

A Hetero-multivalent Covalent Strategy for Enhanced Pulmonary Fibrosis Therapy

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Abstract

Drug combination therapy offers substantial advantages over single-agent treatments. However, discovering synergistic drug pairs remains challenging, as conventional approaches are often time-consuming, costly, and ill-suited for high-throughput screening. Here, we present an innovative strategy that enables efficient discovery of synergistic covalent drug combinations through hetero-multivalent engagement of KEAP1, a cysteine-rich redox sensor in mammalian cells. First, a conformation-sensitive fluorescent reporter for real-time monitoring of microenvironmental changes in KEAP1 was developed, enabling high-throughput discovery of a synergistic covalent drug combination, omaveloxolone (OXO) and cryptotanshinone (CTS). This combination (CTS and OXO) markedly activated the NRF2 cascade and attenuated fibrotic phenotypes in lung fibroblasts, lung organoids, and animal models. Mechanistically, OXO and CTS hetero-bivalently modify two distinct cysteine residues on KEAP1, which in turn synergistically disrupt the KEAP1-NRF2 interaction through a unique allosteric mechanism. Collectively, this work establishes a scalable framework for efficient discovery of covalent drug combinations and offers a new paradigm for enhanced anti-fibrosis therapy.

Introduction

Drug combination therapy is widely used for the treatment of complex diseases, including cancer, viral infections, hypertension, and inflammatory disorders¹⁻³. Compared to single-agent therapies, drug combinations offer substantial benefits, such as enhanced therapeutic efficacy, reduced off-target toxicity, and a greater capacity to circumvent drug resistance⁴. However, the discovery of effective drug combinations has primarily focused on identifying agents that modulate distinct protein targets or converge on complementary biological pathways^{5, 6}. This paradigm has relied extensively on resource-intensive phenotypic screening in cellular or animal models to identify synergistic drug pairs and optimize their dose ratios^{7, 8}. Nevertheless, such experimental-driven approaches are inherently constrained by low throughput, high cost, and limited scalability, rendering them ill-suited for large-scale drug combination screening and efficient dose-ratio optimization⁹. These limitations underscore the urgent need for scalable, high-throughput strategies to efficiently identify synergistic drug combinations and optimize their therapeutic ratios in translational medicine.

The human genome encodes numerous cysteine-rich proteins featuring multiple reactive thiols, such as KELCH-like ECH-associated protein 1 (KEAP1, containing 27 cysteines), Heat shock 70 kDa protein 4 (HSPA4, containing 15 cysteines), and NACHT, LRR and PYD domains-containing protein 3 (NLRP3, containing 45 cysteines)¹⁰⁻¹⁴. Notably, these cysteine-rich proteins are more likely to engage in protein-protein interactions *via* disulfide bonds, positioning them as critical regulators of disease pathogenesis and attractive targets for therapeutic intervention^{15, 16}. Under physiological conditions, the majority of cysteine residues within these proteins remain in a reduced thiol state, rendering them highly susceptible to electrophilic attack. A prime example is KEAP1, in which most cysteine residues are susceptible to covalent modification by electrophilic moieties, while a subset (e.g., Cys77, Cys151, Cys273, Cys288, and

Cys434) play crucial roles in orchestrating nuclear factor erythroid 2-related factor 2 (NRF2) through a network of hyper-reactive cysteine sensors¹⁷⁻²⁰. However, monovalent engagement of a single cysteine residue on KEAP1 often fails to induce profound conformational changes or trigger robust functional modulation. This results in limited therapeutic efficacy and often necessitates higher drug doses, thereby increasing the risk of off-target toxicity. These facts prompted the exploration of a more effective strategy to modulate cysteine-rich proteins through cooperative engagement by multiple covalent agents, aiming to induce robust conformational changes and ultimately achieve enhanced therapeutic outcomes²¹⁻²³.

In the past few decades, a variety of small molecules bearing electrophilic moieties have been reported to covalently modify KEAP1 at distinct cysteine residues²⁴⁻³⁰. In principle, the covalent agents that modify distinct residues on a target protein may induce more substantial conformational changes, thereby eliciting enhanced pharmacological effects^{31, 32}. To validate this concept, an innovative strategy was proposed for the efficient discovery of synergistic drug combinations *via* cooperative covalent engagement of the target protein KEAP1 by hetero-ligands (**Fig. 1**). First, a conformation-sensitive fluorescent reporter was developed for real-time monitoring the microenvironment changes of KEAP1. Upon covalent modifications, the microenvironment changes in the substructure or local conformational state of KEAP1 could be real-time monitored and then converted into quantifiable fluorescence signals, enabling dynamic tracking of the conformational changes of the target protein³³. Subsequently, this fluorescence-based assay was integrated with a suite of pharmacological tests and complementary mechanistic studies (such as chemoproteomic profiling and molecular dynamics simulations), aiming to identify covalent drug combinations for synergistically activating NRF2 cascade and to gain deeper insights into multivalent engagement of KEAP1 (**Fig. 1**)^{34, 35}. All these efforts established a new scalable framework for discovering synergistic covalent drug combinations with enhanced therapeutic efficacy and improved safety profiles, addressing a key challenge in combination therapy development.

Results

Development of a conformation-sensitive fluorescent reporter for KEAP1

To develop a conformation-sensitive fluorescent reporter capable of monitoring real-time microenvironmental changes in KEAP1, we initially screened an in-house library of 50 fluorescent probes. Among all tested candidates, probe **28** (also termed 3-amino-10-(diethylamino)-5,6-dihydrobenzo[c]xanthen-12-ium, **ADDX**) exhibited a markedly fluorescence enhancement upon binding to KEAP1 under physiological conditions (**Figs. 2A, 2B, and S1A**)^{36, 37}. Following stepwise optimization, the optimal assay conditions of **ADDX**-based assay were determined as 0.5 mg/mL KEAP1 and 20 μ M **ADDX** (**Figs. S1B and S1C**). Notably, both omaveloxolone (OXO, an FDA-approved KEAP1 covalent modifier) and bardoxolone methyl (CDDO-Me, a potent NRF2 activator *via* covalent modifying KEAP1) induced dose- and time-dependent increases in the fluorescence signals of the **ADDX**-KEAP1 complex (**Figs. 2C and S1D-S1F**). The fluorescence enhancement triggered by both OXO and CDDO-Me indicated that the

covalent modifiers of KEAP1 elicit time- and dose-dependent changes in the local microenvironment or conformational state of this target protein. These findings establish **ADDX** as a practical, conformation-sensitive fluorescent reporter for real-time monitoring of the microenvironment changes in KEAP1 (**Extended Data Figs. 1 and 2**).

Discovery of KEAP1-targeting synergistic drug combinations

ADDX was subsequently used to construct a fluorescence-based assay for high-throughput screening of the adaptive compounds that synergistically enhance the fluorescence signals of **ADDX**-KEAP1 complex in the presence of OXO (5 μ M) (**Fig. 2D**). Among all tested adaptive compounds, formononetin (FMN), calycosin (CCS), and cryptotanshinone (CTS) triggered the most pronounced fluorescence enhancement, suggesting that these agents induce more pronounced microenvironment changes cooperatively with OXO in KEAP1 (**Fig. 2E**). The **ADDX**-based fluorescence assay was then used to characterize the dose- and ratio-dependent responses induced by these drug combinations. As shown in **Figs. 2F-2H**, these combinations including FMN/OXO (FOC), CCS/OXO (CSOC), and CTS/OXO (COC), all elicited clear ratio-dependent microenvironment changes in KEAP1. The optimal molar ratios of FMN, CCS, and CTS to OXO were determined as 1.25:1, 0.625:1, and 5:1, respectively. At these optimal ratios, three tested drug combinations differentially modulated thermal stability of KEAP1 with a clear ratio dependence (**Figs. 2I and 2J**). Specifically, FOC greatly decreased the melting temperature (T_m) by 3.78°C, while CSOC slightly reduced T_m by 1.54°C. In sharp contrast, COC increased T_m by 6.40°C. Notably, the optimal FMN/OXO, CCS/OXO, and CTS/OXO ratios determined by differential scanning fluorimetry (DSF) were 1.5:1, 0.66:1, and 5:1, respectively, which were highly consistent with the results from **ADDX**-based fluorescence assays. These findings confirm that the **ADDX**-based fluorescence assay is a robust and practical method for detecting the ligand-induced microenvironment changes of KEAP1.

