Screening approaches for the identification of Nrf2-Keap1 protein-protein interaction inhibitors targeting hot spot residues

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ABSTRACT
Protein–protein interactions (PPIs) play a crucial role in most biological processes and are important targets in the development of therapeutic agents. However, small molecule drug discovery that targets PPIs remains very challenging. Targeting hot spot residues is considered the best option for inhibiting such interactions, but there are few examples of how knowledge of hot spots can be used in high throughput screening to find hit compounds. A substrate adaptor protein for a ubiquitin ligase complex, Kelch-like ECH-associated protein 1 (Keap1), negatively modulates the expression of genes involved in cellular protection against oxidative stress. Here, we focused on three arginine hot spot residues in the Keap1 substrate binding pocket (Arg380, Arg415, and Arg483), and screened the carboxylic acid library owned by Japan Tobacco Inc. for compounds that interact with the arginine residues in differential scanning fluorescence assays. Furthermore, we identified several small molecule compounds that specifically bind to the Keap1 Kelch domain hot spots by comparing binding to alanine mutant proteins (R380A, R415A, and R483A) with binding to the wild-type protein using surface plasmon resonance (SPR) screening. These compounds inhibited the protein–protein interaction between the Keap1 Kelch domain and the nuclear factor erythroid 2-related factor 2 (Nrf2) peptide, and the ubiquitination of Nrf2 catalyzed by the Cul3/RINGBox 1 E3 ligase. In addition, the binding mode of one compound (Compound 4) was determined by X-ray crystallography after validation of binding by isothermal titration calorimetry, native mass spectrometry, and nuclear magnetic resonance. Compound 4 had favorable thermodynamic properties, and noncovalently bound to Keap1 with a stoichiometry of 1:1. Our results suggest that Compound 4 could potentially be developed into effective therapeutic or preventive agents for a variety of diseases and conditions such as oxidative stress response, inflammation, and carcinogenesis. We believe that the use of a set of complementary biophysical techniques including the SPR assay with single alanine mutant of hot spots provides opportunities to identify hit compounds for developing inhibitors of PPIs.

Short abstract
Identification of Keap1/Nrf2 Inhibitors.

1. Introduction

Protein–protein interactions (PPIs) play a pivotal role in regulating biological processes, particularly cellular and signaling pathways [1].

Abbreviations: ARE, antioxidant response element; DSF, differential scanning fluorimetry; IPTG, isopropyl β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; Kd, dissociation constant; Keap1, Kelch-like ECH-associated protein 1; MS, mass spectrometry; Neh, Nrf2-ECH homology; NMR, nuclear magnetic resonance; Nrf2, nuclear factor erythroid-2 related factor 2; NTA, nitrilotriacetic acid; PDB, protein data bank; PPI, protein–protein interaction; Rbx1, RING box protein 1; RU, resonance unit; SPR, surface plasmon resonance; STD, saturation transfer difference; TCEP, Tris (2-carboxyethyl) phosphine; Tm, melting temperature; UbcH5c, human ubiquitin-conjugating enzyme H5c; Ube1, ubiquitin-activating enzyme E1; WT, wild-type.

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However, because protein–protein interfaces are not well-suited for effective small molecule binding, small molecule drug discovery targeting protein-protein interactions is still highly challenging [2,3]. Residues in protein–protein interfaces do not contribute equally to the binding energies, and hot spot residues (often large amino acids such as tyrosine, arginine, and tryptophan) make the major contribution to the binding energy [4,5]. These residues can be detected by alanine scanning mutagenesis experiments [4], but many recent studies have focused on identifying hot spots by computational prediction [6,7].

High-throughput screens (HTS) are commonly employed to identify PPI inhibitor candidates, but these HTS alone do not identify the binding sites of compounds on the proteins. In general, the binding sites of inhibitors are revealed by crystallographic analysis, which is extremely expensive and time consuming. Therefore, it is important to confirm that inhibitor candidates bind to hot spots before moving on to crystallographic analysis.

In the Nrf2 antioxidant defense mechanism, Keap1 is a substrate adaptor protein in a ubiquitin ligase complex that targets the Nrf2 transcription factor for degradation. Nrf2 remains at a low cellular concentration under unstressed conditions and is negatively regulated by Keap1 via proteasome-mediated degradation. In response to oxidative stress, Keap1 is deactivated so that Nrf2 escapes from Keap1 mediated degradation and translocates into the nucleus to transcriptionally activate the ARE-dependent antioxidant genes [8]. It is, therefore, a reasonable strategy to look for inhibitors of the Keap1-Nrf2 interaction as potential therapeutic agents against oxidative stress-mediated diseases [9–11]. The Neh2 domain at the Nrf2 N-terminus binds to the Keap1 Kelch domain [12]. The Neh2 domain contains DLG and ETGE motifs, which are essential for the interactions with Keap1. Both the higher affinity ETGE peptide motif (protein data bank [PDB]: 2FLU) and the lower affinity DLG peptide motif (PDB: 2DYH) bind in the central cavity of the Kelch domain and interact with serine, arginine, and tyrosine side chains [13,14]. In particular, Arg 380, 415, and 483 in the substrate binding pocket of Keap1 could be hot spots identified in previous alanine scanning and computational hot spot mapping studies [15,16].

