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Neo-cysteine Molecular Glues for Targeting Mutated SMAD4 Protein

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Abstract

Neo-protein-protein interactions (neoPPIs), directed by genetic mutation-encoded neo-amino acid residues, represent a promising class of precision medicine targets. Small molecules can mimic genetic mutational effects, creating neo-surfaces and acting as molecular glues to mediate neoPPIs and reprogram biological circuitry. This convergence of genomic alterations and chemical interventions highlights a strategy for targeting disease-associated mutations using neo-amino acid residue-directed molecular glues. Among these, neo-cysteine at the protein-protein interaction (PPI) interface represents unique opportunities to develop covalent molecular glues. Despite this promise, identifying neo-cysteine molecular glues (neoCMGs) remains challenging. Here, we report the discovery of a neoCMG through a systematic unbiased chemical screening approach, using SMAD4, a frequently mutated tumor suppressor gene, as a model system. We established a robust PPI biosensor assay for high-throughput chemical screening, leading to the identification of neoCMG101. Biophysical and biochemical characterization revealed that neoCMG101 selectively and covalently modifies the neo-C361 residue on SMAD4, enhance SMAD4-R361C/SMAD3 PPI and restore SMAD-dependent transcriptional activity. This work establishes the feasibility of leveraging neo-cysteine-directed molecular glues to restore mutant PPIs, supporting a generalizable strategy for identifying neoCMG hits through unbiased screening.

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Author Contributions

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Declaration of interests

The authors declare no competing interests.

Such an approach provides a framework for targeting mutation-disrupted signaling networks in cancer and other diseases.

Keywords

activity-based protein profiling; covalent drugs; neo-cysteine; molecular glues; protein–protein interactions

Introduction

Neo-protein-protein interactions (neoPPIs) are an emerging paradigm for understanding how genetic mutations drive disease progression and phenotypic diversity^[1, 2]. Mutations often alter protein-protein interaction (PPI) interfaces by introducing neo-amino acid residues, which can create new interaction surfaces or disrupt existing ones. These mutation-encoded neo-residues act as, or induce, unique molecular epitopes, enabling interactions that wild-type proteins cannot mediate^[2]. Such neoPPIs can rewire signaling pathways, leading to altered cellular behavior, oncogenic transformation, and therapeutic resistance¹. The concept of neoPPIs highlights the intersection of genetic alterations and protein network dynamics, presenting a framework to investigate mutation-specific disease mechanisms. As these interactions are uniquely associated with the mutated state, neoPPIs offer promising avenues for precision medicine, enabling therapeutic strategies that selectively target disease-relevant interactions^[1].

Molecular glues are a novel class of small molecules that induce or stabilize PPIs, reprogramming biological processes through proximity-based pharmacological mechanisms^[3–7]. These molecules bind to one or both proteins at the PPI interface, enhancing molecular interactions or creating neoPPIs. Notably, molecular glues often mimic the effects of genetic mutations, recapitulating neoPPIs driven by mutation-encoded neo-residues^[8, 9]. For example, mutations in proteins such as KBTBD4^[8, 9] or targets of thalidomide analogs^[10] create neo-substrate interfaces that molecular glues emulate to redirect biological processes. By chemically reproducing mutation-driven interactions, molecular glues provide a powerful strategy to selectively target disease-associated pathways, expanding the scope of druggable targets and enabling innovative therapeutic approaches.

The convergence of genomic alterations and chemical interventions in inducing neoPPIs has inspired a promising strategy to discover neo-amino acid-directed molecular glues^[1]. Advances in cancer genomic studies, for example, have revealed a vast landscape of tumor-specific neo-amino acid residues^[11], offering new therapeutic insights for novel precision medicine-based strategies. Among these, neo-cysteine mutations are among the most enriched alterations in both oncogenes and tumor suppressor genes^[12–14]. These mutations are particularly intriguing due to the unique chemical reactivity of the thiol group, which provides opportunities for covalent drug discovery^[12–14]. Covalent drugs targeting cysteines, such as third-generation EGFR tyrosine-kinase inhibitors^[15] and sotorasib targeting KRAS-G12C^[16], have demonstrated significant clinical benefits. However, the inherent reactivity

of cysteines poses selectivity challenges^[17, 18], as off-target effects from undesired cysteine modifications can complicate the design of highly selective covalent drugs.

Structural biology studies revealed that most neo-cysteines are located on the protein surface and cluster at 3D PPI interfaces^[19, 20], often resulting in the disruption of tumor suppressor complexes^[2, 6]. A notable example is the SMAD4-R361C mutation, which disrupts its interaction with SMAD3 and impairs Transforming Growth Factor beta (TGF- β)-mediated tumor suppressive signaling, a pathway critical in many tumor types^[21–23]. In this study, we address the challenge of restoring disrupted PPIs caused by neo-cysteine mutations through the discovery of neo-cysteine-targeted covalent molecular glues (neoCMGs) (Fig. 1A).