COC synergistically activates NRF2 and markedly attenuates OXO-induced cytotoxicity

To further investigate the functional consequences of three selected drug combinations, their NRF2-agonistic effects were tested utilizing the antioxidant response element-luciferase (ARE-Luc) gene reporter assay (**Fig. 3A**). As shown in **Fig. 3B**, all three combinations (FOC, CSOC, and COC) activated NRF2 transcriptional activity in a clear ratio-dependent manner at a fixed total dose of 200 nM. The maximum activation folds compared to control were 5.47 ± 0.37 , 6.05 ± 0.11 , and 7.76 ± 0.48 , for FOC, CSOC, and COC, respectively. Remarkably, COC sustained over 7-fold activation across a relatively broad range of CTS/OXO molar ratios (from 3.5:1 to 5:1), with peak NRF2-agonistic activity achieved at a 4.5:1 ratio. At this optimized stoichiometry, COC exhibited superior NRF2-agonistic activity compared to either CTS or OXO alone (**Fig. 3C**). Meanwhile, COC substantially improved biocompatibility relative to the marketed drug OXO (**Fig. 3D**). Specifically, at a total dose of 2.5 μ M, COC preserved high cell viability ($94.27 \pm 5.50\%$), whereas OXO alone induced pronounced cytotoxicity. These findings highlight that COC synergistically activates NRF2 signaling and substantially attenuates OXO-induced cytotoxicity, underscoring the therapeutic potential as a combinatorial strategy.

Both agents in COC show high pulmonary exposure

To identify the optimal therapeutic indication for COC, the *in vivo* biodistribution of the two constituent agents was characterized. Following intraperitoneal administration of COC at the optimized molar ratio (CTS/OXO = 4.5:1) to male C57BL/6J mice, both CTS and OXO showed high pulmonary exposure (**Figs. 3E and 3F**). Remarkably, the CTS/OXO ratio in lungs remained within the synergistic window for NRF2 activation, measuring 4.01:1 and 4.08:1 at 0.5 h and 1 h post-administration, respectively. These favorable characteristics (high pulmonary exposure and preservation of the synergistic ratio *in vivo*) suggest that COC is particularly well-suited for treating pulmonary diseases driven by NRF2 dysfunction. Idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) exemplify such conditions, both being oxidative stress-driven pulmonary disorders characterized by impaired NRF2 signaling³⁸⁻⁴⁰. Among these pulmonary disorders, IPF has a median survival of only three to five years after diagnosis, yet well-tolerated and effective therapies remain lacking⁴¹⁻⁴³. In this context, pharmacological activation of NRF2 by COC may confer broad protective effects against pulmonary fibrosis by suppressing inflammation, oxidative stress, and fibroblast activation.

COC shows potent anti-fibrotic effects *in vitro* by activating the NRF2 signaling

Next, the anti-fibrotic effects of COC were evaluated in transforming growth factor- β 1 (TGF- β 1)-stimulated human fetal lung fibroblasts (MRC5) and bleomycin (BLM)-challenged mouse lung epithelial cells (MLE-12). As shown in **Figs. S2A and S2B**, COC dose-dependently inhibited cell migration in TGF- β 1-stimulated MRC5 cells, demonstrating a superior anti-fibrotic effect compared to either CTS or OXO alone. In BLM-challenged MLE-12 cells, COC significantly elevated the cellular levels of glutathione (GSH), and markedly reduced the levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) (**Figs. S2C-S2I**). In parallel, COC significantly upregulated the downstream antioxidant response genes of Nrf2 in MLE-12 cells, including *Gclc*, *Gclm*, *Ho-1*, and *Nqo1*, and markedly prompted Nrf2 nuclear translocation in MLE-12 (**Figs. 3G-3I, and S2J-S2M**). Co-immunoprecipitation (CoIP) assays revealed that COC dose-dependently disrupted the formation of Keap1-Nrf2 complex and significantly decreased intracellular reactive oxygen species (ROS) levels (**Figs. 3J, and S2N**). These observations clearly demonstrate that COC exerts potent anti-fibrotic effects by activating the Nrf2 signaling, thereby enhancing antioxidant defense and suppressing the inflammatory cascade in lung fibroblasts.

COC shows potent anti-fibrotic and anti-oxidative stress effects in murine lung organoid models

Next, the anti-fibrotic and anti-oxidative effects of COC were evaluated using both TGF- β 1-stimulated and BLM-induced murine lung organoids. In TGF- β 1-stimulated organoids, the fibrotic markers, including fibronectin (FN1) and collagen type I (COL1), were significantly upregulated to 16.2-fold (FN1) and 3.8-fold (COL1) above vehicle controls, respectively (**Figs. 3K, 3L, S3A, S3B**). Notably, COC dose-dependently suppressed these fibrotic markers, with substantially greater potency than either CTS or OXO alone. At a low dose (0.5 μ M, COCL), COC reduced FN1 and COL1 to 2.1-fold and 1.2-fold respectively, whereas high-

dose COC (1 μ M, COCH) totally restored both to near-baseline (1.1-fold and 0.8-fold vs. vehicle group). A consistent anti-oxidative stress effects was observed in BLM-induced organoids. BLM triggered a robust 2.4-fold increase in ROS levels compared to vehicle group, and COC again outperformed the individual compounds (**Figs. S3C, S3D**). Low-dose COC (0.5 μ M, COCL) greatly reduced ROS levels to 1.4-fold, while high-dose COC (1 μ M, COCH) further lowered it to 1.3-fold. Flow cytometry showed a lower percentage of FITC-A+ cells in the COCH group (21.7%) than in the BLM group (42.6%) (**Fig. S3E**). Furthermore, BLM induced a comprehensive oxidative and inflammatory homeostasis as evidenced by decreased GSH levels and increased MDA, LDH, IL-1 β , and TNF- α levels, with COC consistently showing greater efficacy than either OXO or CTS alone (**Fig. S3F-S3J**). These findings clearly demonstrate that COC synergistically attenuates both TGF- β 1-induced fibrosis and BLM-induced oxidative stress in murine lung organoids, supporting its potential as a combinatorial therapeutic for pulmonary fibrosis.

COC significantly ameliorates PFI in BLM-challenged murine model

Encouraged by the synergistic anti-fibrotic effects observed in pulmonary cells and organoids, the *in vivo* therapeutic effects of COC were further evaluated in a BLM-induced pulmonary fibrosis (BIPF) murine model (**Fig. 4A**). As shown in **Figs. 4B and 4C**, intratracheal instillation of BLM led to a 21-day survival rate of only 50%, but high-dose COC (COCH, 6.8 mg/kg/day) significantly improved survival to 87.5% and completely prevented the progressive body weight loss observed in BIPF model. In contrast, either OXO or CTS alone conferred only partial improvement in survival. The FDA approved anti-fibrotic drug pirfenidone (PFD) increased survival to 62.5% but failed to fully prevent the sustained decline in body weight. At day 21 post-BLM instillation, fibrotic lungs showed marked consolidation and overt edema, accompanied by increased lung wet/dry ratio and elevated lung index (**Figs. S4A-S4C**). These pathological alterations were completely reversed by COCH to the levels comparable to those achieved with PFD ($P < 0.0001$). Notably, even low-dose COC (COCL, 3.4 mg/kg/day) was more effective than OXO alone (6.8 mg/kg/day) in ameliorating these pathological parameters, whereas CTS-treated mice showed no significant improvement (**Figs. S4B and S4C**).

Histological examination and immunofluorescence staining consistently demonstrated that BIPF mice developed severe PF, characterized by robust upregulation of fibrotic markers including FN1, COL1, collagen type III (COL3), and α -smooth muscle actin (α -SMA), along with increased hydroxyproline (HYP) content and elevated transcriptional levels of *Acta2*, *Fn1*, and *Col1a1* (**Figs. 4D, S4D-S4K**). COCH nearly completely reversed these fibrotic changes, restoring them to levels comparable to those achieved with the positive drug PFD. In contrast, either OXO or CTS alone failed to normalize these indicators, underscoring the superior anti-fibrotic efficacy of COCH *in vivo*. Hematoxylin and eosin (H&E) staining showed severe alveolar architectural destruction and pronounced inflammatory infiltrates in BLM-exposed lungs (**Fig. S5A**). Fibrotic lungs showed reduced GSH by 2.82-fold, CAT by 2.63-fold, and T-SOD by 1.67-fold, alongside elevated MDA by 3.57-fold (**Figs. S5B-S5E**). COCH reversed these oxidative stress markers more effectively than PFD, while OXO or CTS alone yielded modest improvements. Consistently, BLM challenge led to elevated mRNA expression of IL-6 by 13.42-fold, IL-1 β by 2.58-fold, and TNF- α by 2.30-fold in lung tissue, alongside increased protein levels of IL-6 by 2.79-fold, IL-1 β by 2.36-fold, and

TNF- α by 1.44-fold in bronchoalveolar lavage fluid (BALF) (**Figs. S5F-S5K**). Both COCH and PFD significantly suppressed these cytokines, with COCH showing superior efficacy, while OXO or CTS alone exhibited weaker effects. These findings clearly demonstrate that COC exerts potent synergistic anti-fibrotic effects *in vivo*, markedly improving survival, preserving lung architecture, suppressing inflammation, and downregulating key fibrotic markers.