We focused on three hot spot arginine residues (Arg380, Arg415, and Arg483) to discover novel small molecule inhibitors of Keap1-Nrf2 interaction and screened the Japan Tobacco (JT) library of carboxylic acid compounds that could be expected to bind to arginine residues in differential scanning fluorimetry (DSF) assays. Furthermore, we identified several small molecule compounds that specifically bind to hot spots by comparing binding to wild-type (WT) Keap1 with that to alanine mutants (R380A, R415A, and R483A) of Keap1 in surface plasmon resonance (SPR) assays. Several classes of identified hit compounds inhibited the protein-protein interaction between the Keap1 Kelch domain and Nrf2 peptide and the ubiquitination of Nrf2 catalyzed by the Cul3/Rbx1 E3 ligase. Furthermore, we confirmed that Compound 4 has relatively potent ubiquitination inhibitory activity and showed that it bound to the hot spots in the Keap1 Kelch domain by X-ray crystallography after the validation of binding by isothermal titration calorimetry (ITC), native mass spectrometry (MS), and nuclear magnetic resonance (NMR). These results indicated that Compound 4 is a novel fully validated hit compound that could potentially be a starting compound for treating a variety of diseases involving this pathway [17]. We believe that the SPR assay using alanine mutants is a very effective way of finding hot spot binders, and our strategy for identifying hot spot binders could contribute to the drug discovery of PPI inhibitors.

2. Materials and methods

2.1. Materials

Peptides were purchased from Sigma-Aldrich Japan. SA sensor chips and CM5 sensor chips of SPR were obtained from Cytiva.

2.2. Peptides

A 9mer ETGE peptide ([76]DEETGEF [80], human Nrf2) was selected as positive control to be used in DSF, the SPR direct binding assay, and ubiquitin ligase activity assay. A 16mer ETGE peptide ([80]AFFAAQLDDEETGEF [84]) attached to lysylsine (KK) at the N-terminal due to improvement of solubility was used for the positive control in the SPR direct binding assay, SPR competition assay, and ITC. N-biotinyl LC-LC-16mer ETGE peptide, where LC = long chain (six carbon linker – amino hexanoic acid), was used as a ligand in the SPR competition assay.

2.3. Protein cloning, expression and purification

Purification of the Keap1 Kelch domain: The human Keap1 Kelch domain (321–609) from the human Keap1 full-length plasmid (Kazusa DNA Research Institute) was amplified by PCR and ligated into pET-15b (Merck KGaA) using primers that encoded an N-terminal His-tag. Constructs were expressed in Escherichia coli BL21 (DE3)pLysS competent cells (Merck KGaA). The expressing clone was inoculated in 2×YT media with ampicillin at 30 °C until an optical density at 620 nm of 0.6 was reached, and then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.5 mM. After 4 h of induction, the cells were harvested by centrifugation and stored at −80 °C. Cells were lysed in 1 mL per 20 mL of original culture volume of lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM Tris (2-carboxyethyl) phosphine (TCEP), and EDTA-free protease inhibitor cocktail) and disrupted by sonication with clearing of the lysate by centrifugation. The Keap1 Kelch domain was purified by Ni-NTA Superflow resin (QIAGEN GmbH) followed by a Resource Q resin (Cytiva). Eluted fractions were further purified with a Superdex 200 10/300 GL column (Cytiva). The final protein buffer contained 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitold, and 10% glycerol.

Purification of biotinylated Keap1 Kelch domain: Keap1 Kelch domain and single point substitutions of R380A, R415A, and R483A were cloned into pETDuet1-hemagglutinin (HA)–BirA with an N-terminal tandem biotin acceptor protein and His-tag. The expressing clones were inoculated in 2×YT media with ampicillin, and then IPTG and biotin were added to a concentration of 10 μg/mL. After 3 h of induction the cells were harvested and stored. Biotinylated Keap1 Kelch domain and mutants were purified as described above.

Purification of isotopically labeled Keap1 Kelch domain: For protein-observed NMR experiments, the Keap1 domain was expressed and purified in a similar manner except for M9 medium containing 1.5 mg/mL 15N-NH4Cl, 2 mg/mL β-glucose–13C6 in D2O used for isotope labeling.

Purification of the Keap1 Kelch domain for Native-MS and crystallography: A synthetic gene of the Kelch domain (309–624) was cloned into the pET15b vector. This vector produces the protein in fusion with the N-terminal His-tag and a thrombin cleavage site between Keap1 and the tag. The His-Keap1 Kelch domain was expressed and purified as described previously [18]. The His-tag was removed by thrombin digestion and gel filtration.