Using the SMAD4 R361C mutation as a model, which disrupts the SMAD4-SMAD3 interaction and impairs TGF- β signaling, we developed a high-throughput time-resolved fluorescence resonance energy transfer (TR-FRET) screening platform to identify compounds capable of restoring this critical interaction. Through systematic screening of a chemical library enriched with known covalent cysteine modifiers, we identified a neoCMG molecule that enhances the interaction between SMAD4-R361C and SMAD3. This neoCMG covalently modifies the neo-C361 residue, effectively inducing the neo-complex of SMAD4-R361C with SMAD3. This work provides a proof-of-concept for using neoCMGs to target neo-cysteine residues and restore mutant PPIs, offering a promising strategy for therapeutic intervention in cancers driven by such mutations.

Results and Discussion

Design and Development of the TR-FRET Assay for NeoCMG Discovery

To enable unbiased chemical screening for neoCMG discovery, a sensitive and scalable high-throughput screening (HTS) platform was developed to monitor the differential SMAD4-SMAD3 PPI dynamics induced by the SMAD4 R361C variant. Toward this goal, we established and optimized a TR-FRET assay in a miniaturized 1,536-well plate format^[6, 24, 25]. The assay uses cell lysates co-expressing His-SMAD4 and Flag-SMAD3, coupled with anti-Flag-Tb and anti-His-D2 antibodies as the donor-acceptor fluorophore pair. In the presence of anticipated neoCMG molecules that induce direct PPI between SMAD4 R361C and SMAD3, the Tb and D2 fluorophores are expected to be brought into close proximity, facilitating energy transfer and the TR-FRET signal generation (Fig. 1B). The cell lysate-based approach not only simplifies assay preparation by eliminating labor-intensive protein purification but also maintains a physiologically relevant environment, which is critical for triaging false-positive hits from non-specific pan-cysteine covalent modifiers.

To evaluate the assay sensitivity in detecting neoCMG-induced changes, we first examined its ability to capture the effect of neo-C361 mutation-driven alterations on the SMAD4-SMAD3 interaction. Using the same protein tags and fluorophore-conjugated antibodies, we compared TR-FRET signals between wild-type (WT) SMAD4 and the R361C mutant in complex with SMAD3. The R361C mutation exhibited a significantly diminished TR-FRET signal compared to WT SMAD4 (Fig. 1C), reflecting a severely disrupted PPI. Quantitatively, the assay showed a differential signal-to-background (S/B) ratio of 7-fold for the R361C mutant versus 37-fold for WT SMAD4 (Fig. 1D). These findings reconfirm the

critical role of the R361 residue in SMAD4-SMAD3 complex formation and highlight the disruptive effect of neo-C361 at the PPI interface. Most importantly, our results demonstrate that this TR-FRET assay achieves high sensitivity in monitoring PPI dynamics at single-amino-acid resolution. The robust assay window and reproducibility establish this platform as an effective tool for the discovery of neoCMG molecules capable of restoring SMAD4-SMAD3 interactions.

Identification of a NeoCMG Hit for SMAD4-R361C

To identify small-molecule neoCMGs capable of restoring the SMAD4 R361C-SMAD3 interaction, we utilized the established TR-FRET assay and screened the Emory Cysteine Binders Library (ECBL), comprising 3,200 covalent modifiers known to target cysteine residues. The ECBL was curated for drug-like properties^[26], and includes diverse thiophilic warheads many of which are found in clinically approved cysteine-targeting drugs^[17, 27, 28]. For the primary screen, we used the His-SMAD4 R361C and Flag-SMAD3 pair under conditions optimized for maximal differential detection sensitivity. Specifically, the EC₉₀ (90% maximal effective concentration) condition for the mutant SMAD4^{R361C}-SMAD3 interaction was selected, which corresponded to the EC₁₀ (10% maximal effective concentration) of the WT SMAD4/SMAD3 PPI. Under this condition, the assay demonstrated robust performance with a signal-to-background (S/B) ratio of 4-fold (Fig. 1E) and a Z' factor exceeding 0.6 across three independent 1,536-well plates (Fig. 1F).

