Allosteric Inhibition of the Tumor-Promoting Interaction Between Exon 2–Depleted Splice Variant of Aminoacyl–Transfer RNA Synthetase-Interacting Multifunctional Protein 2 and Heat Shock Protein 70

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Received May 27, 2021; accepted September 1, 2021

ABSTRACT

Although protein-protein interactions (PPIs) have emerged as an attractive therapeutic target space, the identification of chemicals that effectively inhibit PPIs remains challenging. Here, we identified through library screening a chemical probe (compound 1) that can inhibit the tumor-promoting interaction between the oncogenic factor exon 2–depleted splice variant of aminoacyl–transfer RNA synthetase-interacting multifunctional protein 2 (AIMP2-DX2) and heat shock protein 70 (HSP70). We found that compound 1 binds to the N-terminal subdomain of glutathione S-transferase (GST–N) of AIMP2-DX2, causing a direct steric clash with HSP70 and an intramolecular interaction between the N-terminal flexible region and the GST–N of AIMP2-DX2, which induces masking of the HSP70 binding region during molecular dynamics and mutation studies. Compound 1 thus interferes with the AIMP2-DX2 and HSP70 interaction and suppresses the growth of cancer cells that express high levels of AIMP2-DX2 in vitro and in preliminary in vivo experiment. This work provides an example showing that allosteric conformational changes induced by chemicals can be a way to control pathologic PPIs.

SIGNIFICANCE STATEMENT

Compound 1 is a promising protein–protein interaction inhibitor between AIMP2-DX2 and HSP70 for cancer therapy by the mechanism with allosteric modulation as well as competitive binding. It seems to induce allosteric conformational change of AIMP2-DX2 proteins and direct binding clash between AIMP2-DX2 and HSP70. The compound reduced the level of AIMP2-DX2 in ubiquitin-dependent manner via suppression of binding between AIMP2-DX2 and HSP70 and suppressed the growth of cancer cells highly expressing AIMP2-DX2 in vitro and in preliminary in vivo experiment.

Introduction

Since many physiologic and pathologic cellular events are controlled by protein-protein interactions (PPIs), modulating these PPIs promises attractive ways to develop drugs for many diseases (Nero et al., 2014; Ivanov et al., 2013). Although extensive efforts have been made to develop PPI inhibitors that target oncogenic interactions, most have failed due to a lack of structural understanding of the binding interface and limited efficacy.
using small molecules (Nero et al., 2014; Ran and Gestwicki, 2018; Li et al., 2017). Recently, a few meaningful advances in PPI inhibitor development have been reported using peptide mimetic molecules with molecular weights over 500 Da, but these molecules have shown limitations due to their molecular features including solubility, stability, and cell penetration (Nero et al., 2014; Wojcik and Berlicki, 2016; Totti et al., 2019).

Aminoacyl–transfer RNA synthetase-interacting multifunctional protein 2 (AIMP2) functions as a scaffold for the assembly of the multisynthetase complex (MSC) (Kim, S. et al., 2011). AIMP2 dissociates from the MSC upon UV damage and transforming growth factor-β, tumor necrosis factor-α, and Wnt signaling and functions as a tumor suppressor via its interaction with p53, FUSE-binding protein, Smurf2, tumor necrosis factor receptor–associated factor 2, and dishevelled-1 (Han et al., 2008; Kim et al., 2003; Kim et al., 2016; Choi et al., 2009). DX2 also independently shows oncogenic functions (Han et al., 2008; Kim et al., 2003; Kim et al., 2016; Choi et al., 2009). Validation of DX2 and HSP70 as an effective can-
The puriﬁcation of AIMP2-DX2-HSP70 interaction was conducted for the minimized structures for 25 ps with a 1 fs time step at 303.15 K. The LINCS algorithm (Hess, B. et al., 1997) was used to constrain the bonds involving hydrogen atoms by their equilibrium bond lengths. Finally, the production runs were performed at a temperature of 303.15 K and a pressure of 1 bar by constant particle number, pressure, and temperature dynamics achieved with the Nose-Hoover thermostat (Hoover, 1985) and Parrinello-Rahman (Parrinello and Rahman, 1981) barostat. The length of the time step was set to 2 fs for the production runs, and the trajectory was saved every picosecond. The cutoff values of short-range electrostatic interactions and van der Waals interactions were set to 12 Å. The particle mesh Ewald method (Essmann et al., 1995) was used for long-range electrostatic interactions. To analyze protein-ligand interactions, the snapshot with the lowest nonbonded energy between the protein and ligand and a highly populated conformation of the ligand during the last 200 ns was selected as a representative structure. To avoid the ligand escaping from the binding site into the bulk solvent region, an upper-wall restraint force was applied to the system when the distance between the center of mass of the GST-N and the center of mass of compound 1 was greater than the cutoff limit (d_{up}) of 12 Å. For the wall, the harmonic potential was set with a force constant \( k = 200 \text{ kJ/mol-nm}^{-2} \).

\[
\text{Bias}_{up} = \begin{cases} 
0 & d < d_{up} \\
 k \cdot (d - d_{up})^2 & d \geq d_{up}
\end{cases}
\]

The g_mmpbsa tool (Kumari et al., 2014) was used to calculate the binding energies of the system with the molecular mechanics Poisson-Boltzmann surface area method. The calculation was performed with 3000 snapshots extracted from the 300 ns trajectory every 100 ps. We calculated the final binding free energy and the energetic contribution of each residue by python scripts “MmPbSaStat.py” and “MmPbSaDecomp.py”, which are the part of g_mmpbsa.