Purification of E1 (ubiquitin-activating enzyme E1 [Ube1]), E2 (human ubiquitin-conjugating enzyme H5c [UbcH5c]), Keap1, Cullin3, Rbx1, and Nrf2 Neh2: Ube1 and UbcH5c were constructed, expressed, and purified as described previously [19]. Full-length human Keap1, human Cullin3, human Rbx1, and human Nrf2 Neh2 domain (1–92) were cloned into pVL1393 (Becton, Dickinson and Company). Recombinant baculoviruses encoding HA-Keap1, Myc-Cullin3, His-Rbx1, and Flag-Nrf2 Neh2 were generated using the BD BaculoGold baculovirus expression system (Becton, Dickinson and Company). Recombinant baculoviruses were amplified using SF9 cells (Thermo Fisher Scientific). E3 ubiquitin-ligase complex composed of HA-Keap1/Myc-Cullin3/His-Rbx1 complex and FLAG-Nrf2 Neh2 was expressed in expressSF+ cells (Protein Sciences Corporation). HA-Keap1/Myc-Cullin3/His-Rbx1 complex was purified from cell lysate using Ni-NTA Superflow resin. FLAG-Nrf2 Neh2 was purified by ANTI-FLAG Affinity Gel (Sigma-Aldrich).
2.4. SPR direct binding assay for hot spot residues analysis

SPR experiments for hot spot residues analysis were carried out on a Biacore 4000 instrument at 25 °C. Interactions between a Keap1 Kelch domain (WT, R380A, R415A, and R483A) immobilized on a Series S SA sensor chip (Cytiva) and peptides were measured in real time. After preconditioning the chip surfaces three times with 50 mM NaOH and 50 mM NaCl injections, biotinylated Keap1 Kelch domain was captured at a concentration of 5 µg/mL to a density of ~6000 resonance units (RU). All surfaces, including an SA surface with no additional protein captured, were blocked with amine-PEG biotin (Thermo Fisher Scientific). The 9mer ETGE peptide, 16mer ETGE peptide, and KK-16mer ETGE peptide were serially diluted in the running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 µM EDTA, 0.05% Tween 20 containing 5% DMSO). The peptides were injected with a 1 min association time and a 3 min dissociation time at a flow rate of 30 µL/min. The obtained screening data were solvent corrected, reference subtracted, quality controlled, and globally fitted to a 1:1 binding model using Biacore 4000 evaluation software.

2.5. DSF binding assay

DSF was performed using MicroAmp optical 384-well reaction plates (Thermo Fisher Scientific) with a final volume of 10 µL according to the method of Kranz and Schalk-Hihi [20]. The reaction solution containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Tween 20, 62.5 µM SYPRO Orange (Thermo Fisher Scientific), 5% DMSO, and 1 µM Keap1 Kelch domain was mixed in a reaction plate and sealed using MicroAmp Optical Adhesive Film (Thermo Fisher Scientific). The fluorescence signals were measured from 40 to 60 °C using a heating rate of 1 °C/min on a real-time PCR detection system (Applied Biosystems 7900). Data evaluation and determination of melting temperature (Tm) values were conducted using TmTool™ software that utilized the Boltzmann Eq. (1).

\[
F(T) = F(\text{pre}) + \frac{[F(\text{post}) - F(\text{pre})]}{1 + e^{-rac{T-Tm}{C}}}
\]

where \(F(T)\) is the fluorescence at a particular temperature, \(F(\text{pre})\) is the fluorescence before the transition or melting at the start of the region of analysis (ROA), \(F(\text{post})\) is the fluorescence after the transition or melting at the end of the ROA, \(Tm\) is the melting temperature, and \(C\) is the enthalpy of the reaction.

2.6. SPR direct binding assay for hit validation

SPR experiments for hit validation were carried out on a Biacore 4000 or Biacore S51 instrument in the format used in hot spot residue analysis. DSF hit compounds were prepared as 10 mM stock solution, diluted in the running buffer, and injected at 50 µM. Every 24 cycles, KK-16mer ETGE peptide was injected at 30 µM to monitor the amount of active Keap1 Kelch domain. All binding level data were calculated by adjusting them to molecular weight and protein activity levels as calculated using the amount of active Keap1 Kelch domain (%R_max). The maximum of binding activity (R_max) can be calculated using Eq. (2).

\[
R_{\text{max}} = \frac{\text{MW}_{\text{compound}}}{\text{MW}_{\text{protein}}} \times \text{immobilized protein amount}
\]

where MW_{compound} is molecular weight of compound and MW_{protein} is molecular weight of protein.

Compounds with above 15% R_max and without ill-behavior (very slow dissociation, nonspecific adsorption to reference spots, discontinuous sensorgram, etc.) or superstoichiometry (>300% R_max) were identified as binders [21,22]. To confirm the affinity of the compounds that were obtained at a single concentration, they were tested for binding to the target protein in dose-response format. Compounds were diluted in the running buffer to 100 µM, and serially diluted with running buffer. The compounds were injected with a 1 min association time and a 1–3 min dissociation time at a flow rate of 30 µL/min. The dissociation constant (K_d) values were determined using the Biacore 4000 or Biacore S51 evaluation software.

2.7. SPR solution competition assay

Biotin-labeled LC-TC-16mer ETGE peptide was immobilized at a concentration of 10 nM for 5 min on a Series S SA sensor chip. Solution competition assays were performed under the same conditions at a Keap1 Kelch domain concentration of 30 nM in the presence of varying concentrations of compounds or the KK-16mer ETGE peptide.