Using these optimized conditions, we performed a primary screen by treating cell lysates co-expressing His-SMAD4 R361C and Flag-SMAD3 with 20 μ M (final) of each ECBL compound (2% final DMSO concentration). Compounds exhibiting >2-fold enhancement in TR-FRET signal relative to the DMSO control were prioritized, resulting in 18 top-ranked primary neoCMG hits for dose-response (DR) studies (Fig. 2A). In TR-FRET DR studies, molecular glue activity was quantified by area under the DR curve (AUC), fold-of-change (FOC) of AUC, and statistical significance (p-values) compared to the DMSO control. From these studies, 13 out of the 18 primary hits demonstrated significant and reproducible dose-dependent molecular glue activity, with FOC > 1.4 and $p < 0.0001$ (Fig. 2B). Hits lacking potency or consistent effects were eliminated at this stage.

To validate and further prioritize these DR-confirmed hits, we evaluated their molecular glue activity using an orthogonal, non-fluorescent GST-pulldown assay. This assay incorporates multiple washing steps to identify hits with strong molecular glue activity. Among the 13 DR-positive hits, compound #3 exhibited robust and reproducible activity in the GST-pulldown assay, with an FOC of 2.41 ± 0.29 -fold and p-value of 0.001 (Fig. 2C–D). Based on these findings, compound #3 was designated as neoCMG101. In the TR-FRET dose-response studies, neoCMG101 demonstrated a dose-dependent molecular glue activity with an EC₅₀ (50% maximal effective concentration) of 3.27 ± 0.40 μ M (Fig. 2E). Consistent dose-dependent activity was also observed in GST-pulldown DR experiments (Fig. 2F), and importantly, neoCMG101 did not induce abnormal hyper-interaction between the WT SMAD4 and SMAD3 (Fig. S1A), confirming both its efficacy and selectivity toward the mutant complex. Together, these results establish neoCMG101 as a potent and selective molecular glue capable of restoring the SMAD4^{R361C}-SMAD3 interaction in biochemical

assays, prompting us to next evaluate whether this restoration translates into functional recovery of SMAD-dependent signaling in a cellular context.

To determine whether the molecular glue activity of neoCMG101 observed in biochemical assays translates into functional restoration of SMAD4 signaling in cells, we employed a SMAD-binding element 4 (SBE4) luciferase reporter assay, which quantitatively measures SMAD4/SMAD3-dependent TGF β transcriptional activity^[29, 30]. HEK293 cells stably expressing the SBE4-luciferase reporter were co-transfected with SMAD3 and either SMAD4-WT or the SMAD4-R361C mutant and treated with TGF β in the presence or absence of neoCMG101. As shown in Figure 2G, SMAD4-WT cells exhibited robust TGF β -induced reporter activation, whereas SMAD4-R361C cells displayed markedly diminished activity, consistent with impaired SMAD4-SMAD3 complex formation. Treatment with neoCMG101 significantly restored SBE4-luciferase activity in SMAD4-R361C cells under both basal and TGF β -stimulated conditions, effectively rescuing transcriptional output toward WT levels (Fig. 2G). Consistent results were obtained in a pair of genetically engineered isogenic HCT116 colon cancer cell lines expressing SMAD4 WT or the R361C mutation (Fig. 2H). In addition, we did not observe acute cytotoxicity of neoCMG101 up to 25 μ M in multiple SMAD4-WT epithelial cancer cell lines (Fig. S1B–E). Together, these findings establish neoCMG101 as a potential molecular glue that restores SMAD4^{R361C}-SMAD3 complex formation and reactivates SMAD-dependent transcriptional signaling in cells, prompting detailed biochemical and biophysical investigations to elucidate its direct binding selectivity, covalent modification mechanism, and structural mode of action.

NeoCMG101 Selectively Binds SMAD4-R361C Protein

The cell lysate-based TR-FRET and GST-pulldown assay demonstrated that neoCMG101 induces the proximity of SMAD4-R361C with SMAD3, supporting its potential selectivity for engaging SMAD4-R361C and/or SMAD3 protein in proteome. To confirm direct target engagement, we first evaluated whether neoCMG101 directly binds to SMAD4-R361C and alters its thermal stability. Using purified human recombinant full-length SMAD4 WT and SMAD4 R361C proteins (Fig. 3A), we performed Nano-Differential Scanning Fluorimetry (NanoDSF) to assess thermal denaturation profiles^[31]. SMAD4-WT exhibited a typical melting curve with a melting temperature (T_m) of 62.0 ± 0.1 °C (Fig. 3B–C). In contrast, the SMAD4-R361C mutant showed a significantly lower T_m of 59.5 ± 0.1 °C (Fig. 3C), suggesting possible conformational changes induced by the neo-C361 residue. Upon treatment with neoCMG101, the T_m of SMAD4-R361C increased to 60.9 ± 0.2 °C (Fig. 3C), but not the WT, suggesting direct binding of neoCMG101 and its stabilization effect on the mutant protein.