COC significantly modulates redox and matrix homeostasis by targeting KEAP1

To profile COC-interacting proteins at the proteome-wide level, a peptide-centric local stability assay (PELSA) was used to identify COC-interacting proteins⁴⁴. PELSA-derived thermodynamic profiles coupling with multi-omics target ranking (OTTER) identified KEAP1 as one of the high-confidence targets of COC ($-\log_{10}(\text{pvalue}) \geq 5$, $\log_2(\text{fold change}) \leq -2$) (**Figs. 4E and 4F**). Transcriptomic analysis of lungs from different treatment groups revealed that COC induced a distinct transcriptional signature, with 59.27% (582/982) of differentially expressed genes (DEGs) being unique to this combination therapy and not observed in either the OXO or CTS group alone (**Figs. 4G and S6A-S6E**). Principal component analysis further demonstrated that COC shifted the global transcriptome toward a normal state, as the COC-treated group exhibited the greatest separation from fibrotic lungs and clustered most closely with healthy controls (**Fig. S6F**). Notably, pathway enrichment analysis of PELSA-identified targets revealed significant involvement in biological processes related to oxidative stress response (**Fig. S7**). Reactome pathway enrichment analysis of DEGs from the COCH vs. Model comparison highlighted extracellular matrix (ECM) organization as a key functional category (**Fig. 4H**). Consistent with these findings, COC significantly alleviated oxidative stress, suppressed collagen synthesis, and markedly modulated ECM in the lungs of BIPF mice (**Fig. 4D, S5, and S6**).

The anti-PF effects of COC are abolished in Nrf2-deficient (*Nrf2*^{-/-}) Mice

To further validate the Nrf2-dependent anti-fibrotic mechanism of COC, the anti-fibrotic effects of this combination were evaluated in a BIPF model established in *Nrf2*^{-/-} C57BL/6J male mice. As shown in **Figs. 5A-5B**, BLM instillation drastically reduced the 21-day survival rate to 50%, whereas COCH (6.8 mg/kg/day) only marginally improved survival to 64.71% (compared to 87.5% in wild-type mice) and failed to significantly ameliorate the progressive body weight loss observed in these mice. At day 21 post-instillation, lungs from BIPF *Nrf2*^{-/-} mice showed substantial consolidation, aggravated fibrosis, severe alveolar destruction, and inflammatory infiltrates, while COCH failed to attenuate these fibrotic pathologies, indicating that Nrf2 was the potential target of COCH (**Fig. 5C-5E**). Consistently, COC did not reverse key fibrotic indicators, including increased lung water content, elevated lung index and higher HYP levels, while did not suppress the elevated fibrotic biomarkers (such as FN1, COL1, and α -SMA) in *Nrf2*^{-/-} mice (**Figs. 5F-5O**). In contrast, all these fibrotic biomarkers were significantly reduced in wild-type mice by COC treatment ($P < 0.001$ vs. Model group, **Figs. S4H-S4K**). Moreover, COC was unable to counteract BLM-induced oxidative stress, as reflected by the non-significant levels of GSH, CAT, SOD, and MDA (**Figs. S8A-S8D**). In contrast, COC significantly restored all these oxidative stress markers in wild-type mice ($P < 0.0001$ vs. Model group, **Figs. S5B-S5E**). Similarly, COC showed negligible reduction in pro-

inflammatory cytokines (IL-6, IL-1 β , and TNF- α) in both lung tissue and BALF from *Nrf2*^{-/-} mice (**Figs. S8E-S8J**). These results clearly demonstrate that the anti-PF effects of COC are largely attenuated in the *Nrf2*^{-/-} mice, establishing Nrf2 activation as a crucial mechanism underlying the potent anti-fibrotic effects of this combination.

COC hetero-bivalent modifies KEAP1 at Cys406 and Cys489

Next, an optimized bottom-up proteomic workflow that enabled 100% peptide coverage of all cysteine residues in KEAP1, was used to identify the key cysteine residues engaged by COC on KEAP1^{19, 45, 46}. Results of chemoproteomic mapping revealed that OXO modification at Cys406, while CTS modification at distinct site, Cys489 (**Figs. 6A-6E and S9A**). To assess the functional relevance of these modifications, three KEAP1 mutants including two single-site (C406A or C489A) and a dual-site (C406A/C489A) KEAP1 mutants, were constructed. surface plasmon resonance (SPR) analysis showed that either C406A or C489A mutation markedly reduced the binding affinity of COC to KEAP1 (18.85-fold for C406A and 23.22-fold for C489A), whereas the dual-site mutation (C406A/C489A) almost completely disrupted this binding, reducing the binding affinity from 2.29 μ M to 188.60 μ M (**Figs. S9B-S9F**). Consistently, SPR further revealed that COC destabilized the KEAP1-NRF2 interaction more potently than either CTS or OXO alone (**Figs. S10A-S10D**). Cellular functional assays further reinforced the necessity of both modifications. In *KEAP1*^{-/-} hepatocytes reconstituted with wild-type KEAP1, COC strongly elevated NRF2 protein levels by disrupting KEAP1-NRF2 complex formation. The dual-site mutation (C406A/C489A) fully abolished this effect, whereas C406A mutation partially attenuated the response and C489A mutation had no significant impact. (**Figs. S10E-S10G**). CoIP assays further confirmed that the dual-site mutation significantly stabilized the KEAP1-NRF2 complex, with partial stabilization observed for C406A and no effect for C489A (**Figs. S10G**). These findings establish that hetero-bivalent modification of both Cys406 and Cys489 is essential for COC-mediated synergistic activation of NRF2 signaling.

COC synergistically disrupted KELCH-NRF2 interaction by a unique allosteric mechanism

To further elucidate the synergistic mechanism underlying COC-mediated NRF2 activation, molecular dynamics simulations of the KELCH domain under three conditions including ligand-free KELCH domain (APO), the domain modified with OXO at Cys406, and the domain modified with both OXO at Cys406 and CTS at Cys489 (COC), were performed (**Figs. S11A and S11B**). As shown in **Figs. S11C-S11D**, both OXO and COC modifications allosterically increased the hydrophobic surface area of the KELCH domain, consistent with the observations that both OXO and COC enhanced ADDX-KEAP1 fluorescence intensity in a time- and dose-dependent manner. MM-PBSA analysis revealed that OXO modification established strong electrostatic interactions with residues 393-395 and 441-449 within the KELCH domain (**Figs. S12A and S12B**). These interactions induced a conformational shift in the χ_1 dihedral angle of Cys489 from -29.13° (APO) to +26.40° (OXO-modified), thereby facilitating covalent engagement of CTS at Cys406 (**Figs. 6E-6G, and S12C-S12E**). Upon CTS modification, additional interactions emerged with residues 471, 479, 491, 496, 500, and 538-542, driven predominantly by electrostatic, van der Waals, and solvation forces (**Figs. S12A and S12B**). Importantly, dual-site modification by COC markedly altered the

binding modes between the KELCH domain and the ETGE motif of NRF2. As shown in **Fig. 6H**, the RMSD of the ETGE motif increased progressively from 0.251 ± 0.047 nm (APO) to 0.268 ± 0.054 nm (OXO-modified) and 0.388 ± 0.154 nm (COC-modified). Both MM-PBSA and RMSF analyses indicated that OXO and COC modifications led to unfavorable binding energies (APO: -25.27 ± 3.39 kJ/mol; OXO-modified: 17.71 ± 3.38 kJ/mol; COC-modified: 188.375 ± 3.667 kJ/mol) and enhanced residue fluctuations within the ETGE motif (**Figs. 6I, 6J, and S12F**). Moreover, hetero-bivalent modification by COC severely disrupted the essential Arg380-Arg415-Arg483 salt-bridge network critical for NRF2 recognition (**Figs. 6K-6N and S12G-S12I**). These findings suggest that hetero-bivalent modification by COC disrupt the NRF2-binding interface of the KELCH domain, thereby impairing KEAP1-NRF2 interaction and synergistically activating NRF2 signaling.

Discussion

This work presents hetero-multivalent modification as an innovative strategy for discovering synergistic drug combinations that covalently engage cysteine-rich proteins. Central to this strategy is the development of a conformation-sensitive fluorescent reporter, **ADDX**, which enables real-time monitoring of the microenvironmental changes within KEAP1⁴⁷. Unlike conventional twisted intramolecular charge transfer (TICT)-based fluorogenic tools that target pre-existing hydrophobic pockets, **ADDX** responds dynamically to alterations in the hydrophobic surface landscape induced by covalent engagement^{33, 48-51}. Upon covalent modification of KEAP1, several initially buried hydrophobic residues (such as Cys489) are exposed, thereby generating a neo-interface that accommodates the TICT-based probe and results in a turn-on fluorescence response^{38, 52}. This mechanism translates covalent ligand engagement into quantifiable optical signals, offering a function-oriented readout that reflects not only the occurrence of modification but also its allosteric consequences⁵³⁻⁵⁶. Although TICT-based probes have previously been employed for protein quantification, the **ADDX**-based assay described here establishes a distinct application (sensing ligand-induced changes in the hydrophobic surface area of a target protein), thereby bridging the gap between chemical modification and functional readout at the molecular level⁵⁷.