High-Throughput Screening

DX2 and HSP70 were cloned into pBtT1.1-N(TK/LgBiT) and pBtT2.1-N(TK/SmBiT), respectively. LgBiT fused with protein kinase A type 2A regulatory subunit (PRKAR2A) and SmBiT fused with protein kinase A catalytic subunit (PRKACA) were obtained from Promega. CHO-K1 cells transfected with LgBiT-DX2 and SmBiT-HSP70 were seeded into 96-well white-bottom plates. After incubation for 24 hours, the cells were treated with 6186 chemicals [5 \times 10^5 M] each, Korea Chemical Bank (KCB) in serum-free media for 4 hours. Luciferase activity was detected using a NanoBiT assay system following the manufacturer’s protocol (Promega). Ninety-nine chemicals showing over 60% inhibition at 5 \times 10^5 M were subjected to secondary screening using LgBiT-PRKAR2A and SmBiT-PRKACA for negative screening via the same experimental procedure as above. Ten compounds that showed no effect on the negative screening were subjected to a third screening involving in vitro pull-down assays and cell viability assays using DX2-inducible cell lines.

Surface Plasmon Resonance

To measure the binding afﬁnity of compound 1 to the DX2 protein, we used a Reichert SR7500DC instrument (Reichert Technologies, Depew, NY). Thioredoxin-tagged DX2 and thioredoxin proteins were immobilized at levels of 11,500 and 4000 response units, respectively, on a carbodiimide dextran chip (Reichert, Depew, NY) with buffer containing 10 mM sodium acetate (pH 5.5). Compound 1 (1.5–50 \mu M) in 2% DMSO-containing PBS binding buffer (pH 7.4) flowed at a rate of 30 \mu l/min. Sensorgrams were ﬁtted to a simple 1:1 Langmuir interaction model (A + B = AB) using the Scrubber 2.0 analysis program (BioLogic Software, Australia, and Kaleida Graph Software, Australia) to calculate the values of \( K_a \) and \( K_d \).
Fluorescence-Based Equilibrium Binding Assay

To determine the binding affinity of compound 1 to the DX2 protein, we performed fluorescence-based equilibrium binding experiments. All titration experiments were conducted at 20 °C using a Jasco FP 6500 spectrofluorometer (Easton, MD). Purified human tag-free DX2 proteins were equilibrated with various concentrations of ligand 1 before fluorescence emission was measured. Ligand stock solutions were titrated into a protein sample dissolved in phosphate buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4. Protein samples were excited at 280 nm, and the decrease in fluorescence emission upon ligand binding was measured at 335 nm as a function of the ligand concentration. All titration data were fitted to a hyperbolic binding equation to obtain the $K_a$ values.

Biomolecular Fluorescence Complement Assay

For the biomolecular fluorescence complement (BiFC) assay, DX2 and HSP70 were cloned into the EcorRI/Xhol sites of the pCE-BiFC-VN173 and pCE-BiFC-VC155 vectors to tag Venus-N173 and Venus-C155, respectively. CHO cells expressing VN173-DX2 and VC155-HSP70 were treated with compound 1 in a dose-dependent manner for 4 hours. After incubation, the cells were washed with cold PBS three times and fixed with 100% cold methanol for 10 minutes, and the nuclei were stained with 4′,6-diamidino-2-phenylindole for cell counting. The BiFC signal was determined by confocal microscopy, and signal-positive cells were counted in the same number of independent experiments. The experiment was independently repeated three times.

In Vitro Pull-Down Assay

DX2 was cloned into the EcorRI/Xhol sites of the pGEX4T-1 vector to express GST-tagged DX2. The purified GST or GST-DX2 proteins were mixed with the HSP70 protein (ADI-NSP-555, Enzo) in 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, and protease inhibitor (Calbiochem), followed by treatment with or without compound 1 for 4 hours. After incubation, proteins coprecipitated with beads were washed three times with incubation buffer excluding 0.5% Triton X-100, subjected to SDS-PAGE, and detected by Coomassie staining. To examine the binding of compound 1 to the DX2 or HSP70 proteins, we incubated compound 15 with 100 μg of tag-free DX2 or HSP70 proteins as starting amounts for 12 hours in incubation buffer and performed the same procedure described above. Biotin alone was used as a negative control compound for product 15.

Immunoprecipitation

The cells were lysed with 50 mM Tris-HCl (pH 7.4) lysis buffer containing 100 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM EDTA, and protease inhibitor (Calbiochem). Two hundred micrograms of total cell lysates as starting amounts were mixed with specific antibodies and preincubated with agarose beads for 12 hours against the proteins of interest. After mixing, proteins coprecipitated with agarose beads were gently washed with cold lysis buffer excluding 0.5% Triton X-100 and 0.1% SDS three times and separated by SDS-PAGE, and detected by Coomassie staining. Proteins were detected by immunoblotting using specific antibodies against the proteins of interest. To determine the compound 1–mediated ubiquitination of DX2, the cells were treated with compound 1 and MG-132 (50 μM) for 12 hours. The cells were then lysed with 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl, 0.5% Triton X-100, 1% SDS, 10% glycerol, 1 mM EDTA, protease inhibitor (Calbiochem), and the pan-deubiquitinase inhibitor PR-619 (Sigma-Aldrich), boiled at 100 °C for 10 minutes, and then diluted in lysis buffer including 0.1% SDS (Crespo-Yáñez et al., 2018). The following experimental procedures were performed as described above.

Xenograft

H460 cells (1 × 10⁷) were subcutaneously injected into the left and right sites of the backs of 7-week-old female BALB/cSLnu/nu mice (Central Laboratory Animal Inc., Korea). Five or 10 μg/kg compound 1 was intraperitoneally administered to the mice ($n = 3$ mice per group) every other day for 10 days. The volumes of the embedded tumors and body weights were measured five times over the experimental period. After 10 days, all mice were sacrificed, and the embedded tumors were excised. The weights of the harvested tumors were measured, and photos of the whole-body embedded tumors and excised tumors were taken. To determine the level of the DX2 protein in the embedded tumors, the excised tumors were homogenized and lysed in PBS containing 1% Triton X-100, 0.1% SDS, and protease inhibitor (Calbiochem). Supernatants after centrifugation at 15,000 rpm at 4 °C for 30 minutes were subjected to SDS-PAGE and immunoblotting using a specific antibody against DX2. Animal experiments were approved and performed in compliance with the University Animal Care and Use Committee guidelines at Yonsei University.