Given that SMAD4-SMAD3 interaction is SMAD3 phosphorylation-dependent^[32], we next examined whether neoCMG101 binds to phosphomimic SMAD3 (p-SMAD3, Fig. 3D). NanoDSF analysis revealed no significant differences in T_m between p-SMAD3 treated with neoCMG101 (72.3 ± 0.8 °C) and DMSO control (72.6 ± 0.2 °C) (Fig. 3E). These results indicate that neoCMG101 selectively binds to SMAD4-R361C but not to p-SMAD3.

We then investigated whether neoCMG101 binding to SMAD4-R361C could stabilize the SMAD4-SMAD3 complex. Using reconstituted SMAD4-SMAD3 complexes, we measured

T_m values with and without neoCMG101 treatment. The WT SMAD4-SMAD3 complex exhibited a T_m of 65.5 ± 0.1 °C (Fig. 3F), while the R361C mutant complex displayed a significantly reduced T_m of 62.6 ± 0.1 °C (Fig. 3F), consistent with neo-C361 residue-induced complex destabilization. NeoCMG101 treatment increased the T_m of the R361C complex to 63.5 ± 0.1 °C (Fig. 3F), but not the WT, indicating stabilization of the R361C complex. Further comparison of ΔT_m (change in T_m) values between SMAD4 alone and the SMAD4-SMAD3 complex revealed a ΔT_m of 3.4 ± 0.002°C for WT SMAD4, compared to 0.6 ± 0.1°C for SMAD4 R361C (Fig. 3G). Treatment with neoCMG101 increased the ΔT_m of SMAD4 R361C complex to 1.5 ± 0.1°C, but not the WT, further supporting its role in stabilizing the disrupted complex.

SMAD4 is a multidomain protein, with the neo-C361 residue located in the Mad homology 2 (MH2) domain that mediates PPI with SMAD3^[33]. To identify the binding domain of neoCMG101, we examined its engagement using recombinant WT and R361C SMAD4 MH2 domain truncations (Fig. 3H). Similar to full-length SMAD4, the SMAD4-MH2-R361C protein showed a left-shifted melting curve with a significantly lower T_m of 65.5 ± 0.1°C compared to the WT counterpart (67.8 ± 0.1 °C, Fig. 3I–J). Treatment with neoCMG101 further decreased the T_m of SMAD4-MH2-R361C (63.3 ± 0.1 °C, Fig. 3I–J), but not the WT, suggesting direct binding to the MH2 domain containing the neo-C361 residue. Altogether, these results demonstrate that neoCMG101 directly binds to SMAD4-R361C, stabilizes its structure, and enhances its interaction with SMAD3, providing biophysical mechanistic insights into its molecular glue activity.

NeoCMG101 Covalently Modifies the Neo-C361 Residue of SMAD4

To determine whether the direct binding of neoCMG101 to SMAD4-R361C involves covalent cysteine modification and to assess its proteome selectivity, we conducted a competitive alkylation-based chemoproteomic profiling. We reasoned that neoCMG101 covalent engagement would competitively reduce iodoacetamide (IAM)-alkylated peptides^[34]. Indeed, analysis of HEK293T lysates expressing His-SMAD4-R361C (Fig. S2A) and genetically engineered HCT116 SMAD4-R361C colon cancer cells (Fig. S2C) revealed significant enrichment of SMAD4-R361C peptides in neoCMG101-treated samples compared to DMSO controls, suggesting covalent modification and selectivity within the proteome. These results suggest SMAD4-R361C as a potential covalent target of neoCMG101 and provide biochemical evidence for its cysteine-reactive molecular glue activity, prompting further investigation into its underlying covalent modification mechanisms.

NeoCMG101 is a small molecule featuring an aminobenzothiazolone core, an alkyne click handle, an iminoamide, and a sulfonamide group (Fig. 4A), suggesting multiple potential mechanisms to covalently modify cysteine residues.^[18] We hypothesized that thiol of cysteine (Cys-SH) may react with neoCMG101 through three possible mechanisms (Fig. 4A): (i) 1,4-nucleophilic addition^[35] followed by a aminobenzothiazolone ring-opening process, (ii) Thiol–Alkyne Reaction^[36], (iii) 1,2-nucleophilic addition at the iminoamide group^[37], each resulting in distinct forms of the SMAD4-R361C-neoCMG covalent cysteine adducts (Fig. 4A).

