A.



B.

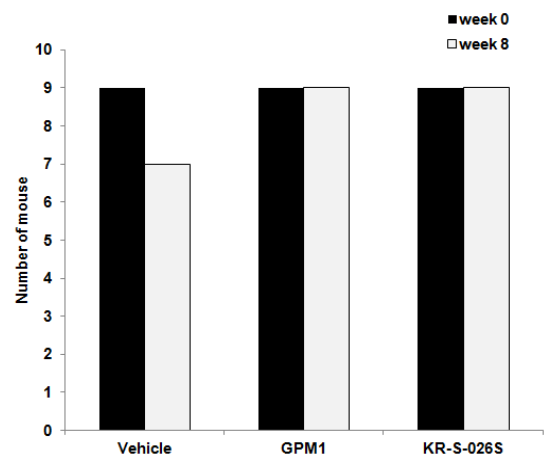
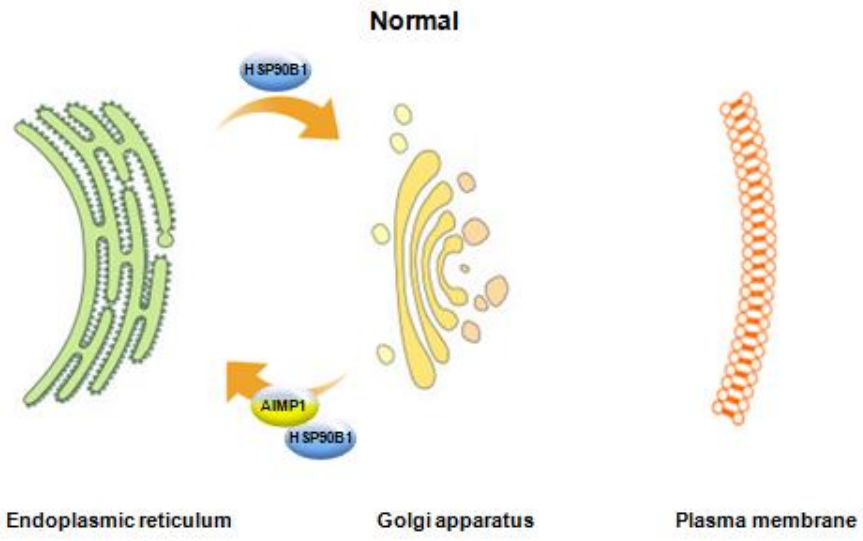


Figure 4. *In vivo* effect of KR-S-026S on autoimmune disease phenotypes in NZB/W F1 mouse model.

(A) Protein concentrations (mg/dL) in the urines, measured from vehicle (n=7), GPM1 (n=9), and KR-S-026S (n=9) treated groups for 8 weeks and proteinuria level from each samples was analyzed by Accute TBA-40FR.

(B) Number of survived mice in vehicle, GPM1 treated and KR-S-026S treated groups. In the vehicle group, one mouse died at the third week and another died at the fourth week.

A.


Normal
HSP90B1
AIMP1
HSP90B1
Endoplasmic reticulum
Golgi apparatus
Plasma membrane

B.

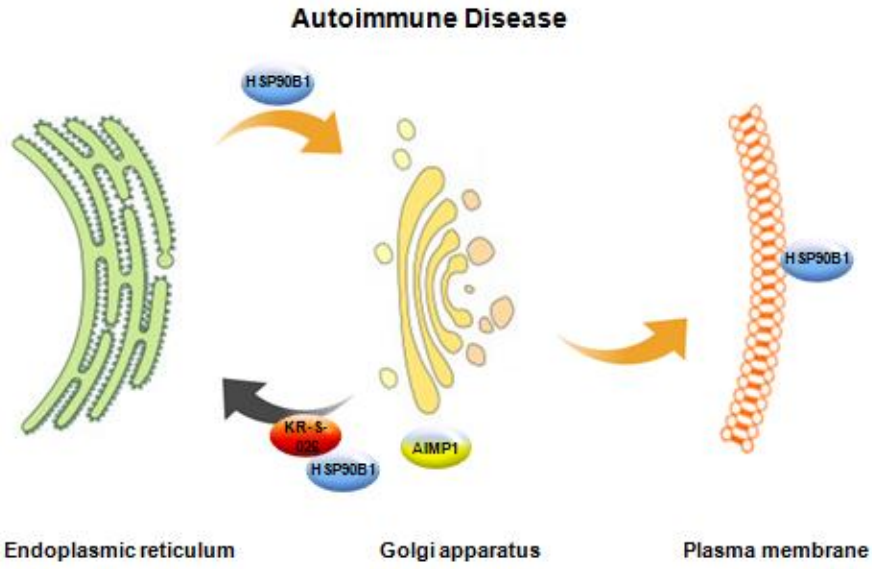

Autoimmune Disease
HSP90B1
KR-S-
020
AIMP1
HSP90B1
HSP90B1
Endoplasmic reticulum
Golgi apparatus
Plasma membrane