Three-Dimensional Culture

H460 cells in two-dimensional culture conditions were detached from the culture plate using Accumax (EMD Millipore). After cell counting using the disposable hemocytometer C-Chip (INCYTO), we diluted the cells to a concentration of 5000 cells per 100 μl in medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were then seeded onto ultralow attachment 96-well three-dimensional (3D) culture plates (Corning). After incubation for 48 hours, the spheres and cancer cells cultivated under 3D culture conditions were treated with compound 1 for 72 hours, and cell viability was determined using CellTiter-Glo 3D cell viability assay (Promega) following the manufacturer's instructions. The experiment was independently repeated three times.

Anchorage-Independent Colony Formation Assay

To define stable cells, we introduced FLAG-tagged DX2 wild type (WT) and mutants into A549 or H460 cells for cultivation in medium containing G418 (800 μg/ml, #G0175, Duchefa) for the selection of cells stably expressing the ectopically introduced plasmids. After culture for two weeks, the settled colonies were selected, and the level of overexpressed DX2 was determined by immunoblotting using an anti-FLAG antibody. Each of the stable cells was subjected to an anchorage-independent colony formation assay using a cell transformation assay kit (Cell Biolabs, Inc.) following the manufacturer’s instructions. The number of colonies stained by hematoxylin (Sigma) was counted. The experiment was independently repeated three times.

Cell Viability Assay

DX2-inducible or empty vector–inducible A549 isogenic cells (2 × 10⁶ cells per well) were cultured in 96-well flat-bottom plates and pre-treated with dox (Sigma) to induce DX2 expression. After pretreatment with dox for 24 hours, the cells were cultured in serum-free medium diluted with chemicals for 48 hours. To check the resistance vulnerability, H460 cells pretreated with compound 1 (0.5 μM) for three weeks were treated with chemical in a dose-dependent manner for 72 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml, Sigma) was added to each well followed by incubation for 1.5 hours. After the medium was discarded, the precipitated formazan crystals were solubilized with DMSO (Duchefa), and the absorbance was measured at 560 nm using a microplate reader (Sunrise, Tecan). All of the experiments were repeated three times independently.

Refolding Assay

Using a Protein Refolding Kit (Boston Biochem) following the manufacturer’s instructions, the effects of the compound on the protein refolding process were examined. Purified proteins HSP70 and heat shock protein 40 and Glow-Fold Substrate were mixed with compound 1 in reaction buffer containing Mg²⁺ ATP, and all of the reagents were incubated for 5 minutes at room temperature. To unfold the substrate,
the mixture was heated at 50°C for 8 minutes and left on ice for 10 minutes. The refolding reaction was conducted at 30°C for 1.5 hours, and the luminescence signal from the refolded substrate was measured with a plate reader (GloMax, Promega). Experiments were independently repeated three times.

Reverse Transcription PCR

Total RNA extracted from H460 cells using the RNeasy Mini Kit (Qiagen) was used for reverse transcription PCR with deoxynucleotide triphosphates, random hexamers, and Moloney murine leukemia virus. To determine the mRNA expression of DX2, AIMP2, and actin, we used 2 μl of cDNA in the PCR with the following specific primers: DX2, CTGGGCAAGTGCAGGATTAGGGG and AAGTGAAATCCCA GCTGATAG; AIMP2, ATGCCGATGTACCAGGTAAAG and CTTAA GGAAGCTTGAGGGCCGT; actin, CCTTCCTGGGCATGGAGTCCT GCTGATAG; AIMP2, ATGCCGATGTACCAGGTAAAG and CTTAA GGAAGCTTGAGGGCCGT; actin, CCTTCCTGGGCATGGAGTCCT

Statistics

Statistical tests were performed with Prism (GraphPad). A value of \( P < 0.05 \) was considered statistically significant. All error bars represent S.D. For quantitative data, statistical parameters are reported in the figure legends.

Results

Selection and Characterization of a Chemical Probe.

Our previous report demonstrated that DX2 is stabilized by its interaction with HSP70 and that inhibition of the interaction between these two proteins reduces DX2-mediated cancer proliferation (Lim et al., 2019). Therefore, we searched for an inhibitor of this interaction to suppress DX2-dependent cancer progression using a luciferase-based complementation assay system (Dixon et al., 2016) established for high-throughput screening (Fig. 1A). In a primary screening using 6186 compounds from KCB, 99 compounds showed over 60% inhibition at 5 μM (Fig. 1B, upper; Supplemental Fig. 1A). The specificity of these 99 compounds for DX2 was determined using the binding pair PRKACA and PRKAR2A (Dixon et al., 2016), and 10 compounds were found to specifically suppress the binding between DX2 and HSP70 but not PRKACA-PRKAR2A (Fig. 1, B and C). Further validation of the 10 compounds was carried out using in vitro pull-down and cell viability assays. An in vitro pull-down assay using the purified HSP70 and GST-DX2 proteins demonstrated that compound 1 significantly inhibited the interaction of these two proteins (Fig. 1D). Furthermore, compound 1 suppressed DX2-dependent cell proliferation in a DX2-inducible system (Fig. 1, B and E, bottom). Using this three-step screening procedure, we identified compound 1 as a potential PPI inhibitor of DX2 and HSP70 (Fig. 1F).