To confirm whether neoCMG101 covalently modifies cysteine and to identify the primary mechanism of its covalent reactivity, we performed an Activity-Based Protein Profiling (ABPP) assay^[38–40] to analyze the SMAD4-R361C-neoCMG adducts. If the 1,4-nucleophilic addition is the predominant mechanism, the resulting SMAD4-R361C-neoCMG adduct would retain a free alkyne handle (Fig. 4B), enabling fluorescent labeling via a bio-orthogonal copper-catalyzed alkyne-azide click reaction (CuAAC) with Rhodamine-azide (Rho-N₃)^[41]. In support of this notion, the ABPP assay revealed a neoCMG101 dose-dependent increase in fluorescence signal from the SMAD4-R361C protein (Fig. 4C). To further validate the specificity of this labeling, we performed a competitive ABPP assay using IAM, a pan-cysteine modifier^[34], to compete with neoCMG101 for covalent cysteine modification (Fig. 4B). IAM treatment significantly reduced neoCMG101-mediated fluorescent labeling (Fig. 4D), suggesting that the fluorescence signal arises specifically from neoCMG101-modified cysteines. Together, these results demonstrate the formation of a SMAD4-R361C-neoCMG adduct retaining a free alkyne group, supporting the 1,4-nucleophilic addition as the most likely covalent modification mechanism.

Next, we performed the same ABPP experiments using the SMAD4 MH2 domain truncation, which contains the neo-C361 residue. Consistent with the full-length protein, we observed a dose-dependent increase in fluorescence signal from the SMAD4 MH2 domain upon treatment with neoCMG101 (Fig. 4E), which was abolished by IAM in the competitive ABPP assay (Fig. 4F). These results confirm that neoCMG101 binds and covalently modifies the MH2 domain of SMAD4-R361C most likely through the 1,4-nucleophilic addition mechanism.

To evaluate the selectivity of neoCMG101 for SMAD4-R361C in the presence of SMAD3, we performed an ABPP experiment using a mixture of SMAD4-R361C and p-SMAD3 at a 1:2 molar ratio. As individual protein controls, robust fluorescent labeling was observed for both SMAD4-R361C and p-SMAD3 upon neoCMG101 treatment (Fig. 4G). However, when the two proteins were combined, significant fluorescent labeling was detected for SMAD4-R361C, but not for p-SMAD3 (Fig. 4G). To further evaluate proteome-wide selectivity, we compared neoCMG101 with a broad-spectrum cysteine-reactive probe, iodoacetamide alkyne (IAA), in HEK293T lysates supplemented with His-SMAD4-R361C using ABPP assay. We found that neoCMG101 selectively labeled SMAD4-R361C with minimal off-target reactivity even at 100 μ M, in sharp contrast to the widespread, non-specific proteome labeling observed with 1 μ M IAA (Fig. S2B). Similarly, using isogenic HCT116 SMAD4-R361C cell lysate, we found that neoCMG101 exhibited much less non-specific labeling as compared to IAA (Fig. S2D). These results suggest the selectivity of neoCMG101 for covalently modifying SMAD4-R361C within the SMAD4-SMAD3 complex, further supporting the specificity of the 1,4-nucleophilic addition of neoCMG101 toward the SMAD4-R361C protein.

To determine whether the neo-C361 is covalently modified by neoCMG101, we analyzed the SMAD4-R361C-neoCMG101 adduct using protein mass spectrometry. If neoCMG101 reacts with the neo-C361 residue via a 1,4-nucleophilic addition mechanism, the resulting adduct would retain a free thiol group available for IAM-alkylation

during sample preparation (Fig. 4H). Based on this mechanism, we hypothesized that neoCMG101-modified peptides would exhibit a mass shift of 428 Da, corresponding to the neoCMG101 adduct, or 486 Da, reflecting the neoCMG101-acetamide form (Fig. 4H). Mass spectrometry analysis of both full-length and MH2 domain SMAD4-R361C identified neo-C361 as a major modification site (Supplementary Data 1). For the full-length protein, the mass spectrum revealed a neo-C361-containing peptide (D-C361-FCLGQLSNVHR+IGVDDLRRRLCILR) with a mass shift of 486 Da, consistent with the neoCMG101-acetamide adduct (Fig. 4I). Similarly, for the SMAD4-R361C MH2 domain truncation, the mass spectrum identified a neo-C361-containing peptide (C361-FCLGQLSNVHR+GIGVDDLRRRLCILR) with a mass shift of 428 Da, corresponding to the neoCMG101 adduct (Fig. 4J). These results suggested that neoCMG101 covalently modifies the neo-C361 residue of SMAD4-R361C through the proposed 1,4-nucleophilic addition mechanism (Fig. 4A).