Concentration–response data were fitted to the Eq. (3) using Spotfire (TIBCO software):

\[
\% \text{ inhibition} = \frac{\text{max} - \text{min}}{1 + 10^{[\mu M \text{ compound}] / K_d}} + \text{min}
\]

In this equation, parameter min equals the baseline of 100% inhibition, max the plateau of 100% activity. IC_{50} gives the transition center. The Hill slope (Hill) determines the slope of the curve at the transition center.

2.8. Ubiquitin ligase activity assay

The procedures for the ubiquitin ligase activity assay were performed as previously described [23]. Briefly, 16 µL of 90 nM His-Ube1, 750 nM His-UbeH5c, 750 nM HA-Keap1/Myc-Cul3/His-Rbx1, and 750 nM FLAG-Nrf2 Neh2 in reaction buffer (75 mM Tris–HCl, pH 8.0, 12 mM MgCl2, 0.1% BSA, 4 mM ATP) and 2 µL of compounds in 50% DMSO were mixed. Then 6 µL of 680 µM ubiquitin was added and incubated at room temperature for 1 h. The reaction was terminated with the addition of 14.4 µL of stop buffer (NuPAGE LDS sample buffer [Invitrogen; final concentration 1X], NuPAGE reducing agent [final concentration 1X], and EDTA [final concentration 3.5 mM]). Reaction products were boiled for 5 min and resolved by SDS-PAGE, followed by immunoblotting with an anti Nrf2 antibody (Thermo Fisher Scientific).

2.9. Native-MS binding assay

All the data have been acquired on an ESI-TOF mass spectrometer (Micro mass LCT) in positive mode using a Triversa Nanomate as infusion robot. For denaturing mass spectrometry analysis, acquisitions and calibration were performed on the m/z range 500–5000 using a 2 µM myoglobin solution as external calibrator. For native mass spectrometry analysis, acquisitions and calibration were performed on the m/z range 500–5000 using a 2 g/L cesium iodide in 2-propanol/water (50/50 v/v) as external calibrator. Native mass spectra were recorded at Vc 50 V and Pi = 5 mbar. The purified Keap1 Kelch domain was buffer exchanged against 50 mM ammonium acetate buffer, pH 7.5, using NAP5 desalting columns (Cytiva). Analysis under denaturing conditions in 50% water/50% acetonitrile/0.5% formic acid revealed a major species with mass 35,204.6 Da, which was attributed to intact Keap1 Kelch domain (theoretical mass: 35,205.6 Da). Characterization of compound binding to the Kelch domain under non-denaturing conditions was then performed in 50 mM ammonium acetate pH 7.5. In Native-MS conditions, the main species showed a +64–65 Da mass shift regarding the expected mass which could be explained by a potential non-covalent binding of an endogenous zinc molecule. For the Native-MS binding experiment, Keap1 and compound 4 were incubated at 50 mM at a 1:5 molar ratio and analyzed at Vc = 50 V, Pi = 5 mbar. Data interpretation was performed using MassLynx v4.1 software (Waters) and masses were calculated using the peak picking method.
2.10. ITC binding assay

ITC titration experiments were performed at 25 °C on an Auto-ITC 200 system (Malvern Instrument). All samples were in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20 containing 5% DMSO. In a typical experiment, 100 µM KK-16mer ETGE peptide or 500 µM compound was titrated in the sample cell containing 10 µM Keap1 Kelch. Each titration consisted of a preliminary 5 µL injection followed by 18 or 36 subsequent 13 µL additions. Binding data were analyzed using the computer program Origin 7 supplied by Malvern Instrument.

2.11. Protein- and ligand-observed NMR binding assay

For protein-observed NMR experiments, samples were prepared with 100 µM [3,15N] labeled Keap1 Kelch domain in buffer containing 10 mM HEPES, pH 7.2, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20, and 5% DMSO-d6. Compounds were serially diluted in 100% DMSO-d6, and added to the sample to a final concentration of 50, 100, 200, 400, or 800 µM. [15N] HSQC experiments were performed at 25 °C on a Bruker Avance III HD 700 MHz spectrometer with a 5 mm cryoprobe. Processing and spectral visualization were performed using Topspin 3.2 and NMRPipe. Kd values were determined from fitting of chemical shift perturbations using Eq. (4) [24].

\[
\Delta \delta_{\text{obs}} = \Delta \delta_{\text{max}} \left( \frac{[P_0] + [L_0] + K_d}{2} \right) - \left( \frac{([P_0] + [L_0] + K_d)^2 - 4[P_0][L_0]}{2[P_0]} \right) \tag{4}
\]

where \(\Delta \delta_{\text{obs}}\) and \(\Delta \delta_{\text{max}}\) are respectively the observed and maximum chemical shift changes, and \([P_0]\) and \([L_0]\) are respectively the total concentration of protein and ligand.