Additionally, the usefulness of compound 1 as a starting point for the optimization of the discovery of an anticancer agent was validated through the examination of preliminary structure-activity relationships on a series of 8 compounds in KCB libraries (Table 1). There were eight compounds (2–9) with enedione moieties in the KCB whose biologic activities on DX2 were evaluated at 5 μM. Compounds 2–4 substituted at the 3 or 4 positions of the R² benzene ring showed 66%, 76%, and 53% inhibition, respectively. Replacement of the ester at R² with amide 5 (56% inhibition), arylketo 8 (58% inhibition), and alkylketo 9 (58%) retained the compound’s ability to inhibit the interaction between DX2 and HSP70. Additionally, the introduction of biaryl groups (compounds 8 and 9) at R³ was tolerable for activity, and a variety of variations at R³ might be possible. Additionally, compound 1 can form a covalent bond with DX2 by a nucleophilic addition. Then, we examined compounds 10 and 11 without the site required for nucleophilic addition among the KCB libraries, resulting in retention of the inhibitory activities (Table 2). Based on this preliminary structure-activity relationship study, we synthesized compound 1 and its biotinylated derivative compound 15 by conventional procedures (Miyatake-Ondozabal and Barrett, 2013; Sun et al., 2009; Ackerman et al., 2018) (Fig. 2) for biologic evaluation, including dose dependency, functional assays in cell lines, and studies on the mode of action. Because compound 4 with a (4-phenylpropoxy)benzyl group at R¹ was active (53% inhibition) in the screening, we primarily attempted to prepare compound 15, where the 4-position of the benzene ring at R¹ is biotinylated. Fortunately, compound 15 showed similar activity to compound 1 and was used to study the mode of action (Supplemental Fig. 3, A and B). In summary, compounds with three or four substituents on the benzoyl moiety retained activity, and the introduction of a biaryl group at C3 instead of a benzene group was tolerable for efficacy. The replacement of the ester group with a more metabolically stable keto or amide group also maintained activity, suggesting that the structure of compound 1 can be broadly modified to improve efficacy and safety and should be useful as a chemical probe to elucidate the mode of action on DX2.

We examined the inhibitory activity of compound 1 for further validation as a chemical probe (Fig. 3). The IC₅₀ value of compound 1 was determined to be 2.9 μM for inhibiting the binding between DX2 and HSP70, but the IC₅₀ was over 100 μM for the inhibition of PRKACA and PRKAR2A binding (Fig. 3A), indicating that compound 1 specifically interrupted the binding between DX2 and HSP70. Next, time-dependent binding inhibition of compound 1 was also confirmed (Supplemental Fig. 2A). Compound 1 did not show time-dependent inhibitory activity on the binding between DX2 and HSP70, which indirectly indicates that inhibition of DX2 by compound 1 is not based on covalent modification. To exclude the possibility of artifacts of our screening system, we determined the effects of compound 1 in a complementary fluorescence-based assay, and the same result was observed (Supplemental Fig. 2B). The endogenous interaction of the two proteins was found to be abrogated by treatment with compound 1 by reciprocal immunoprecipitation, implying its physiologic function (Fig. 3B). We also examined whether depletion of compound 1 induces the binding of DX2 and HSP70. The compound 1-mediated decrease in the binding of the two proteins was recovered by adding fresh medium (Fig. 3D), indicating that compound 1 inhibits the binding of the two proteins. Together, these observations indicate that compound 1 specifically interferes with the binding of DX2 with HSP70.

Because inhibition of the interaction between DX2 and HSP70 has been reported to lead to the turnover of DX2 via ubiquitination (Lim et al., 2019), we examined whether compound 1 destabilizes the DX2 protein. First, we monitored the
The signif

... interferences with HSP70 access, resulting in DX2 turnover. 

cal for the compound 

expression, and there was no decrease in DX2 despite treat-
down, the level of DX2 basically increased without HSP70 

of DX2 was also investigated by knockdown of Siah1 via its spe-

(Fig. 3E; Supplemental Fig. 2C, upper). We also determined the 

are AIMP2-interacting proteins in the MSC (Kim et al., 2011) 

Lim et al., 2019), and decreased binding to HSP70 (Fig. 3F), 

by its increased binding to Siah1, an E3 ligase against DX2 

treatment with compound 

3E). We also observed increased ubiquitination of DX2 upon 

reduced the DX2 protein level but not the mRNA level (Fig. 

Fig. 1. Identification of compound 1, a PPI inhibitor of DX2 and HSP70. (A) NanoBiT-based screening system. (B) Flowchart of the binding inhibi-
tor screening. Ninety-nine chemicals showing over 60% inhibition at 5 μM were selected from the primary screening. To examine the specificity, we used the binding pair PRKACA and PRKAR2A as a secondary screening, and 10 chemicals were chosen. (C) Comparison of the inhibitory effects of the 10 chemicals (10 μM) on the binding of DX2-HSP70 and PRKACA-PRKAR2A via the NanoBiT assay. (D) Inhibitory efficacy of the chemicals on the direct binding of DX2 and HSP70 determined by an in vitro pull-down assay. Quantified values of binding are presented at the bottom of the gel image. (E) Cell viability was determined upon treatment with the 10 chemicals (10 μM) in DX2-inducible A549 cells. DX2 was induced by treatment with dox. (F) Structure of compound 1. The experiments in (C) and (E) were independently repeated three times with error bars denoting S.D. Student’s two-tailed t test was performed for statistical analysis. *P < 0.05; **P < 0.01; ***P < 0.005. EV, empty vector.

protein and mRNA levels of DX2 upon treatment with different 

doses of compound 1 and found that compound 1 specifically 

reduced the DX2 protein level but not the mRNA level (Fig. 

3E). We also observed increased ubiquitination of DX2 upon 

treatment with compound 1, which was found to be mediated 

by its increased binding to Siah1, an E3 ligase against DX2 

(Lim et al., 2019), and decreased binding to HSP70 (Fig. 3F), 

implying that compound 1 induces the binding of Siah1 to DX2 

by interfering with HSP70 access, resulting in DX2 turnover. 

The significance of Siah1 on compound 1–mediated degradation 

of DX2 was also investigated by knockdown of Siah1 via its spe-
cific small interfering RNA (siRNA). When Siah1 was knocked 
down, the level of DX2 basically increased without HSP70 expression, and there was no decrease in DX2 despite treat-

ment with compound 1 (Fig. 3G), suggesting that Siah1 is criti-

cal for the compound 1–dependent decrease in DX2. We further 

validated that compound 1 did not affect the protein level of 

AIMP2 or other aminoacyl–transfer RNA synthetases, which 

are AIMP2-interacting proteins in the MSC (Kim et al., 2011) 

(Fig. 3E; Supplemental Fig. 2C, upper). We also determined the 

level of CDK4, a known downstream molecule of HSP70 (Lim 
et al., 2019), and the protein-folding activity of HSP70 upon 
treatment of compound 1 to elucidate the possibility that com-

pound 1 could inhibit the interaction of DX2 and HSP70 based 

on the interaction with HSP70. We confirmed that there was 

no effect on the level of CDK4 or on the folding activity of 

HSP70 (Supplemental Fig. 2, C, bottom, and D), suggesting the 
PPI inhibition based on the interaction with DX2, not on the 
interaction with HSP70 of compound 1. Altogether, these data 
led to the conclusion that compound 1 inhibited the interaction 

between DX2 and HSP70, resulting in DX2 degradation via 

recruitment of Siah1.