NeoCMG101 Restores SMAD4-R361C–SMAD3 Interaction by Re-Establishing Critical Electrostatic Interactions

To assess the structural basis of neoCMG101's molecular glue activity, we performed computational molecular modeling studies to analyze the interaction between SMAD4-R361C and SMAD3 in the presence of neoCMG101. As shown in the wild-type SMAD4-SMAD3 MH2 domain complex crystal structure, the R361 residue of SMAD4 forms complex-stabilizing salt bridge and hydrogen bond with D408 of SMAD3 (Fig. 5A). In contrast, the AlphaFold-predicted structure of the SMAD4-R361C-SMAD3 complex revealed that the SMAD4-R361C mutation disrupts this interaction by increasing the distance between SMAD4-C361 and SMAD3-D408 to 6.74 Å, thereby destabilizing the complex (Fig. 5B). Covalent docking followed by molecular dynamics simulations suggested that neoCMG101 covalently modifies the neo-C361 residue, adopts an open and extended conformation, and re-establishes the disrupted SMAD4-R361C and SMAD3 interface (Fig. 5C). Specifically, the sulfonamide group of neoCMG101 mimics the R361 residue, re-forming electrostatic interaction with D408 of SMAD3, while introducing new stabilizing interactions with K409 of SMAD3 through additional hydrogen bond (Fig. 5C–D). These results predicted a structural basis for the molecular glue activity of neoCMG101, demonstrating its ability to restore protein-protein interactions disrupted by the SMAD4-R361C mutation.

To confirm this predicted model, we examined the impact of D408 and K409 residues of SMAD3 on neoCMG101's molecular glue activity. We performed site-directed mutagenesis to generate single (D408A, K409A) and double (D408A/K409A) mutants of SMAD3 and evaluated their interaction with SMAD4-R361C in the presence of neoCMG101 using GST pull-down assays. Consistent with the computational predictions, mutation of D408 and/or K409 on SMAD3 significantly compromised the neoCMG101-induced stabilization of the SMAD4-R361C/SMAD3 interaction (Fig. 5E–F). These results further suggest that neoCMG101 re-establishes critical electrostatic interactions with D408 and K409 residues of SMAD3, which are essential for its molecular glue activity in stabilizing the mutation-disrupted SMAD4-R361C/SMAD3 complex.

Characterization of neoCMG101 analogs for initial SAR evaluation

The structural modeling suggested that the sulfonamide group of neoCMG101 is essential for the molecular glue activity (Fig. 5C–D). In contrast, the benzothiazole ring on the opposite end does not directly engage in significant interactions at the protein interface, suggesting potential room for optimization. To explore structure-activity relationships (SAR), we designed two neoCMG101 analogs, neoCMG102 and neoCMG103 (Fig. 6A). They share a common aminobenzothiazolone core structure, the sulfonamide group and the alkyne handle, but differ in their substituted aromatic rings: neoCMG102 features a naphthalene moiety, while neoCMG103 contains a cyano-substituted phenyl group (Fig. 6A). These modifications allow us to explore how the distinct electronic, steric and hydrophobicity profiles of the aromatic ring substitutions may alter the essential covalent binding to neo-C361 and the molecular glue activity.

To determine whether these aromatic ring substitutions affect binding capability, we performed thermal shift assays using both full-length SMAD4-R361C and the MH2 domain truncation. NeoCMG101, 102, and 103 all induced comparable thermal stabilization of both the full-length SMAD4-R361C protein and the MH2 domain truncation (Fig. 6B–C), suggesting that the aromatic ring substitutions did not impair their biophysical binding to SMAD4-R361C. Next, we assessed whether the analogs retained their covalent thiol-reactivity. Leveraging the shared alkyne handle in ABPP assays, we observed robust fluorescent labeling signals for neoCMG102 and 103, comparable to those of neoCMG101, in both the full-length SMAD4-R361C protein and the MH2 domain truncation (Fig. 6D–E). These findings indicate that the aromatic ring substitutions in neoCMG102 and 103 preserve their biochemical activity to covalently modify cysteine residues on SMAD4-R361C.

While neoCMG102 and 103 retained comparable biophysical binding and covalent thiol-reactivity to neoCMG101, their distinct aromatic substitutions may still lead to altered molecular conformations or orientations at the PPI interface, potentially impacting their molecular glue activity. To explore how these substitutions influence their molecular glue activity, we conducted molecular dynamics (MD) simulations^[42] to examine potential conformational differences among neoCMG101 and its analogs when bound to the SMAD4-R361C/SMAD3 complex. The Root Mean Square Deviation (RMSD) analysis at binding site residue suggested distinct interface dynamics between neoCMG101 and its analogs (neoCMG102 and 103) when bound to SMAD4-R361C. NeoCMG101 showed a high RMSD values, suggesting a dramatic conformational change induced by neoCMG101 that re-establishes the SMAD4-R361C-SMAD3 interface (Fig. 6F). In contrast, neoCMG102 and 103 exhibited lower RMSD values, suggesting a less dramatic conformational change which prevents effective interface restoration (Fig. 6F). These *in silico* results indicate that neoCMG102 and 103 may primarily adopt a closed and folded conformation, preventing the re-establishment of the SMAD4-R361C and SMAD3 interface (Fig. 6G).