For ligand-observed NMR experiments, samples were prepared with 10 µM non-labeled Keap1 Kelch domain in buffer containing 50 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 5% DMSO-d6. Compounds were diluted in 100% DMSO-d6, and added to the sample to a final concentration of 250 µM. All experiments were performed at 10 °C on a Bruker Avance III HD 700 MHz spectrometer with a 5 mm cryoprobe. For saturation transfer difference (STD) experiments, the reference spectrum was acquired with the off-resonance saturation at 30 ppm, and the saturation spectrum was acquired with the on-resonance saturation at 0 ppm. The duration of the saturations was 2 s for both on- and off-resonance experiments. All STD experiments were recorded with 16 scans. Processing and spectral visualization were performed using Topspin 3.2.

2.12. X-ray protein structure determination

Each compound was dissolved in the precipitant solution to reach a final concentration of 1 mM for soaking. The apo crystal in 0.1 M sodium acetate, pH 5.0, 1.5 M di-ammonium sulfate was transferred into the soaking drop and left for 4 h at 4 °C. Crystals were cryoprotected with ammonium sulfate and flash-frozen in liquid nitrogen. The crystallographic experiments were performed on the Proxima-1 beamline at Soleil Synchrotron, Paris, France. The structure has been deposited in the Protein Data Bank database (PDB: 8IVG) [17].

3. Results

3.1. Hot spot residue analysis by SPR direct binding assay

Before screening the library, we confirmed that Arg380, 415, and 480 are hot spots by evaluating the binding of ETGE peptides (9mer, 16mer, and KK-16mer) to immobilized Keap1 Kelch domain using the SPR direct binding assay. ETGE peptide bound to wild-type Keap1 Kelch domain in a dose-dependent and saturable manner (Fig. 1). Their Kd values were 1.7 µM for the 9mer, 93 nM for the 16mer, and 86 nM for the KK-16mer ETGE peptides, and these values were comparable to reported data [25]. On the other hand, the binding levels of all ETGE peptides against alanine mutants (R380A, R415A, and R483A) greatly decreased, and the Kd values were > 30 µM for the 9mer, > 1 µM for the 16mer and the KK-16mer ETGE peptides for all mutants (Fig. 1B). The results indicated that Arg380, 415, and 480 are crucial hot spot residues in the Keap-1/Nrf2 interaction.

3.2. DSF focused library screening

We first assessed whether the melting temperature (Tm) of Keap1 Kelch domain could be measured and used for compound screening. The fluorescence intensity (RFU) was plotted as a function of temperature (Fig. 2A). By fitting the fluorescence intensity curves using the Boltzmann equation, the Tm of the Keap1 Kelch domain (Tm = 47.6 °C) was obtained. Next, we examined the effect of 9mer ETGE peptide (previously reported Kd 0.8 µM [25]) on the Tm of the Keap1 Kelch domain. The peptide showed stabilization of the Kelch domain in a concentration-dependent manner (Fig. 2B). These findings ensured the assay’s feasibility and provided the conditions for performing thermal unfolding experiments using potential small molecule inhibitors.

We used DSF to screen 28,000 compounds with carboxylic acid groups from our JT library to identify compounds that stabilize Keap1. For the primary screen, compounds were measured at a single concentration of 100 µM. A screen of 80 plates yielded Z’ factors ranging from 0.70 to 0.95, which we interpreted as indicative of excellent assay performance (Fig. S1). DSF screening of the JT carboxylic acid library showed that the vast majority of compounds had little or no effect on the Tm of the Keap1 Kelch domain. We chose 180 compounds showing an increase in Tm > 1 °C (five standard deviations, Fig. 2C and 2D). Among them, 60 compounds showed thermograms that exhibit a high initial fluorescence that slopes downward or a small transitional increase by fluorescence quenching (black triangles in Fig. 2D). In most cases this may be a false positive, but there is something of a gray area. Since we can rule out false positives by orthogonal assays such as the SPR assay, we proceeded to perform a reproducibility assay. The primary hits were restested using the same protocol in triplicate. Seventy-nine compounds confirmed to stabilize protein were further characterized by SPR.

3.3. Hit validation by the SPR direct binding assay

The objective of performing the SPR direct binding assay for hit compounds from the DSF screen is to distinguish actual binding from non-specific binding. This assay was conducted in the format used in the evaluation of the ETGE peptides against WT and alanine mutants (R380A, R415A, and R483A). Seventy-nine DSF hit compounds were injected at 50 µM. Twenty-two compounds with above 15% Rmax and without ill-behavior or superstoichiometry were selected as described in Materials and Methods. These compounds were tested for binding to the Keap1 Kelch domain in a dose-response format. Furthermore, because we focused on the hot spot arginine residues 380, 415, and 483 in Keap1, we tested the direct binding to mutant proteins (R380A, R415A, and R483A) at the same time as WT proteins in dose response format. Eight compounds bound to the Keap1 Kelch domain in a dose-dependent and saturable manner and bound to at least one mutant protein at less than half the level of binding to the WT protein at 100 µM (Figs. 2C and S2).