The **ADDX**-based fluorescence assay offers a dual advantage in combination therapy development. Beyond enabling the identification of synergistic drug pairs through ligand-induced conformational changes in KEAP1, this assay directly addresses another critical bottleneck, drug ratio optimization. It is well-known that the conventional approaches to determining optimal stoichiometry rely primarily on labor-intensive cellular or animal experiments⁵⁸⁻⁶⁰. By contrast, the conformational-sensing mechanism of the **ADDX**-based assay enables high-throughput discovery of synergistic combinations alongside simultaneous optimization of their stoichiometry⁶¹. Notably, this strategy is particularly well-suited for targeting therapeutically relevant yet challenging proteins that function through protein-protein interactions or harbor multiple reactive residues⁶²⁻⁶⁶. For example, proteins such as 3C-like protease (3CL^{Pro}), NLRP3, stimulator of interferon genes (STING), and HSPA4, all of which exhibit intrinsic structural flexibility and contain multiple cysteines^{12, 67-71}. In this work, KEAP1 serves as a representative target protein to illustrate how inherent conformational flexibility enables hetero-

multivalent modification to achieve more profound functional regulation, thereby providing a generalizable framework for modulating previously intractable protein systems. Furthermore, this strategy is readily adaptable to multivalent engagement on other nucleophilic residues (including lysine, serine, and threonine) or multiple nucleophilic residues in a target protein of interest, underscoring its broader utility beyond cysteine-reactive ligands^{4, 45, 56, 72-75}.

Covalent drug development has historically been met with caution due to concerns over off-target reactivity and the associated toxicity⁷⁶⁻⁷⁸. Advances over the past decade have demonstrated that rationally designed or structurally optimized covalent agents can achieve target-specific engagement with limited off-target reactivity, revitalizing interest in this modality^{77, 79-81}. Different from the strategies of rational design or structural modification, this work introduces a distinct paradigm (hetero-multivalent modification) that enables more profound functional perturbation of the target protein, achieving enhanced efficacy at substantially lower individual doses⁸²⁻⁸⁴. The dose reduction further minimizes the potential off-target effects, addressing a longstanding challenge in covalent drug development^{85, 86}. Furthermore, conventional methods for discovering synergistic drug combinations are based on phenotypic outcomes, relying largely on trial-and-error experimentation or serendipitous findings⁸⁷⁻⁸⁹. As a result, the molecular basis of synergistic interactions often remains unclear². In contrast, this work identifies synergistic drug combinations by monitoring microenvironment changes in the conformational state of the target protein, enabling rational, mechanism-driven combination discovery. This transition from empirical to mechanism-based logic constitutes not merely a methodological advance, but a deeper reconceptualization of synergistic action itself⁹⁰.

From a clinical perspective, this mechanism-driven approach offers distinct advantages for optimizing existing covalent therapeutics. As exemplified by the hetero-multivalent combination of COC, this strategy achieves enhanced therapeutic efficacy at individual doses of both CTS and OXO while circumventing the dose-limiting toxicities typically associated with high-dose OXO monotherapy⁹¹. This dual benefit supports a translational paradigm in which approved covalent drugs are repurposed for new indications in a mechanism-guided manner, avoiding the high costs and protracted timelines associated with *de novo* drug development. For complex refractory diseases or chronic conditions requiring long-term management, such as sepsis, autoimmune diseases and pulmonary fibrosis, the simultaneous attainment of improved efficacy and reduced toxicity offers particularly compelling clinical value^{92, 93}. More importantly, this work provides a translatable blueprint for advancing combination therapy into clinical practice by broadening the therapeutic window, improving patient safety, and expanding the clinical utility of existing therapeutic agents⁹⁴.

Materials and Methods

Chemicals and reagents

The compounds BLM (CAS No. 11056-06-7) and PFD (CAS No. 53179-13-8), a positive control for PF, were sourced from MedChemExpress (NJ, USA), while OXO and CTS were provided by Shanghai Standard Technology Co., Ltd (Shanghai, China). Exogenous TGF- β 1, utilized for fibroblast stimulation, was procured from PeproTech (NJ, USA). Cell viability was assessed using a CCK-8 obtained from Yeasen Biotechnology Co., Ltd (Shanghai, China). For the evaluation of oxidative stress, a ROS assay kit, Steady-Glo® Reagent, and dimethyl sulfoxide (DMSO) was purchased from Beyotime Biotech Inc (Shanghai, China), and the quantification of fibrosis and antioxidant biomarkers—including HYP, MDA, GSH, and T-SOD—was performed using detection kits ordered from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The NRF2 fragment (residues 1-85) was custom provided by Suzhou Jinweizhi Biotechnology (Suzhou, China).

Synthesis and structural characterization of ADDX

A solution of 6-amino-3,4-dihydro-1(2*H*)-naphthalenone (1.61 g, 10 mmol) and 4-(diethylamino)salicylaldehyde (1.95 g, 11 mmol) in concentrated sulfuric acid (10 mL) was heated under reflux at 90°C for 5 h. After cooling to room temperature, the reaction mixture was carefully poured into ice-water, followed by the addition of perchloric acid (70%, 2 mL). The mixture was extracted with dichloromethane/methanol, and the organic layer was dried over anhydrous sodium sulfate. The crude product was purified by silica column chromatography (dichloromethane/methanol = 25:1) to afford the dark red product (2.2 g, 52.5%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.34 (s, 1H), 8.04 (d, *J* = 8.8 Hz, 1H), 7.77 (d, *J* = 9.1 Hz, 1H), 7.32 (s, 2H), 7.21 (dd, *J* = 9.2, 2.5 Hz, 1H), 7.16 - 7.10 (m, 1H), 6.71 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.56 (d, *J* = 2.1 Hz, 1H), 3.60 (q, *J* = 7.0 Hz, 4H), 2.98 - 2.88 (m, 4H), 1.21 (t, *J* = 7.0 Hz, 6H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.32, 157.55, 157.05, 153.84, 147.21, 145.14, 131.19, 130.37, 120.59, 115.50, 115.17, 114.47, 113.38, 112.90, 96.32, 55.36, 45.30, 27.35, 25.53, 12.85.

Fluorescence-based screening assay for KEAP1 modulators and synergistic drug pairs

To screen for small-molecule modulators or synergistic drug pairs targeting KEAP1, a fluorescence-based biochemical assay was established using 96-well black polystyrene microplates. First, 2 μ L of a 50 $\times$ stock solution of the test compound(s) was dispensed into each well. Subsequently, 100 μ L of an assay mixture, composed of 1 $\times$ KEAP1 protein (0.5 mg/mL final concentration) and 1 $\times$ ADDX probe (20 μ M final concentration) prepared in binding buffer (25 mM Tris-HCl, pH 8.0), was added to initiate the reaction. The plate was then incubated at 37°C for 30 min with orbital shaking at 210 rpm to ensure equilibration of the binding reaction. Following incubation, fluorescence intensity was measured continuously using an Agilent BioTek Synergy H1 microplate reader at excitation and emission wavelengths of 580 nm and 630 nm, respectively. To correct for background signal, the fluorescence of ADDX alone (in the absence of KEAP1) was subtracted from all sample readings. Compounds or combinations that elicited a statistically significant change in fluorescence intensity, relative to vehicle-treated controls, were identified as primary hits.

Assessment of NRF2 agonist activity using an ARE-Luc reporter assay in HEK-293T cells

HEK-293T cells stably transfected with a HEK-293T-ARE-Luc reporter construct were used to evaluate NRF2 agonist activity. The cells were seeded into white-walled, clear-bottom 96-well plates at a density of 1.0×10^5 cells per well in 100 μ L of complete culture medium. The plates were then incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂ to allow for cell adhesion and stabilization. Following the stabilization period, the culture medium was carefully aspirated and replaced with fresh medium containing varying concentrations of the test compounds. Specifically, cells were treated with either individual drugs or synergistic drug pairs prepared at a series of molar ratios. After 24 h of co-incubation under standard culture conditions, the luciferase activity was measured. Briefly, an equal volume (100 μ L) of Steady-Glo® Luciferase Assay Reagent (Promega) was added to each well, and the plates were incubated at room temperature for 5 min with gentle shaking to ensure complete cell lysis and stabilize the luminescent signal. The bioluminescence intensity was then quantified using a SpectraMax iD3 multimode plate reader (Molecular Devices) with an integration time of 1000 ms. All experimental conditions were performed in triplicate, and the relative luciferase activity was normalized to the vehicle-treated control group (set as 1.0) to determine the fold change in NRF2 activation.