To experimentally validate the computational prediction, we performed TR-FRET dose-response assays comparing neoCMG101 with neoCMG102 and 103 (Fig. 6H). Consistent with modeling predictions, neoCMG102 and 103 exhibited significantly reduced molecular glue activity, suggesting that their aromatic substitutions did not engage additional stabilizing residues but instead constrained their conformational and orientational flexibility,

impairing their ability to restore SMAD4-R361C/SMAD3 interactions. To further confirm whether altered steric hindrance and hydrophobicity at the aromatic ring substitution consistently impair molecular glue activity, we tested seven additional neoCMG101 analogs bearing diverse aromatic substitutions and observed similarly decreased molecular glue efficacy across all analogs (Supplementary Data 2). These results underscore the critical role of optimal molecular conformation in determining molecular glue efficacy and highlight the precise structural requirements for designing effective neo-cysteine-targeted molecular glues.

Discussion

Protein-protein interactions are central to maintaining signaling networks that regulate essential cellular processes, including proliferation, differentiation, and apoptosis^[1, 43]. Perturbation of these interactions by genetic mutations is a hallmark of cancer development and therapeutic resistance, positioning PPIs as critical yet challenging targets for drug discovery^[1, 20, 44]. Hypomorphic PPIs (hypoPPIs), caused by mutations that weaken or disrupt interaction interfaces, represent an underexplored therapeutic opportunity due to the inherent difficulty of stabilizing transient or destabilized interactions^[6]. In this study, we established a systematic, unbiased screen for neo-cysteine-targeted covalent molecular glues (neoCMGs), expanding our earlier platform that identified hypoPPI-directed molecular glues.^[6] Using the SMAD4-R361C mutation as a prototypical model, we demonstrated that neoCMG101 covalently modifies the neo-C361 residue and stabilizes its interaction with SMAD3. This work highlights neoCMG101 as a proof-of-concept for leveraging mutation-specific cysteine reactivity to address hypoPPIs and underscores the therapeutic potential of neoCMGs as a precision oncology strategy.

Molecular glues have emerged as a transformative modality for drug discovery, enabling the modulation of PPIs through stabilization or the creation of neo-protein-protein interactions (neoPPIs)^[3–5]. These small molecules reprogram biological pathways by inducing proximity between proteins, thereby expanding the druggable proteome to include targets once considered “undruggable.” Covalent molecular glues advance this concept further by utilizing electrophilic warheads to selectively and stably modify specific residues^[45–47], such as cysteine, allowing for prolonged activity and precise control of protein interactions. NeoCMG101 exemplifies this paradigm by selectively modifying the neo-C361 residue in SMAD4-R361C, restoring its interaction with SMAD3 and offering a framework for targeting other mutation-driven disruptions. Recent advances, such as template-assisted covalent stabilization^[46, 48] and cysteine-targeted warheads^[17], illustrate the growing versatility of covalent molecular glues. For instance, a 14-3-3 stabilizer covalently modifying cysteine residues has been shown to enhance PPIs, demonstrating how covalent glues can modulate complex signaling networks^[48, 49]. Similarly, EN450 targeting NF- κ B exemplifies the potential of covalent glues to reprogram protein behavior by inducing neoPPIs^[47]. By integrating chemical reactivity with structural specificity, neoCMG101 expands the application of molecular glues to mutation-directed therapeutic opportunities.

A distinguishing feature of neoCMG101 is its mutation selectivity, a significant advancement over existing covalent molecular glues that typically lack mutation specificity

and target residues irrespective of their disease context. NeoCMG101 selectively engages the neo-C361 residue introduced by the SMAD4-R361C mutation, providing a mutation-directed strategy for re-engineering disrupted PPIs. This mutation-specific approach not only restores SMAD4-R361C and SMAD3 interaction but also underscores the potential of combining cancer genomics and structural insights to identify actionable neo-cysteines. Similar approaches have been taken for developing KRAS-G12C inhibitors through G12C-directed molecular glue to recruit FKBP12 and cyclophilin, which has been showing promising pre-clinical efficacies^[50]. The cancer genome sequencing has revealed a wealth of neo-cysteine mutations^[13, 14], particularly in tumor suppressor genes, where loss-of-function mutations disrupt critical PPIs^[11, 19, 20]. Additional examples include FBXW7-R465C/CCNE in colon and endometrial cancers^[2, 51, 52], KEAP1-R470C/NRF2 in lung cancer^[53], and SPOP-Y87C/BRD4 in prostate cancer^[2, 54, 55], which further illustrate the potential for targeting neo-cysteines at PPI interfaces across diverse malignancies using covalent molecular glues. By addressing these neo-cysteines, neoCMGs offer a scalable and precise framework for targeting mutation-driven vulnerabilities, extending the utility of covalent molecular glues beyond canonical targets^[56].