**Mode of Action Study of Compound 1.** To unveil the 

mode of action of compound 1, we used biotin-conjugated com-

pound 15. First, we compared the function of compound 15 

with that of the original compound 1. Treatment of H460 cells 
with compound 15, similar to compound 1, reduced the binding 
of HSP70 to DX2 and destabilized the DX2 protein (Supplemental Fig. 3, A and B), suggesting that the conjugated 

biotin compound retained its function. We then examined
whether compound 15 bound directly to DX2 or HSP70 using an in vitro pull-down assay and observed direct binding between the compound and DX2, not HSP70 (Fig. 4A). We further performed a competitive binding assay using compounds 15 and 1 to determine the specific binding of the chemical to DX2 or HSP70. The DX2 protein, not HSP70, was pulled down by compound 15, and the amount of coprecipitated DX2 was reduced by the addition of compound 1, implying the specific binding of compound 1 to DX2 and not HSP70 (Fig. 4B). We also confirmed the competitive binding of compounds 1 and 15 to endogenous DX2 proteins as above via the treatment of H460 cells with the two compounds (Fig. 4C). Next, we measured the binding affinity of compound 1 to DX2 via a surface plasmon resonance assay and determined a $K_D$ value of 12 μM (Fig. 4D), which was similar to the IC₅₀ value obtained for the inhibition of the DX2-HSP70 interaction (Fig. 3A), implying the significance of the binding of the compound to DX2 for inhibition of the DX2-HSP70 interaction. We further confirmed the binding affinity via a fluorescence-based equilibrium binding experiment (Breen et al., 2016) and obtained a binding affinity similar to that stated above (Supplemental Fig. 3C). The results from two other binding assays suggested that direct binding of compound 1 to DX2 could affect protein function. We also determined which domain of DX2 bound to compound 1. DX2 was divided into the NFR, GST domain (GST), and C terminus of the GST domain (Lim, S. et al., 2019) (Fig. 4E, right), and each fragment was mixed with compound 1. The in vitro pull-down assay using streptavidin-Sepharose beads revealed that the NFR and GST-N, a region known for binding with HSP70 (Lim et al., 2019), were bound by compound 1 (Fig. 4E, left), further validating compound 1 as a PPI inhibitor. All of the binding data imply that compound 1 inhibits the interaction between DX2 and HSP70 via direct binding with DX2.

To investigate the binding mode of compound 1 with DX2, we performed a molecular modeling study because the disordered NFR was not visible in other structural assays (Lim et al., 2019). Due to the disordered nature of the NFR, the replicated run of 300 ns MD simulations was conducted three times. The initial pose of compound 1 for MD simulation with the highest docking score of 34.94 was obtained from molecular docking simulation using CDOCKER. To increase the chance of determining the binding event and to properly adjust the position of compound 1 to fit into the GST-N, we performed MD simulations with the upper-wall restraint force. Because ligand binding is a rare event in the simulation considering the protein flexibility in the solvent system, this binding event was observed in only one trajectory among three replicated systems with the upper wall. From this trajectory, we found that compound 1 sought the proper hydrophobic fit into the GST-N for the beginning 100 ns (Fig. 5A). The DX2 was well stabilized at around 0.45 nm of Cα-root mean square deviation (Supplemental Fig. 4). Although the Cα-root mean square deviation values are relatively fluctuating during beginning 200 ns, the binding conformation of compound 1 was well maintained in the GST-N for the last 200 ns, showing stable interactions with three significant residues, Y47, N56, and K129 (Fig. 5B). Cα-RMSF on DX2 represented the significant conformational change of the N-terminal region during the beginning 200 ns simulation time. Not only the most residues in binding region but also the N-terminal region, which is generally fluctuating, seemed to be very stable during the last 100 ns (Supplemental Fig. 4B). The snapshot at 211.3 ns, having the lowest nonbonded energy (Supplemental Fig. 4C) between DX2 and a highly populated conformer of compound 1 during the last 200 ns (Supplemental Fig. 4A, inset), was selected as a representative structure for further interaction analysis. For the energetics of the binding mode between compound 1 and DX2, we calculated the total binding energy using the molecular mechanics Poisson-Boltzmann surface area method. The binding structure was

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Preparation of the biotinylated compound 15 reagents and conditions: (a) BBr₃ 2 equiv, CH₂Cl₂, −15°C, 1.5 hours, 82%; (b) piperidine 0.5 equiv, benzaldehyde 1.2 equiv, toluene, reflux, 43 and 25%; (c) (COCl)₂ 1 equiv, cat. dimethylformamide, CH₂Cl₂, 0°C; (d) pyridine 1 equiv, CH₂Cl₂, room temperature, overnight, 10%.
stabilized from initial $-21.33 \text{ kJ/mol}$ to an average $-37.21 \pm 13.08 \text{ kJ/mol}$ of total binding energy over the last 100 ns (Supplemental Fig. 4C). The energy of representative structure at 211.3 ns was $-46.31 \text{ kJ/mol}$. To validate the important amino acids in the protein-ligand interaction, per-residue energy decomposition analysis was performed with the 300 ns simulation trajectory. From this analysis, we found that the three key residues (Y47, N56, and K129) are located at the lowest nonbonded interaction energies (Supplemental Fig. 4D), which is well correlated with the mutation analysis. The carbonyl groups of the benzoyl and ester moieties on compound 1 interact with the side chain amines of N56 and K129, respectively, by H-bonding (Supplemental Fig. 4B). There was a $\pi-\pi$ interaction between the styrene of compound 1 with Y47 of DX2. To validate the MD simulation results, we generated alanine mutants of the DX2 amino acids that were suggested to be critical for the binding of compound 1. First, we examined the binding of the alanine mutants to the chemicals using compound 15 via an in vitro pull-down assay. DX2 WT and most of the mutants showed strong binding, but the binding of the DX2 mutants Y47A, N56A, and K129A to compound 15 was significantly reduced (Fig. 5C). Next, we determined whether compound 1 diminished the binding between HSP70 and DX2. Based on the immunoprecipitation results using the expressed mutants in 293T cells, we determined that compound 1 abrogated the interaction between HSP70 and DX2. On the immunoprecipitation results using the expressed mutants in 293T cells, we determined that compound 1 abrogated the interaction between HSP70 and DX2.