Establishment of alveolar organoids

Mouse lung organoids were established as a physiologically relevant *ex-vivo* model to quantify the anti-fibrotic activity of CTS, OXO, or their pairwise combination. Distal lung epithelial tips were micro-dissected from 13.5-day embryonic mouse lungs, minced into ~ 1 mm³ fragments, and enzymatically dissociated with Accutase (Invitrogen, USA) at 37°C for 40 min under 5% CO₂. Digestion was terminated by adding complete medium containing 10% fetal bovine serum (FBS). The suspension was sequentially triturated, filtered through a 70 μ m cell strainer to remove undigested tissue, and centrifuged at 1,000 rpm for 3 min. The pellet was resuspended in 1 mL of lung-organoid medium and seeded at 6,000 cells per well into 96-well ultra-low-attachment U-bottom plates (Corning, USA). Cultures were maintained at 37°C, 5% CO₂, and 95% humidity. The lung-organoid medium consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, 1:1; Invitrogen, Carlsbad, CA, USA) supplemented with 0.1% fatty-acid-free bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 1% (v/v) Insulin-Transferrin-Selenium-G supplement (ITS; Invitrogen), and 100 ng/mL recombinant human fibroblast growth factor 7 (FGF-7; PeproTech). Organoid morphology was monitored daily by phase-contrast microscopy, and the entire medium was refreshed every 48 h. For the anti-fibrotic efficacy assessment, mouse lung organoids were treated with either 10 ng/mL TGF- β 1 or 10 μ g/mL BLM in conjunction with vehicle, CTS (1 μ M), OXO (1 μ M), the COC (0.5 or 1 μ M), or PFD (100 μ M) for 48 h. Whole-mount immunofluorescence was performed to assess the expression of FN1 and COL1. ROS levels were assessed using the DCFH-DA probe. Fluorescence imaging was conducted using an Operetta CLS high-content screening system. Z-stack layer scanning mode was employed with a scanning distance of 400 μ m and a step size of 4 μ m. The final images were generated as maximum intensity projections to eliminate limitations of single-layer focusing. Exposure time was set at 400 ms. The depth of field for FITC channel was 16.27 μ m, and for Alexa Fluor 555 channel was 16.88 μ m. Fluorescence intensity was quantified using ImageJ software. The MFI was calculated as Mean (Mean gray value) = IntDen/Area, where IntDen represents

the total fluorescence intensity of the specific channel, and Area is the field of view area under the same magnification (the image area of each picture). The fluorescence intensity was normalized to untreated controls, which were set as 1-fold. Quantification of fluorescence intensity was performed on 8-bit processed images, with a gray value range of 30-255 selected for analysis.

Animal tests

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (IACUC, Approval No. PZSHUTCM2507240018). Male C57BL/6J mice (6-8 weeks old, 20-22 g) were obtained from Shanghai Jihui and housed under specific pathogen-free conditions with a 12 h light-dark cycle, $25 \pm 2^{\circ}\text{C}$ temperature, $60 \pm 10\%$ humidity, and ad libitum access to food and water. *Nrf2*^{-/-} mice were kindly provided by Prof. Lili Ji (Institute of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine). Mice were randomly assigned to seven experimental groups (n = 12 per group): (1) Control; (2) Model (BLM alone); (3) CTS (BLM + CTS, 6.8 mg/kg/day); (4) OXO (BLM + OXO, 6.8 mg/kg/day); (5) COCL (BLM + COC at a low dose, 3.4 mg/kg/day); (6) COCH (BLM + COC at a high dose, 6.8 mg/kg/day); and (7) PFD (BLM + PFD, 200 mg/kg/day) as a positive control. PF was induced by intratracheal injection of BLM (2 mg/kg). For treatment groups, intraperitoneal administration of the respective compounds or pirfenidone was initiated three days post-BLM instillation and continued daily until the end of the experiment. Body weights were monitored daily throughout the experimental period. All mice were euthanized on day 21 after BLM administration, and lung tissues were collected for subsequent histopathological and biochemical analyses.

Covalent docking and MD simulations

To elucidate the covalent binding mechanisms of KEAP1 ligands, a computational workflow comprising covalent docking, MD simulations, and binding free energy calculations was employed. Covalent docking was performed using MOE 2019.01 with the KELCH domain of human KEAP1 (PDB: 1U6D). Ligands OXO and CTS were constructed in MarvinSketch and covalently tethered to Cys406 and Cys489, respectively. The top-ranked pose by GBVI/WSA dG scoring was selected for subsequent MD simulations. Force field parameters for the non-standard cysteine-ligand adducts were generated using AmberTools20 with the amber99sb-ildn force field and RESP charges. Each system was solvated in a TIP3P water box, neutralized with 0.15 M NaCl, and converted to GROMACS format using ACYPE. Following energy minimization, each system was equilibrated under NVT and NPT ensembles for 100 ps at 310 K, followed by a 500 ns production MD run at 310 K and 1 bar with a 2-fs time step. Long-range electrostatics were treated using the PME method. Post-simulation, trajectory clustering was performed to identify representative conformations. Binding free energies were calculated using the MM/PBSA method over the final 10 ns of each trajectory. Detailed parameters are provided in the Supporting Information.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 8.0. Intergroup comparisons were conducted through one-way analysis of variance (one-way ANOVA) followed by Dunnett's test, with a *P* value of less than 0.05 considered statistically significant.

Declarations

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Figures

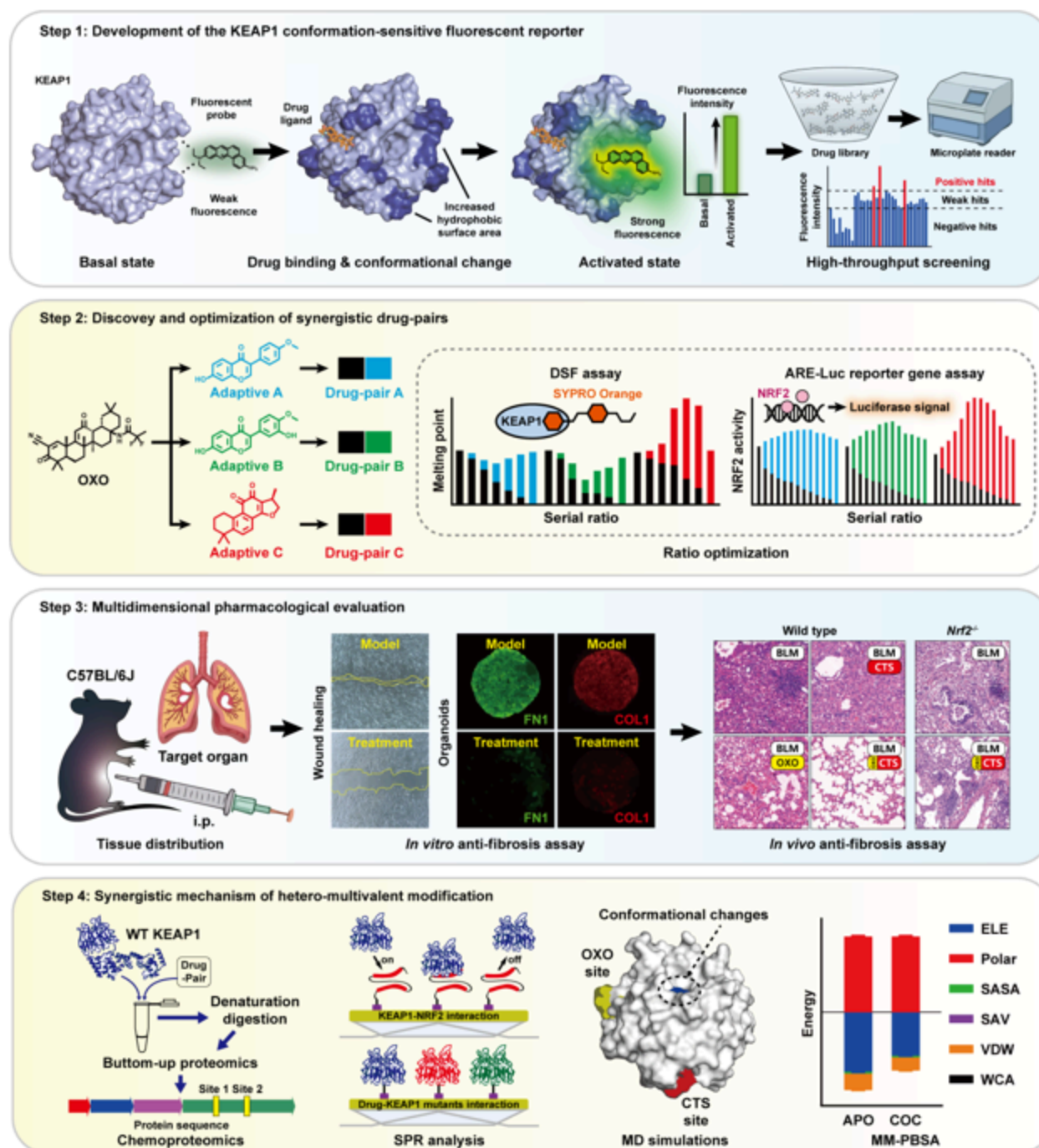


Figure 1

An overview of the development of a conformation-sensitive fluorescent reporter for real-time monitoring of microenvironment changes in KEAP1 and for discovering synergistic covalent drug combinations to mitigate pulmonary fibrosis.