Figure 5. A schematic model representing function of KR-S-026S on autoimmune disease environment.

(A) In normal environment, HSPB1 circulates between ER and golgi by retrograde transport supported by AIMP1.

(B) In autoimmune disease environment, HSPB1 no longer retrograde back to the ER and translocates to the plasma membrane instead. Surface translocation of HSPB1 induces autoimmune disease which is relieved by treatment of KR-S-026S supporting retrograde transport of HSPB1 to the ER.

DISCUSSION

Autoimmune disease is characterized by production of autoantibodies attacking normal tissues. Clinically, corticosteroids are commonly used for treatment of autoimmune disease. Corticosteroids relieve the symptoms by suppressing production of immune cells and inflammation (23). They were introduced to relieve symptoms for autoimmune disease and decreased mortality rate of autoimmune disease (24). However, long term usage of corticosteroid is associated with severe side effects such as infection, hypertension, hyperglycema, avascular necrosis, osteoporosis, cataracts, glaucoma, and myopathy (23). Therefore, developing target or mechanism based therapy for autoimmune disease has significant implication.

HSP members, HSP60, HSP70, HSP90 families are known to be linked with immune stimulation (25, 26). Studies have found that HSP90 families are overexpressed in patients with autoimmune disease (27). One of the members of HSP90 family, HSP90B1 or gp96 is highly associated with autoimmune disease that its cell surface localization induces aberrant activation of immune cells and consequently induces autoimmune disease (21, 22, 28). Previously, we have found that AIMP1 supports retrograde transport of HSPB1 to the ER; we investigated compound that is able to substitute for the role of AIMP1, supporting retrograde transport of HSPB1 to the ER

We have previously identified GPM1 as a lead compound. However, due to poor chemical stability, we composed GPM1 derivatives with different chemical structures (Fig 1B). We selected KR-S-026S with most effective chemical in

decreasing cell surface level of HSPB1 by flow cytometry (Fig 1C). For further verification of effectiveness of KR-S-026S, we evaluated HSPB1 in cell surface level with immunofluorescence which showed remarkable decrease in HSPB1 at the plasma membrane of RAW 264.7 cells (Fig 2B). Furthermore, cytosol and plasma membrane of RAW 264.7 cells were fractionated and HSPB1 in plasma membrane decreased dramatically (Fig 2A).

Biotransformation is the chemical modification of biological system upon foreign substrate. Many drugs fail to sustain in the body system or lose their capability in biological system. For instance, half-life of a drug can be affected depending on its metabolic biotransformation (29). In order to confirm the chemical stability of KR-S-026S, we checked microsomal stability, permeability, and cell viability upon treatment of KR-S-026S. Microsomal stability of GPM1 was poor that it could not stay in the body system more than 30 minutes, indicating less effectiveness as a compound. However, KR-S-026S showed high microsomal stability both in human and rat, meaning that it is able to stay in the human or rat body and present its effectiveness to the target (Fig 3B). Furthermore, KR-S-026S had no adverse effect on cell viability, demonstrating that it has little toxic effect on RAW 264.7 cell (Fig 3A). KR-S-026S also had high level of permeability on MDCK cell, epithelial cell (Fig 3C). These data imply that chemical properties of KR-S-026S in absorption and stability were favorable; and it had little effect on cell proliferation.