### TABLE 1
Inhibitory effects of derivatives of compound 1 on the DX2-HSP70 and PRKACA-PRKAR2A interactions.

| Compound | R<sup>1</sup> | R<sup>2</sup> | R<sup>3</sup> | % Inhibition (5μM) |
|----------|--------------|--------------|--------------|-------------------|
| 1        | O           | O            | O            | 59.95 ± 2.66      |
| 2        | O           | O            | O            | 66.12 ± 4.18      |
| 3        | O           | O            | O            | 76.31 ± 2.36      |
| 4        | O           | O            | O            | 53.27 ± 6.72      |
| 5        | O           | O            | O            | 55.69 ± 5.40      |
| 6        | O           | O            | O            | 34.45 ± 6.31      |
| 7        | O           | O            | O            | 49.43 ± 4.33      |
| 8        | CF<sub>3</sub> | O            | O            | 57.53 ± 3.11      |
| 9        | CH<sub>3</sub> | CH<sub>3</sub> | O            | 57.53 ± 3.11      |

### TABLE 2
Inhibitory effects of quaternary derivatives of compound 1 on the DX2-HSP70 interactions.

| Compound  | % Inhibition (5μM) |
|-----------|--------------------|
| Michael Acceptor | 59.95 |
| Quaternary carbon 1 | 63.43 |
| Quaternary carbon 11 | 60.87 |
Chemical Inhibition of AIMP2-DX2-HSP70 Interaction

In this study, we characterized the binding between DX2 and HSP70 and the binding between PRKACA and PRKAR2A upon treatment with compound 1. The experiments were independently repeated three times with error bars denoting the S.D. Student's two-tailed t test was performed for statistical analysis (**P < 0.005). Inhibitory effects of compound 1 on the endogenous binding of DX2 with HSP70. HSP70 (left) or DX2 (right) from the same cells were precipitated with a specific antibody. Actin was used as a loading control. (C) In vitro pull-down assay showing the inhibitory effects of compound 1 on the interaction between DX2 and HSP70. Quantified values of binding are presented at the bottom of the gel image. (D) H460 cells were treated with compound 1, and then the chemicals were removed by addition of fresh medium at the indicated time. The cells were precipitated by using an anti-HSP70 antibody. (E) Protein and mRNA levels from H460 cells treated with compound 1. (F) Ubiquitination assay of H460 cells treated with compound 1. (G) Significance of Siah1 on HSP70- or compound 1-dependent DX2 levels. H460 cells knocked down by introducing specific Siah1-specific siRNA (si-Siah1) were ectopically expressed with HSP70 (upper) or treated with compound 1 (bottom). The quantification of the protein level for each experiment is presented at the bottom of each blot. EV, empty vector; IP, immunoprecipitation; WCL, whole cell lysates; Ub, ubiquitin.

From the ubiquitination assay, we observed that DX2 WT was ubiquitinated upon treatment with compound 1, resulting in a decreased level of DX2, but DX2 ubiquitination and DX2 protein expression of the tested mutants were not affected by treatment with compound 1 (Fig. 5E). Together, these observations indicate that the pocket surrounded by Y47, N56, and K129 in the NFR and GST-N of DX2 is critical for binding with compound 1.

The NFR was shown to be involved in the binding of compound 1 to the GST-N of DX2 (Fig. 6; Supplemental Movies 1 and 2). Among the three significant interacting residues, Y47 is located in the NFR and has a stable hydrophobic interaction with compound 1. Moreover, a stable hydrophobic interaction between L50 and T117 was shown in the chemically bound system but not in the apo system (Fig. 6A), indicating that the disordered NFR containing L50 has no chance to interact with the GST-N containing T117 without compound 1 binding. Interestingly, along with being involved in the binding of compound 1, the NFR binds with the surface of DX2 through intramolecular hydrophobic interactions, meaning that the binding of the NFR to GST-N could mask the L97 and T117 residues, which are significant residues for the binding to HSP70 (Lim et al., 2019). In particular, hydrophobic interactions of G40, Y47, and L50 in the NFR with L97 and T117 on the surface of GST-N were observed in the representative structure of the system (Fig. 6B). The induced binding of the NFR and GST-N in the presence of compound 1 seemed to significantly increase the area of the bumping region to interfere with the interaction between DX2 and HSP70 (Fig. 6C). These analyses revealed that the interaction of compound 1 in the pocket of DX2 surrounding Y47, N56, and K129 induces a sterical clash against HSP70 by direct binding interference and a conformational change in DX2, resulting in turnover of the DX2 protein.