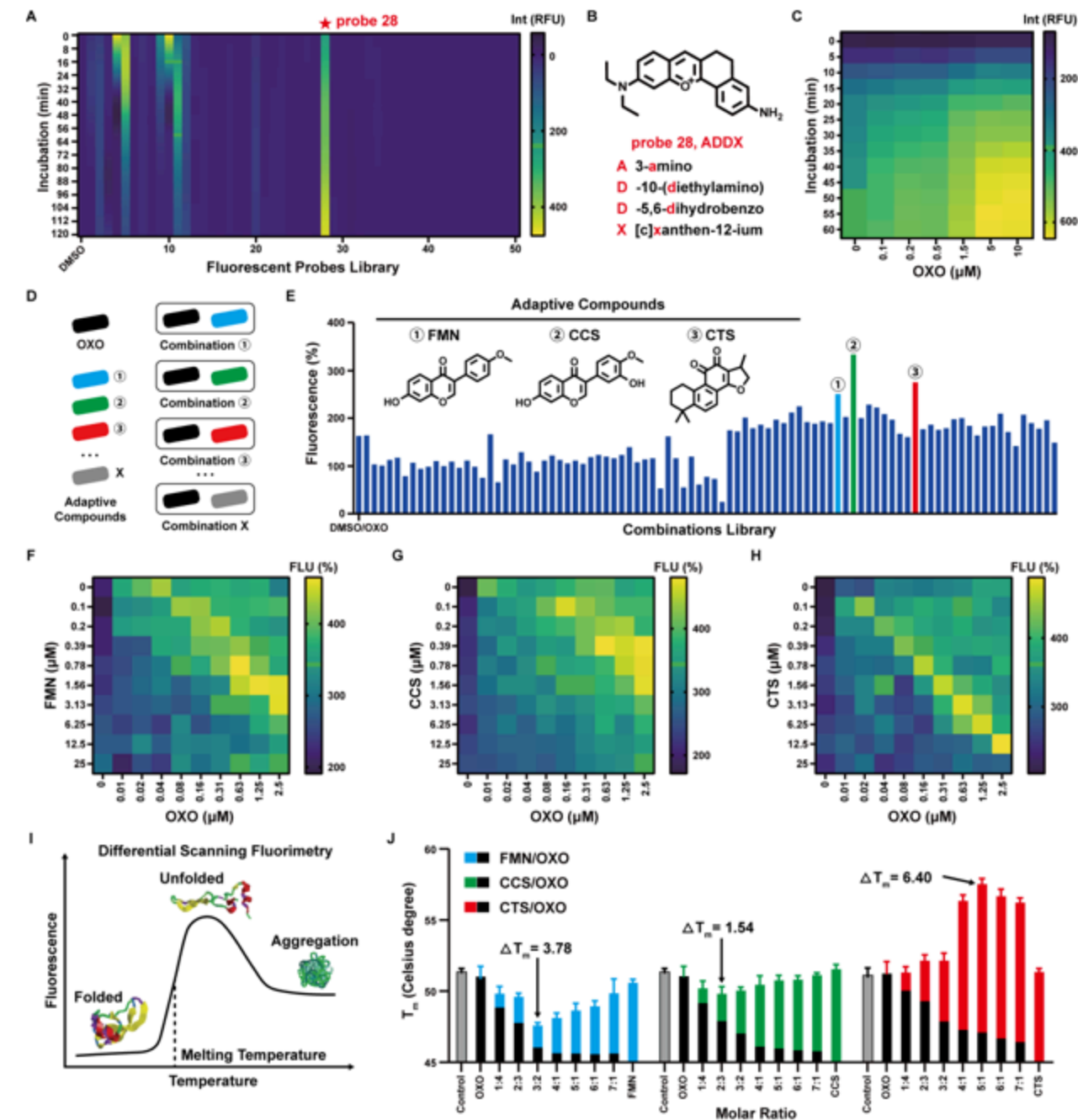


Figure 2

High-throughput discovery of the synergistic drug-pairs targeting KEAP1. (A) A fluorescence probe library was screened to identify a conformation-sensitive reporter for KEAP1. (B) The chemical structure of probe 28 (3-amino-10-(diethylamino)-5,6-dihydrobenzo[c]xanthen-12-ium, ADDX) was shown. (C) Time- and dose-dependent conformational modulation of KEAP1 by OXO (a known covalent KEAP1 binder)

was measured using ADDX as the fluorescent reporter. (D) A schematic representation of drug-pair combinations using OXO as the fixed agent. (E) ADDX-based fluorescence assay was used to screen potential synergistic drug-pairs targeting KEAP1. (F-H) Dose- and ratio-dependent conformational modulation of KEAP1 by drug-combinations (FOC, CSOC, and COC) was demonstrated. (I) A schematic of the DSF assay for testing the effects of drug combinations on KEAP1 stability. The T_m shift indicated ligand binding. (J) The DSF melting curves to illustrate the ratio-dependent effects of three combinations on KEAP1 thermal stability.

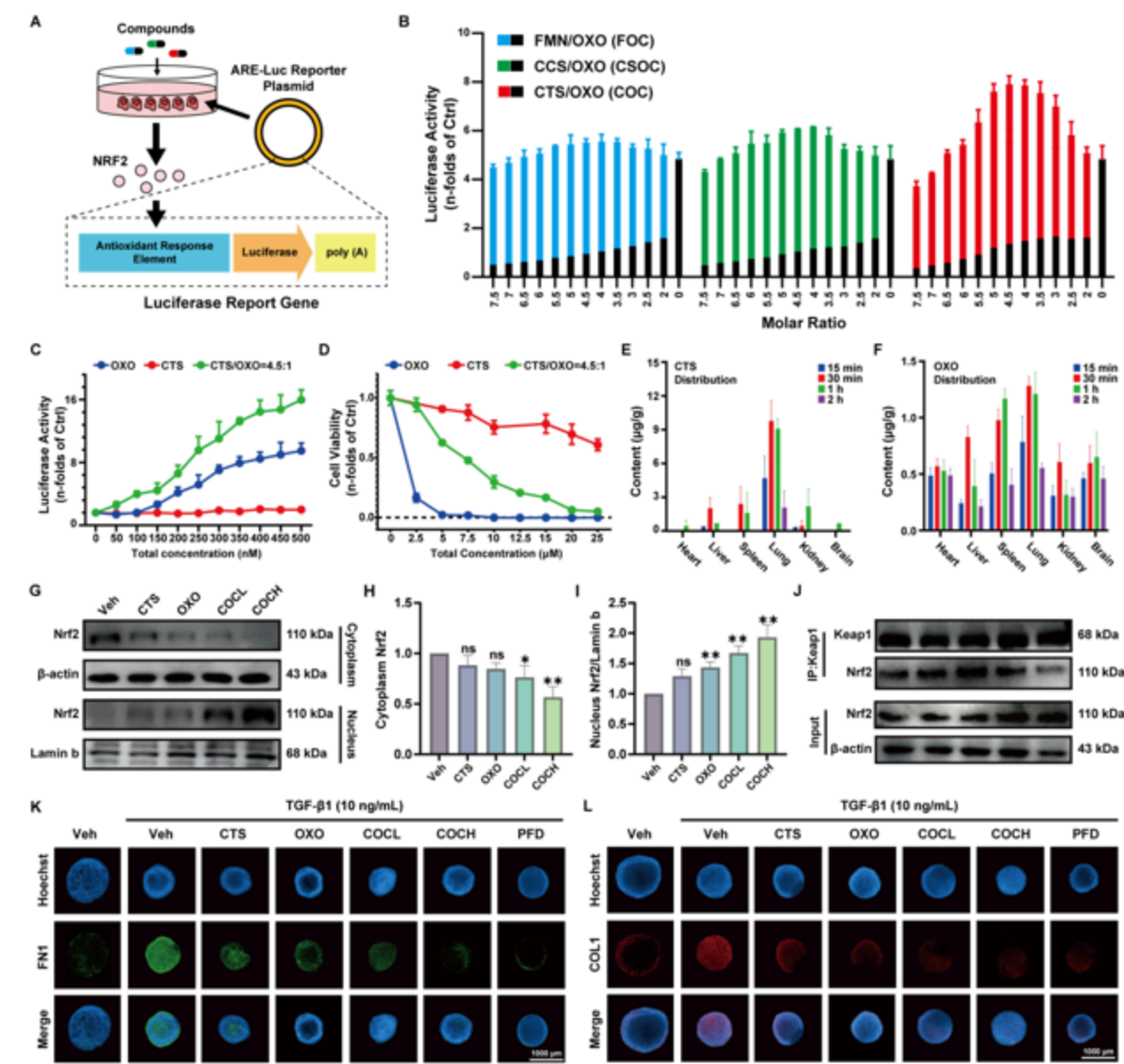


Figure 3

CTS and OXO synergistically activate NRF2 signaling and show potent anti-fibrotic effects *in vitro*. (A) The ARE-luciferase reporter assay used to assess NRF2 agonistic activity. (B) NRF2-agonistic effects of three drug-combinations (FOC, CSOC, and COC) at different ratios. (C) CTS, OXO, and COC dose-dependently activated NRF2 transcriptional activity. (D) Cell viability assays of CTS, OXO, and COC, which