Previous *in vivo* data showed effect of GPM1 on HSPB1tm transgenic mice in which HSPB1 surface level is chronically enhanced (22). Upon treatment with

GPM1, mouse had reduced proteinuria, serum levels of nuclear antigen-specific, and double stranded DNA-specific autoantibodies. We used NZB/W F1 mouse which develop autoimmune disease from the age of 18 weeks (30). In order for mouse model to develop severe autoimmune disease, we started the chemical treatment from the age of 24 weeks for 2 months. NZB/W F1 mouse model developed high level of proteinuria, which is a hallmark of severe autoimmune disease (31). And as chemicals were treated, level of proteinuria of KR-S-026S treated group did not increase as much as control group or GPM1 treated group (Fig 4A). Moreover, two mice died in DMSO treated group presumably due to severe autoimmune disease whereas no mouse died in the other groups, indicating that GPM1 and KR-S-026S have beneficial effect in survival of NZB/W F1 mouse model (Fig 4B).

We selected KR-S-026S from GPM1 derivatives due to its ability to reduce cell surface level of HSPB1 with enhanced chemical property. We showed that treatment of KR-S-026S ameliorated symptoms of autoimmune disease and survival rate in NZB/W F1 model. Therefore, we can conclude that KR-S-026S is a promising therapeutic compound in autoimmune disease by controlling immunological activity through regulation of HSPB1 cell surface level (Fig 5A, B).

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요약 (국문초록)

HSP90B1 세포표면 이동의 화학적 억제와 자가면역질환에 대한 효과

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임종하

자기 면역 질환은 자신의 면역세포에 의해 공격받는 질병으로 알려있다. HSP90B1 혹은 gp96은 소포체와 골지 사이를 순환하는 샤프론 단백질이다. 하지만 자기 면역 질환의 모델에서 HSPB1은 소포체와 골지 사이를 순환하지 않고 세포막으로 이동하여 면역세포를 활성화 시키는 것으로 연구가 되어있다. 세포막에 존재하는 HSPB1은 수지상 세포를 과도하게 활성화 시키고 그러한 수지상 세포들은 T 세포와 B 세포의 활성을 돕게 된다. 마지막으로 T와 B 세포들은 자기 조직을 공격하게 되어 자기 면역 질환을 유도하게 된다. 따라서 HSPB1의 세포막 이동은 자기 면역 질환에서 중요한 역할을 하며 이러한 질병에서 세포막에 위치하는 HSPB1의 양이 증가하는 것으로 알려져 있어 자기 면역 질환의 타깃으로 고려될 수 있다. 그리고 HSPB1의 세포막 이동을 막는 역할을 한다는 단백질로써 AIMP1이라는 단백질이 알려져 있다. 정상적인 상태에서 AIMP1은 HSPB1과 결합하여 소포체에 머무르도록 도와주는 역할을 하지만 자기 면역 질환에서는 AIMP1과 HSPB1의 결합이

분리되어 있다. 그러기 때문에 AIMP1의 역할은 자기 면역 질환에서 HSPB1의 세포막 이동을 조절함으로써 매우 중요한 역할을 한다.

따라서 우리는 AIMP1을 기능적으로 모방하는 화합물을 스크리닝 하였다. 화합물들 중 HSPB1의 세포막 레벨을 가장 많이 낮추고 독성이 없는 화합물로써 GPM1이 선정이 되었다. 자기 면역 질환이 유도된 쥐를 이용한 생체 내 실험에서 GPM1 처리 군에서 질병이 완화된 것을 확인할 수 있었다. 하지만 화합물의 안정성에 있어서 GPM1은 매우 불안정하였다. 불안정한 화합물은 채 내에서 약효가 나타나기 전에 분해되어 버리기 때문에 화합물의 발달 과정에 있어서 매우 중요한 단계이다. 따라서 우리는 GPM1과 유사한 구조를 갖은 파생물들을 합성하였다. 그리고 파생물들 중 가장 효과적으로 HSPB1의 세포막 레벨을 감소 시키는 화합물을 선정하여 KR-S-026S라는 화합물이 선정되었다. 이 화합물은 세포에서 HSPB1의 세포막 레벨을 감소시키는 것 뿐만 아니라 자기 면역 질환이 유도된 쥐에서, GPM1 보다 더욱 극적인 질환 개선 효과를 보였다. 뿐만 아니라, KR-S-026S는 GPM1과는 다르게 높은 안정성을 보였고 삼투성과 독성효과를 보았을 때에도 매우 양호한 화합적 요소를 갖추고 있다. 따라서 AIMP1의 역할을 모방하며 HSPB1의 세포막 이동을 막는 KR-S-026S는 자기 면역 질환 치료에 매우 촉망되는 화합물이다.

주요어 : 자가면역질환, HSPB1, AIMP1, GPM1, 의약품