**Inhibition of Cancer Development via Compound 1.** Because compound 1 leads to the degradation of DX2 via inhibition of binding with HSP70, we examined whether compound 1 suppresses cancer cell proliferation. H460 cells showing a high level of DX2 were treated with compound 1, and cell viability was shown to decline in a dose-dependent manner (Fig. 7A, red circle). We further determined whether compound 1–mediated suppression of cell viability was dependent on the level of DX2. After examining the levels of DX2 and HSP70 in various lung cells (Supplemental Fig. 5A), the cells were subjected to a cell viability assay upon treatment with compound 1, and the 50% inhibition concentration (EC_{50}) of cell proliferation was calculated. The cells with the highest levels of DX2, H460 and H358, showed the lowest EC_{50} values among the tested cell lines (Fig. 7A, red), and the EC_{50} values were very similar to the IC_{50} and KD previously determined for the inhibition of binding between DX2-HSP70, implying that suppression of the interaction between DX2 and HSP70...
using compound 1 leads to cell death. Normal (WI-26) and cancerous (H1650) lung cells, those with the lowest level of DX2, were not affected by compound 1 (Fig. 7A, yellow), further suggesting that DX2 is required for compound 1 to affect cell death. The significance of the DX2 level on compound 1-meditated suppression of cell proliferation was also determined in DX2-knockdown H460 and H358 cells by introducing specific siRNA (Supplemental Fig. 5B). A time-dependent inhibitory effect of cancer cell proliferation via compound 1 was also confirmed (Supplemental Fig. 5C). Before determining the in vivo efficacy of this compound, we treated H460 cells with compound 1 in a 3D cell culture system. Compound 1 dose-dependently decreased the proliferation of H460 spheroids in the 3D environment (Fig. 7B), implying that compound 1 could function in an in vivo model. The IC50 of compound 1 in 3D culture was much higher than that in the two-dimensional system, which might be due to the poor penetration of compound into the spheroids as mentioned in publications (Park et al., 2016; Langhans, 2018). We further validated the effects of compound 1 on cancer cell progression in vivo via a xenograft model using H460 cells. Compound 1 was intraperitoneally injected at doses of 5 and 10 mg/kg, and tumor growth and body weight were monitored over the experimental periods. Even though these data are preliminary due to small number of mice per each group, administration of compound 1 significantly reduced tumor size in a dose-dependent manner with little effect on body weight (Fig. 7C, left; Supplemental Fig. 5D). A reduction in tumor weight was also observed (Fig. 7C, middle). We further validated the endogenous levels of the DX2 and HSP70 proteins in the excised tumors from the mice. The level of DX2, but not HSP70, was found to be decreased after injection of compound 1, and the quantified level is shown as a graph excluding the confusion from object variation (Supplemental Fig. 5E), implying that treatment with compound 1 led to inhibition of cancer proliferation via degradation of the DX2 protein.

To unveil the dependency of the effects of compound 1 on its binding with DX2, we generated A549 cell lines stably expressing DX2 WT or binding-defective mutants Y47A, N56A, and K129A and treated these cells with various doses of compound 1. The EC50 value of these A549 stable cells expressing DX2 WT (0.92 μM) was very similar to the EC50 found in H460 cells (0.97 μM) (Fig. 7D, red; Fig. 7A), emphasizing the significance of the DX2 level on the function of compound 1. However, there was no effect from compound 1 in cell lines expressing DX2 binding-defective mutants (Fig. 7D, blue), suggesting that the decreased cell viability induced by treatment with compound 1 is mediated by its binding with DX2. We further examined the significance of the binding of compound 1 to DX2 via an anchorage-independent colony-forming assay. As expected, the number of colonies and the efficacy of compound 1 both increased in cells stably expressing DX2 WT but not in the mutant cell lines (Fig. 7E). The above two assays showed that agglutination of the binding between DX2 and compound 1 prevented the chemical-mediated inhibition of cancer cell development, leading us to the conclusion that the binding of compound 1 to DX2 is critical for the function of compound 1. To examine the resistance vulnerability of compound 1, we determined the EC50 of compound 1 in H460 cells pretreated with chemicals for three weeks and compared it with that of native H460 cells. A small decrease in EC50 was observed, but compound 1 efficiently decreased cell viability even though it was used as a pretreatment (Supplemental Fig. 5F), meaning that compound 1 could also function for a long time.

**Discussion**

DX2 is considered a novel target for cancer therapeutics because of its oncogenicity, and specific siRNA and small molecules targeting DX2 have been studied as therapeutic strategies for DX2-expressing tumors (Choi et al., 2011; Lee et al., 2013). Despite many trials, none of the examined tools have been shown to efficiently target DX2. Recently, HSP70 was shown to significantly affect the protein level of DX2 by
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Fig. 5. Prediction and validation of the binding site of compound 1. (A) Distance distribution of compound 1 with three significant residues displaying stable interactions during the last 200 ns. The blue, magenta and green lines represent the distances of ligand:O3, ligand:C13, and ligand:O1 toward K129:NZ, Y47:CG, and N56:ND2, respectively. Compound 1 is represented as green sticks. (B) Binding mode of compound 1 with DX2 obtained from the molecular modeling study. The electrostatic surface model of DX2 taken from the representative structure at 211.3 ns (right) and the zoomed-in view showing the detailed interactions with compound 1 (green) with the three significant binding residues (red) shown by the stick model (left). (C) Binding of compound 1 to DX2 via mutagenesis studies. Cell extracts expressing each of the FLAG-tagged DX2 mutants were mixed with compound 15 and subjected to an in vitro pull-down assay. PD denotes pull-down. (D) DX2-HSP70 binding inhibition of compound 1 via mutagenesis studies. 293T cells expressing FLAG-tagged DX2 were incubated with or without compound 1 for 6 hours and then subjected to immunoprecipitation. Actin was used as a loading control. The quantification of the protein level for each experiment is presented as a bar graph on the right of (C) and (D). (E) Ubiquitination assay using DX2 mutants. The amounts of ubiquitinated DX2 were assessed by SDS-PAGE and immunoblotting using an anti-ubiquitin (Ub) antibody. The quantification of the protein level for each experiment is presented as a bar graph on the right of (C) and (D). IP, immunoprecipitation; WCL, whole cell lysates.