were conducted in HEK293T cells following 24-h incubation. Tissue distribution of CTS (E) and OXO (F) in C57BL/6J mice after intraperitoneal injection of COC at a single dose of 25 mg/kg. (G) Nrf2 protein levels in the cytoplasmic and nuclear fractions of MLE-12 cells treated with vehicle (Veh), CTS (1 μ M), OXO (1 μ M), or COC (low dose COCL, 0.5 μ M; high dose COCH, 1 μ M) were evaluated. (H and I) Quantitative analysis of Nrf2 band intensities was conducted using ImageJ software. (J) Co-immunoprecipitation (Co-IP) analysis of Keap1-Nrf2 complex formation was performed in MLE-12 cells treated with vehicle (Veh), CTS (1 μ M), OXO (1 μ M), a low-dose COC (COCL, 0.5 μ M), or a high-dose COC (COCH, 1 μ M). Immunoprecipitation was conducted using anti-Keap1 or anti-Nrf2 antibodies, and anti- β -actin antibody was used as a control. (K and L) Immunofluorescence staining was used to detect and quantify the MFI of FN1 and COL1 expression in mouse lung organoids treated with vehicle (Veh), CTS (1 μ M), OXO (1 μ M), a low-dose COC (COCL, 0.5 μ M), or a high-dose COC (COCH, 1 μ M). Data in B-I, K, and L, $n = 3$. Data are presented as mean $\pm$ standard deviation. Statistical significance: $*P < 0.05$, $**P < 0.01$ vs. Veh group; *ns*, not significant (one-way ANOVA).

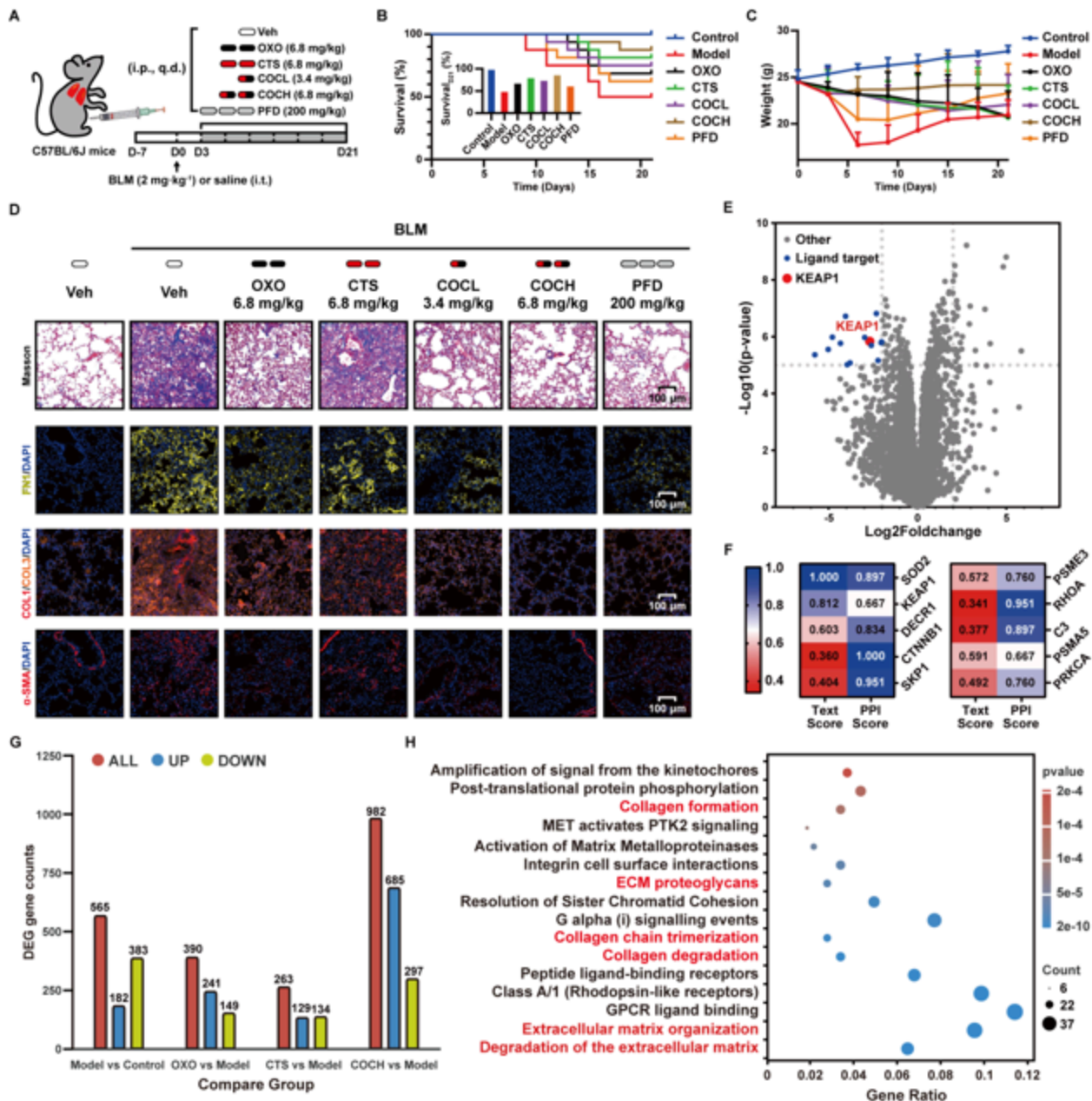


Figure 4

COC targets KEAP1 and shows synergistic anti-fibrotic effects *in vivo*. (A) Experimental timeline: male C57BL/6J mice received a single intratracheal instillation (i.t.) of BLM on day 0. From day 3 onward, daily intraperitoneal (i.p.) injections of vehicle, CTS (6.8 mg·kg⁻¹), OXO (6.8 mg·kg⁻¹), low-dose COC (COCL, 3.4 mg·kg⁻¹), high-dose COC (COCH, 6.8 mg·kg⁻¹), or PFD (200 mg·kg⁻¹) were administered. (B) Kaplan-Meier survival curve presented the survival proportion up to 21 days post-BLM, the final survival rate at day 21 was indicated. (C) Body weight changes were measured every 3 days from day 0 to 21. (D) Representative micrographs of 5 μm lung sections obtained on day 21 were shown, including Masson's trichrome staining, immunofluorescence for FN1 (yellow), dual immunolabeling for COL1 (red) and collagen-3 (COL3, orange), and immunolabeling for α-SMA (red). Nuclei were counterstained with 4',6-

diamidino-2-phenylindole (DAPI, blue). Scale bar: 100 μ m. (E) A volcano plot displayed the binding targets regulated by COCH vs. Model, as determined by PELSA. Ligand-stabilized peptides ($-\log_{10}(\text{pvalue}) \geq 5$, $\log_2(\text{fold change}) \leq -2$) were shown as blue dots, the KEAP1 peptide as a red dot, and other quantified peptides as grey dots. (F) The top 10 targets from PELSA data (COCH vs. Model), ranked by OTTER scores and filtered for "oxidative stress," were listed. (G) The counts of differentially expressed genes (DEGs) ($-\log_{10}(\text{pvalue}) \geq 5$, $|\log_2(\text{fold change})| \geq 2$) were presented for Model vs. Control, OXO vs. Model, CTS vs. Model, and COCH vs. Model. (H) Reactome pathway enrichment analysis was performed for DEGs identified in the COCH vs. Model comparison.