3.4. Hit identification by the SPR solution competition assay and ubiquitin ligase activity assay

We then evaluated whether SPR hit compounds could inhibit the binding between the Keap1 Kelch domain and ETGE peptide using the SPR-based solution competition assay. After 16mer ETGE peptide was immobilized, the Keap1 Kelch domain was injected. The Keap1 Kelch domain was confirmed to bind to the immobilized 16mer peptide at a Kd of 100 nM. For the competitive binding assay, varying concentrations of the KK-16mer ETGE peptide were incubated with the Keap1 Kelch...
domain protein at a fixed concentration (30 nM: concentration set below the $K_D$ value of the Keap1 Kelch domain and the KK-16mer ETGE peptide) and injected over surfaces with immobilized 16mer ETGE peptide, and the binding levels of the Keap1 Kelch domain were measured. The calculated IC$_{50}$ value for inhibition by the KK-16mer ETGE peptide was 50 nM (Fig. S3). Next, a competition assay of eight hit compounds was performed, and three compounds (Compound 1, 2, and 3) showed an IC$_{50} \leq 50 \mu$M (Fig. 3 B).

Recombinant Cullin3–Rbx1–Keap1 protein complex has been reported to mediate the ubiquitination of Nrf2 [23]. We evaluated whether the 9mer ETGE peptide inhibits Keap1-mediated ubiquitination of Nrf2. Western blotting showed that the 9mer ETGE peptide dose-dependently suppressed Nrf2 Neh2 ubiquitination (Fig. S4). Furthermore, it was found that Compound 1 was able to inhibit ubiquitination of Nrf2 Neh2 (Fig. 3 C).

3.5. Characterization of compound 4 by Native-MS, ITC, and NMR

We chose Compound 1, which exhibited relatively high inhibitory activity among the HTS hit compounds, in a ubiquitin ligase assay. Since Compound 1 was a racemic mixture, it was separated into individual enantiomers, the (S)-enantiomer of Compound 4 and (R)-enantiomer of Compound 5. It was found that Compound 4 was active (IC$_{50} = 39 \mu$M) while Compound 5 was inactive (IC$_{50} > 100 \mu$M) (Fig. 4A and 4B). SPR experiments determined that Compound 4 bound to the WT, R380A mutant, and R415A mutant proteins in a dose-dependent and saturable manner. Their $K_D$ values were 25 nM for WT, 13 nM for R380A, and 33 nM for R415A, respectively. On the other hand, Compound 4 did not bind to the R483A mutant protein (Fig. 4C). To further confirm binding stoichiometry of the HTS hit compound, we conducted native MS analysis. Under non-denaturing conditions, the Keap1 Kelch domain displayed a narrow charge state distribution detected in the mass range of $m/z$ 2800 to 3800, which indicated that the protein remained folded in 50 mM ammonium acetate, pH 7.5, and the most abundant charge state was 11+$^+$. When Keap1 and HTS hit compounds were incubated at a 1:5 molar ratio, the spectra revealed masses corresponding to a 1:1 binding stoichiometry for Compound 4 in a high fraction of ligand-bound protein (93%) (Fig. S5).

To reveal the thermodynamic signature of binding and optimize it for the HTS hit compound, we performed ITC analysis of Compound 4 binding to the Keap1 Kelch domain. Prior to analyzing the HTS hit compound, we first examined the KK-16mer ETGE peptide. Keap1–KK-16mer ETGE peptide binding was driven by favorable binding enthalpy but opposed by unfavorable binding entropy change ($\Delta H = -56 \text{kJ/mol}, -T\Delta S = 14 \text{kJ/mol}$) (Fig. S6). We next assessed the thermodynamic properties of Compound 4. ITC showed a $K_D$ of 12 µM and favorable...
Fig. 2. Primary screening via Differential scanning fluorimetry. (A) Temperature denaturation curves of the Keap1 Kelch domain (1 µM) in the presence of 0, 0.67, 2, 6, 18, and 54 µM 9mer ETGE peptide monitored by SYPRO Orange. Melt curves are plotted as relative fluorescence versus temperature (°C). (B) The increases in the thermal stability of the Keap1 Kelch domain (1 µM) following the addition of 9mer ETGE peptide (0.67, 2, 6, 18, and 54 µM). Data are shown as the means of triplicate measurements, and the error bars show the standard error of the mean. (C) Flowchart and summary of the screening campaign. (D) Scatter plots of primary screening data and selection of hit compounds exhibiting ΔTm ≥ 1 °C (shown in black) at a single concentration of 100 µM. Thermograms that are compromised by fluorescence interference such as high initial fluorescence and small transitional increase by fluorescence quenching are indicated by black triangles.

Fig. 3. Inhibitory activity for Keap1-Nrf2 interaction and Nrf2 ubiquitination of Compound 1–3. (A) Chemical structures of Compounds 1, 2, and 3. (B) Concentration-dependent inhibition of the binding of Compounds 1, 2, and 3 (0.3, 1, 3, 10, 30, and 100 µM) competitively with Keap1 (30 nM) to immobilized 16mer ETGE peptide. Data are shown as the means of quadruplicate measurements. (C) Concentration-dependent inhibition of polyubiquitin chain formation by Compounds 1, 2, and 3. His-Ube1 (90 nM), His-UbcH5c (750 nM), HA-Keap1/Myc-Cul3/His-Rbx1 (750 nM), and FLAG-Nrf2 Neh2 (750 nM) with compounds (0, 38, 150, and 600 µM) were mixed. After ubiquitin (680 µM) was added and incubated at room temperature for 1 h, the boiled reaction products were applied to SDS-PAGE followed by Western blotting using an antibody specific to Nrf2.
enthalpy and entropy on binding ($\Delta H = -18$ kJ/mol, $-T\Delta S = -10$ kJ/mol) (Fig. 5A).