Blocking the access of Siah1, a specific E3 ligase, leading to the suggestion that a PPI inhibitor between DX2 and HSP70 may be a good tool to target DX2 (Lim et al., 2019). Here, we suggest compound 1 as a novel PPI inhibitor for the binding of DX2 and HSP70 and fully address the functional mode of action of this compound. We identified compound 1 as a hit compound for a PPI inhibitor targeting the interaction of DX2 and HSP70 using a luciferase-based complementation system (NanoBiT) (Lim et al., 2019). Compound 1 has the possibility of forming a covalent bond with DX2 by nucleophilic addition. Then, we examined compound 13 and compound 14 [5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-y]pentanoic acid] without the site for nucleophilic addition, resulting in retention of the inhibitory activity (Table 2). Additionally, compound 1 did not show time-dependent inhibitory activity on the binding of DX2 and HSP70 (Supplemental Fig. 2A). These results indirectly indicate that inhibition of DX2 by compound 1 is not based on covalent modification. Compound 1 was found to inhibit and induce the binding of HSP70 and Siah1 to DX2, respectively, resulting in ubiquitination-dependent degradation of DX2. Furthermore, compound 1 was shown to reduce cancer cell development in vitro and in preliminary in vivo experiment in a DX2-dependent manner. We further analyzed the mode of action of compound 1. The binding of compound 1 to DX2 is important for DX2 cellular function, and DX2 residues Y47, N56 and K129 appeared to be significant for binding via MD simulation and mutational analysis. Two modes of action of compound 1 were proposed: allosteric modulation of the DX2 structural conformation and direct steric clashing of DX2-HSP70 binding.

Because a structural understanding of target proteins is critical for drug discovery, many target proteins have been analyzed using techniques such as X-ray crystallography and NMR analysis. However, assessing the structure of the full-length protein via the above traditional analyses is difficult because many proteins have an unstructured flexible region. This flexible region has been reported to be significant for protein function (Babu, 2016); therefore, there have been many efforts to overcome this limitation. DX2 has a disordered NFR consisting of 50 amino acids. To determine whether the mode of action of compound 1 was associated with the NFR, we conducted MD simulations. The MD simulations suggested that compound 1 binds the pocket surrounding the NFR (Y47) and GST-N (N56 and K129), which was indirectly confirmed via mutagenesis experiments. Through the MD study, we also unveiled the mode of action of compound 1, which directly interferes with access to HSP70 and masks the HSP70

Table 2. Docking scores and binding affinities for compound 1 and the hit compounds.

| Compound | Docking Score (kcal/mol) | Binding Affinity (nM) |
|----------|--------------------------|-----------------------|
| Compound 1 | -7.3 | 1.00 |
| Compound 13 | -6.8 | 1.94 |
| Compound 14 | -6.5 | 0.97 |
| Compound 15 | -6.2 | 1.01 |
| Compound 16 | -5.9 | 1.06 |
| Compound 17 | -5.6 | 1.53 |
| Compound 18 | -5.3 | 1.35 |
| Compound 19 | -5.0 | 1.77 |

Note: The docking scores were calculated using Autodock Vina, and the binding affinities were determined using the fluorescein isothiocyanate (FITC)-labeled compound.
binding surface of DX2 via a hydrophobic interaction between the NFR and the GST-N binding site in the bound state of compound 1. From these results, we demonstrated that the NFR and GST-N might be critical for the binding of DX2 to HSP70, further supporting the described mode of action of compound 1. Although some DX2 exists in its free form, the possibility that DX2 could be constitutively bound to HSP70 still exists. Therefore, structural analysis with compound 1 via MD simulations needs to be considered with HSP70 bound to DX2 for competitive binding, and this will be our further study.

Many of the 650,000 PPIs estimated from proteomics tools have recently been reported to be oncogenic (Ran and Gestyawicki, 2018; Li et al., 2017). Therefore, PPIs have been considered to be a significant therapeutic target, especially for cancer, and many trials have sought to discover PPI inhibitors that interfere with oncogenic PPIs (Nero et al., 2014; Ivanov et al., 2013). The discovery of small molecule PPI inhibitors is now a research focus because of the importance of PPIs in cancer. Here, we identified compound 1, with a molecular weight of less than 500 Da, as a PPI inhibitor that efficiently suppresses the oncogenic binding between DX2 and HSP70. Our results showing PPI inhibition using a small molecule with a molecular weight of less than 500 Da could provide new encouragement to continue the search for the discovery of PPI inhibitors using small molecules, which exhibit fewer problems in the clinical setting than large molecules. Cancer patients suffer from the side effects of therapeutic agents against cancer. Treatment with compound 1 did not affect normal cell proliferation (Supplemental Table 1) or the body weights of the tested mice, suggesting that compound 1 has no or only small side effects. This advantage may be due to the PPI inhibition mode of action; specific inhibition of the interaction between DX2 and HSP70 led cancer cell regression without affecting normal cells. We also evaluated metabolic stability of compound 1. The remaining amount of compound 1 was determined using liquid chromatography–tandem mass spectrometry analysis (mass spectroscope: Agilent6460; high pressure liquid chromatograph: Agilent1261) upon incubation with rat and human liver microsomes for 30 minutes, which provided 8.76% and 7.02%, respectively (Supplemental Table 2). The metabolic stability of compound 1 is very poor, and further optimization is needed to
improve its physicochemical properties as well efficacy for the discovery of a sustainable lead on antitumor agent based on the inhibition of DX2 and HSP70 interaction.

**Conclusion**

We identified compound 1 to inhibit the tumor-promoting protein-protein interaction between DX2 and HSP70 through allosteric modulation as well as competitive binding. We performed MD simulations, mutagenesis analyses, and characterization of biologic function using compound 1 and its biotin-conjugated chemical 15 to elucidate the mode of action of compound 1. Compound 1 seems to induce allosteric conformational changes in the DX2 protein and direct binding clashes between DX2 and HSP70. Compound 1 reduced the DX2 protein level in a ubiquitin-dependent manner via suppression of the binding between DX2 and HSP70 and suppressed the growth of cancer cells highly expressing DX2 in vitro and in preliminary in vivo experiment. This paper presents a novel chemical route to interfere with oncogenic PPIs.

**Authorship Contributions**

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*Contributed new reagents or analytical tools:* D.G. Kim, Lim, Park.

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*Wrote or contributed to the writing of the manuscript:* D.G. Kim, Huddar, Y. Lee, Park, S. Lee, S. Kim.

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