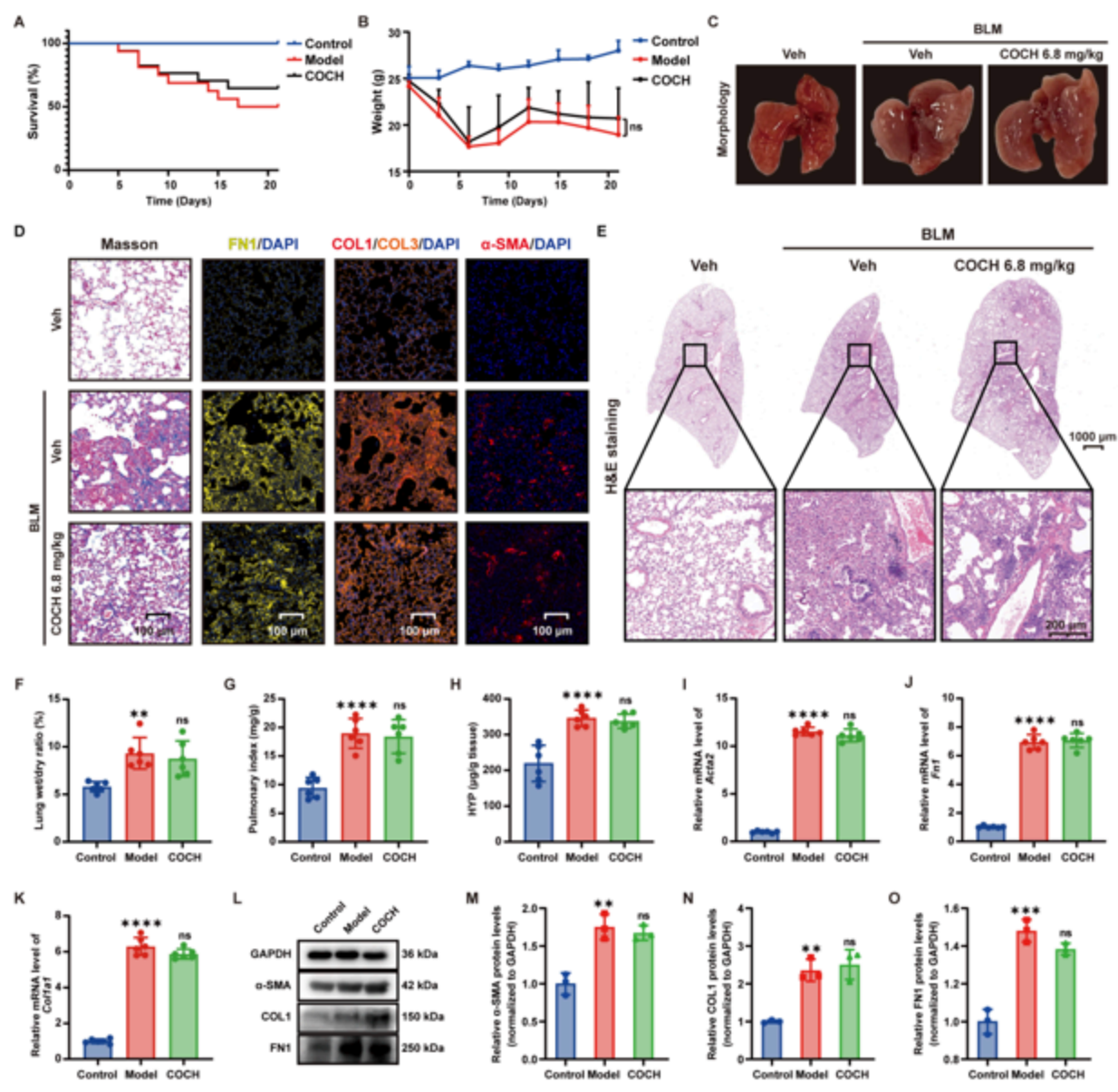


Figure 5

COC ameliorates PF *via* activation of Nrf2 signaling *in vivo*. (A) Kaplan-Meier survival curves for *Nrf2*^{-/-} mice 21 days post-BLM, with final survival rate at day 21. (B) The changes in body weights were

measured every 3 days from day 0 to 21 in *Nrf2*^{-/-} mice. (C) Representative gross lung images from *Nrf2*^{-/-} mice at day 21 (cranial-caudal view). (D) Representative micrographs of lung sections (5 μm) from *Nrf2*^{-/-} mice at day 21: Masson's trichrome; immunofluorescence for FN1 (yellow); dual immunolabeling for COL1 (red) and COL3 (orange); immunolabeling for α-SMA (red). Nuclei counterstained with DAPI (blue). Scale bar: 100 μm. (E) Representative H&E-stained lung sections from *Nrf2*^{-/-} mice at day 21. Scale bar: 1000 μm (overview) and 200 μm (detail). (F) Pulmonary edema assessed by wet-to-dry weight ratio. (G) Lung index: (lung wet weight / body weight) × 100. (H) HYP content in lung tissue. (I-K) Relative mRNA levels of *Acta2*, *Fn1*, and *Col1a1* in lung tissue. (L) Western blot analysis of α-SMA, COL1, and FN1 in lung tissue. (M-O) Quantification of western blot band intensity using ImageJ. Data in A-K: n = 6, and data in L-O: n=3. Data are mean ± standard deviation. ##*P* < 0.01, ####*P* < 0.0001 vs. Control group; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs. Model group; *ns*: not significant (one-way ANOVA).

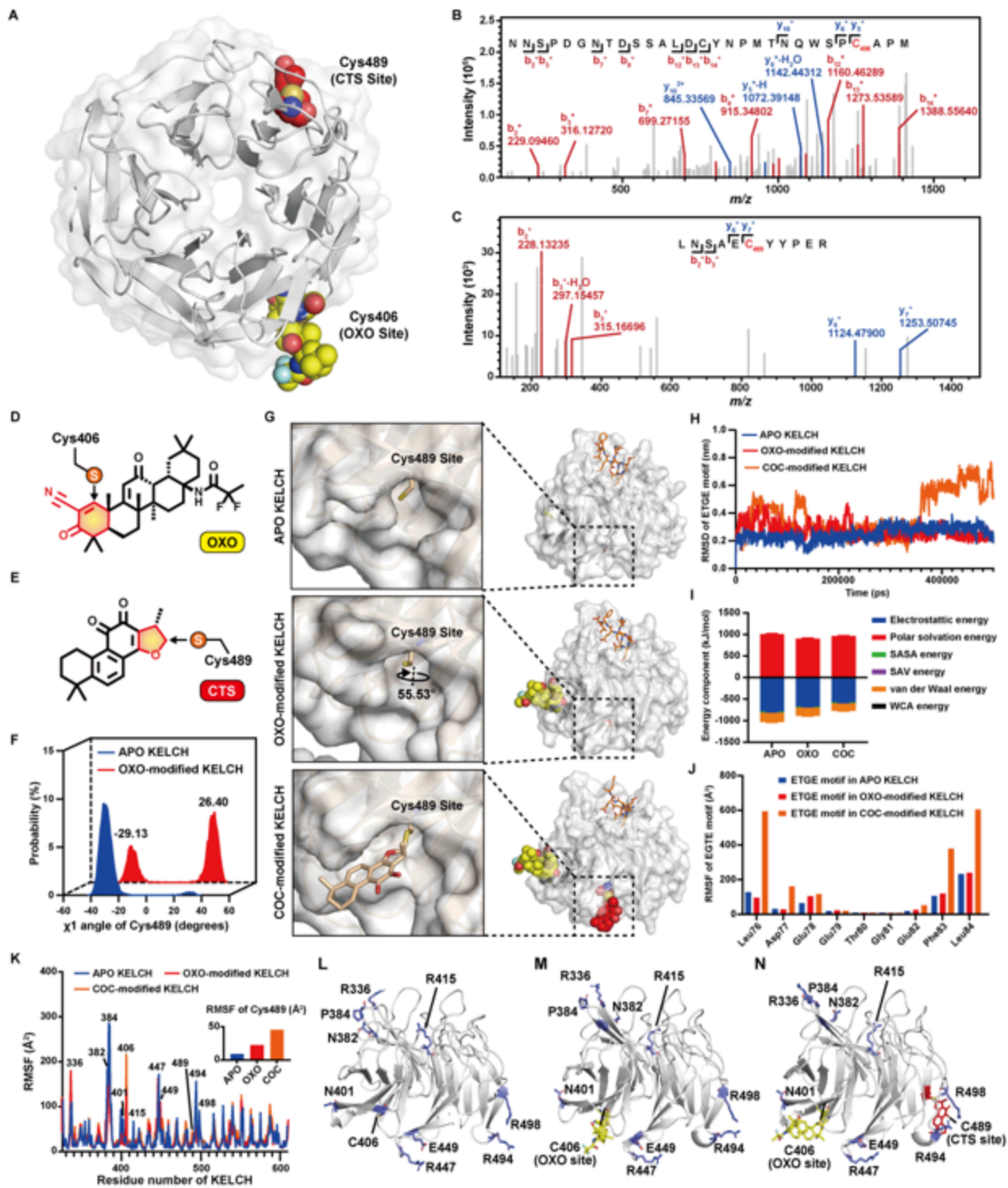


Figure 6

The unique hetero-bivalent engagement mechanism of COC. (A) Overview of KELCH domain modified with OXO at Cys406 and CTS at Cys489. (B and C) Bottom-up proteomics identified OXO- and CTS-modified peptides at Cys406 and Cys489, respectively. (D and E) The proposed KEAP1-modification mechanisms of OXO and CTS. (F) χ_1 -dihedral angles of Cys489 in APO- vs. OXO-modified KELCH. (G) Structural comparison revealed that OXO modification induced a 55.53° rotation of the Cys489 thiol group, which facilitated the subsequent modification by CTS at the thiol group of Cys489. (H) RMSD of

NRF2 ETGE motif bound to APO, OXO-modified, or COC-modified KELCH. (I) MM-PBSA binding free energies for ETGE motif with APO-, OXO-, and COC-modified KELCH. (J) RMSF of ETGE motif during 500-ns simulations with each KELCH variant. (K) RMSF of each KELCH variant during 500-ns simulations, highlighting the fluctuations of Cys489 specifically. (L-N) Structures of APO KELCH (L), OXO-modified KELCH (M), and COC-modified KELCH (N). Proteins in white cartoon; OXO (yellow) and CTS (red) as sticks. Residues with significant RMSF changes in slate blue.

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