Protein-based and ligand-based NMRs were performed for further validation of the folding state of the Keap1 Kelch domain and the binding of Compound 4. Protein-based $^{15}$N-$^1$H HSQC NMR using stable-isotope-labeled Keap1 Kelch domain showed well dispersed amino acid signals (Fig. 5B). This indicated that the Keap1 Kelch domain protein was in a rigid folding state rather than a disordered state. In addition, several amino acid peaks showed a Compound 4 concentration-dependent shift and saturation at high concentrations. Plotting the shift distances of six arbitrarily selected amino acid peaks, we obtained a $K_D$ of 25 $\mu$M from chemical shift perturbation analysis (Fig. 5B), which was close to the $K_D$ of 12 $\mu$M for ITC. The binding of Compound 4 to the Keap1 Kelch domain was also evaluated by ligand-based STD NMR, and STD signals were observed at 2.2 ppm corresponding to H-1 (Me) and 6.9 ppm corresponding to H-2 and/or H-3 of p-tolyl moiety (Fig. 5C). Assignment of STD signals revealed that protons of p-tolyl moiety were in close proximity to the Keap1 Kelch domain.

3.6. X-ray analysis of compound 4

The structure of human Keap1 with Compound 4 (PDB: 8IVG) was solved at 1.79 Å resolution with a packing similar to the packing described for mouse Keap1 (PDB: 1X2J) [15, 17]. Human Keap1 had a cylindrical-shaped structure with a typical $\beta$ propeller topology. Interactions between Compound 4 and Keap1 included four direct electrostatic interactions and one indirect electrostatic interaction as well as hydrophobic contacts (Fig. 6). The carbonyl oxygen of the amide of Compound 4 formed a hydrogen bond with the side chain of Ser555. The carbonic acid of Compound 4 formed a salt bridge with the side chain of Arg415, and hydrogen bonds with Ser508 and Arg415 via a water molecule. In addition, the p-tolyl moiety was immersed deeply in the hydrophobic region at the center of the $\beta$-propeller tunnel.

4. Discussion

PPIs are considered challenging targets for conventional drug discovery because drug-like small molecules often cannot inhibit the interactions. Indeed, we previously performed HTS of a 300,000-compound library using a Keap1/Nrf2 inhibition assay, but disappointingly, we were unable to identify validated hits. Several studies have established that the PPI surface can be dominated by a few amino acid residues, termed hot spots, and that disrupting those interactions could be a potentially effective strategy for drug design [27].

The interaction between Nrf2 and Keap1 proteins has been characterized at the molecular level. Two separate regions within the Nrf2 Neh2 domain, the “DLG” motif and the “ETGE” motif, interact with the Keap1 Kelch domain via formation of salt bridges between acidic Asp- and Glu-residues in the Nrf2 Neh2 domain and Arg 380, 415, and 483 in the Keap1 Kelch domain. Furthermore, previous alanine scanning and computational hot spot mapping studies have indicated that these arginine residues in the Keap1 Kelch domain could be hot spots [15, 16].

In this work, we focused on these arginine residues in the Keap1 Kelch domain and through various biophysical screens we attempted to discover small molecules that directly bind to these residues and disrupt the interaction between two proteins (Figs. 3B, 3C, and 4B). After confirming by SPR assay that Arg 380, 415, and 483 were hot spot residues, we endeavored to find compounds that bind to arginine residues by first collecting 28,000 compounds with carboxylic acid groups expected to interact with these residues from our JT library and adapting them for primary screening. We chose DSF as the primary screening method because of its medium throughput [28]. Indeed, the DSF assay allowed us to screen 28,000 compounds in nine days. We selected 180 compounds showing an increase in $T_m \geq 1^\circ C$ (Fig. 2D). Among them, 60 compounds showed thermograms that are compromised by fluorescence interference. Since the DSF assay detects fluorescence signals, auto-fluorescent compounds often interfere with the assay detection system.
Although SYPRO Orange has a higher excitation wavelength (470 nm), 60 of 180 compounds in the primary assay and 31 of 79 compounds in the reproducibility assay interfered with the spectral properties (high initial fluorescence intensity or small transitional increase by fluorescence quenching) because the compounds were at concentrations of 100 µM. Coyle and Walser reported that genuine binder Tm shift values were ~7 °C for some ligandable binding sites, and none of several compounds that generated Tm shift values of ~15 °C (big shifters) were found to be hits by any other method [29]. In our study, compounds showing an increase in Tm ≥ 7 °C interfered with the spectral properties and big shifters were false positives. These compounds were eliminated by secondary orthogonal binding analysis using SPR. Further, SPR solution competition and direct binding assays showed that three compounds (Compounds 1, 2, and 3) had an IC₅₀ ≤ 50 µM for WT protein and bound to at least one mutant protein at less than half the level of binding to the WT protein (Figs. 3B and S2). This indicated these compounds specifically bound to the WT protein and interacted with hot spots. Meanwhile, the patterns of inhibition of Compounds 2 and 3 towards SPR solution competition were linear (Fig. 3B). Their inhibitory activities in the ubiquitin ligase assay were weaker than expected. Sensorgrams of Compounds 2 and 3 for WT proteins in SPR experiments included slow dissociation that may be nonspecific binding (Fig. S2), which could be the reason for the linear inhibition. We focused on Compound 1 which showed a relatively high inhibitory activity in the ubiquitin ligase assay and is a racemic mixture, and confirmed that the (S)-enantiomer of Compound 4 was active (Fig. 4B).

Compound 4 was further characterized using biophysical tools such as Native-MS, ITC, NMR, and X-ray, because cross-validation provides
increased confidence. Good agreement among the SPR-, ITC-, and NMR-derived Kᵦ values and IC₅₀ values in the solution-based assay was observed (Figs. 4B, 4C, 5A, and 5B).

Crystal structure analysis showed that Compound 4 formed a salt bridge with the side chain of Arg483 and a hydrogen bond with Arg415 via a water molecule. On the other hand, no interaction with Arg380 was observed (Fig. 6). In the SPR assay, no binding between Compound 4 and the R483A mutant protein was observed, but binding to the R380A mutant protein, as to the WT protein, was observed (Fig. 4C). These SPR results were in reasonable agreement with the crystallographic analysis. On the other hand, Compound 4 also formed a hydrogen bond with Arg415 via a water molecule, but the SPR assay showed binding to the R415A mutant protein as well as the WT protein. Since the crystal structures represent a snapshot of just a few conformational states, the contribution of the interaction between Arg415 and Compound 4 may be small. Thus, the contribution of the interaction between compounds and each residue can be elucidated by combining SPR analysis with crystal structure analysis.

Having access to the thermodynamic signature in the early stages of the drug discovery process will provide critical information towards the selection of the best drug candidates for development [30,31]. Keap1–KK-16mer ETGE peptide binding was driven by favorable binding enthalpy but opposed by unfavorable binding entropy change (Figure S6). This enthalpy-driven and unfavorable entropy characteristic has also been reported for the interaction between Keap1 Kelch and 9mer ETGE peptide [32]. Whereas Compound 4 exhibited favorable binding enthalpy and entropy change in the binding to Keap1 (Fig. 5A), X-ray crystal analysis indicated the formation of electrostatic interactions between Compound 4 and Arg415, Arg483, Ser508, and Ser555 (as mentioned above, the contribution of R415 may be small) as well as hydrophobic contacts (Fig. 6). In addition, the STD NMR showed that the p-tolyl moiety of Compound 4 bound more proximal to the entrance of the β-propeller tunnel, forming a hydrophobic interaction [33] (Fig. 5C). These results suggested that the favorable enthalpy change was obtained from electrostatic interactions, and the favorable entropy change resulted from hydrophobic effects. These favorable thermodynamic contributions represent an attractive starting point for future medicinal chemistry exploration because optimal geometry for polar interaction provides favorable binding enthalpy, and higher affinity can be achieved if both enthalpy and entropy contribute favorably to the Gibbs energy of binding [31].

Binding stoichiometry has been used to validate and prioritize hit compounds [34]. Generally, the binding stoichiometry can be obtained from ITC experiments. However, we could not elucidate the binding stoichiometry because of the low c-value (<1) in the ITC experiment on Compound 4, and we could only determine thermodynamic parameters (ΔG, ΔH, and −ΔTS) using the low-c ITC method [26,35]. Instead, we used Native-MS which is a powerful tool for determining the stoichiometry. It established that Compound 4 bound to the Keap1 Kelch domain with 1:1 binding stoichiometry [36] (Fig. S5).

Overall, we have successfully identified several hits through the screening of targeted hot spots in PPI. The DSF primary binding assay and characterization of HTS hit compounds by biophysical techniques such as SPR, ITC, NMR, and Native-MS provided an efficient way to generate PPI inhibitors. The SPR direct binding assay was particularly effective for identifying the compounds which bind to hot spot residues and estimating the contribution of the interaction between the compound and each residue. Among hit compounds, Compound 4 had the favorable characteristic of 1:1 binding to Keap1 Kelch domain and favorable enthalpy and entropy contributions. The mutant study also revealed that Compound 4 dominantly bound to the hot spot Arg483 residue in the Keap1 Kelch domain. Therefore, it could serve as a promising starting point for oxidative stress-mediated diseases, and indeed our subsequent work has shown improved activity of Compound 4 [17]. Furthermore, we believe that the SPR direct binding assay using alanine mutants is very effective for finding hot spot binders, and could contribute to the drug discovery of PPI inhibitors.

Authors’ contributions

W.A. performed the DSF and NMR experiments. R.H. performed the SPR, ITC, and ubiquitin ligase activity assay. T.U. prepared the purified proteins. F.D. performed Native-MS experiments. A.N., K.Y., and T.A. synthesized compounds. W.A. and R.H. authored the paper. Y.H. performed the X-ray crystallography and data analysis. K.O. and K.H. directed the study. All authors approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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