Cysteine-targeting covalent warheads remain a cornerstone of covalent drug discovery due to the chemical adaptability of the thiol group^[12, 13, 17, 57]. Representative warheads include acrylamides, which form covalent bonds through Michael addition and are widely used in FDA-approved drugs like osimertinib^[58]. Other classes, such as nitriles and cyanoacrylamides, provide enhanced reactivity and hydrolytic stability^[17, 18, 59], while heteroaromatic compounds like benzoxazines and diazoketones broaden the chemical space for cysteine covalent inhibitors^[17, 18, 59]. These diverse warhead chemistries reflect a range of reactivity mechanisms, including 1,4-conjugate addition, nucleophilic aromatic substitution, and strain-release reactions. NeoCMG101 introduces a novel aminobenzothiazole-based covalent warhead that engages the neo-C361 residue via a proposed 1,4-nucleophilic addition followed by ring opening. Proteome-wide cysteinome profiling further revealed that neoCMG101 exhibits favorable selectivity toward the neo-C361 site, supporting its mutation-specific covalent engagement and fine-tuned thiol reactivity. In addition to SMAD4-R361C, our chemoproteomic analyses also revealed several potential off-target hits, such as HSP90, HSC70, CMBL and DAZAP1 (Fig. S2A&C, Supplementary Data 3–4). These results need to be further validated and may provide valuable information for guiding future SAR optimization to further refine scaffold selectivity. This covalent modification expands the understanding of cysteine reactivity and provides a new framework for designing selective covalent molecular glues. Further mechanistic validation is ongoing to confirm the proposed reaction pathway. By integrating mutation-specific targeting with innovative warhead chemistry, our findings highlight the potential of aminobenzothiazole-derived warheads for addressing the complex challenges of cysteine covalent drug discovery.

Covalent drugs have achieved significant clinical success, as demonstrated by osimertinib for EGFR T790M-mutant lung cancer^[58], ibrutinib for BTK-driven malignancies^[60], and sotorasib for KRAS G12C-driven cancers^[16, 61, 62]. These drugs exemplify the power of leveraging cysteine's reactivity for selective, irreversible targeting of disease-relevant proteins. Advances in structure-guided design and chemoproteomics

have enabled the rational discovery of covalent inhibitors with exceptional specificity, addressing longstanding concerns about off-target effects^[17, 28]. Our work builds on these advancements by introducing neoCMG101 as a mutation-directed covalent molecular glue. By targeting neo-cysteines such as SMAD4-R361C, neoCMG101 establishes a new approach for addressing hypoPPIs, paving the way for a next-generation strategy in precision oncology. This study highlights the potential of integrating cancer genomics, structural biology, and covalent warhead chemistry to unlock new therapeutic opportunities in mutation-driven cancers. Beyond restoring hypoPPIs, the neoCMG framework also lays the chemical foundation for engineering mutation-selective proximity-based systems—such as PROTACs^[63], RIPTACs^[64], LOCKTACs^[65], and TCIPs^[66], enabling allele-specific targeting and functional modulation of mutant proteins with improved precision.

Conclusion

This study introduces a generalizable concept and approach for discovering mutation-directed neo-cysteine covalent molecular glues that restore disrupted protein-protein interactions. Using SMAD4-R361C as a model, we identified neoCMG101, a first-in-class neo-cysteine-targeted molecular glue that covalently modifies the neo-C361 residue through a unique 1,4-nucleophilic addition and ring-opening reaction. NeoCMG101 selectively stabilizes the SMAD4-R361C/SMAD3 complex and restores downstream TGF β /SMAD signaling in cells, demonstrating its mutation-specific molecular glue activity. The discovery of neoCMG101 establishes a broadly applicable concept for targeting mutation-induced hypoPPIs through neo-residue-directed covalent chemistry. This work expands the chemical and biological space of “undruggable” targets and highlights the potential of neo-cysteine molecular glues as precision tools to re-engineer disease-altered signaling networks. Future optimization of scaffold design and electrophilic warhead chemistry may further enable the rational development of mutation-selective therapeutics in cancer and other disorders driven by pathogenic PPIs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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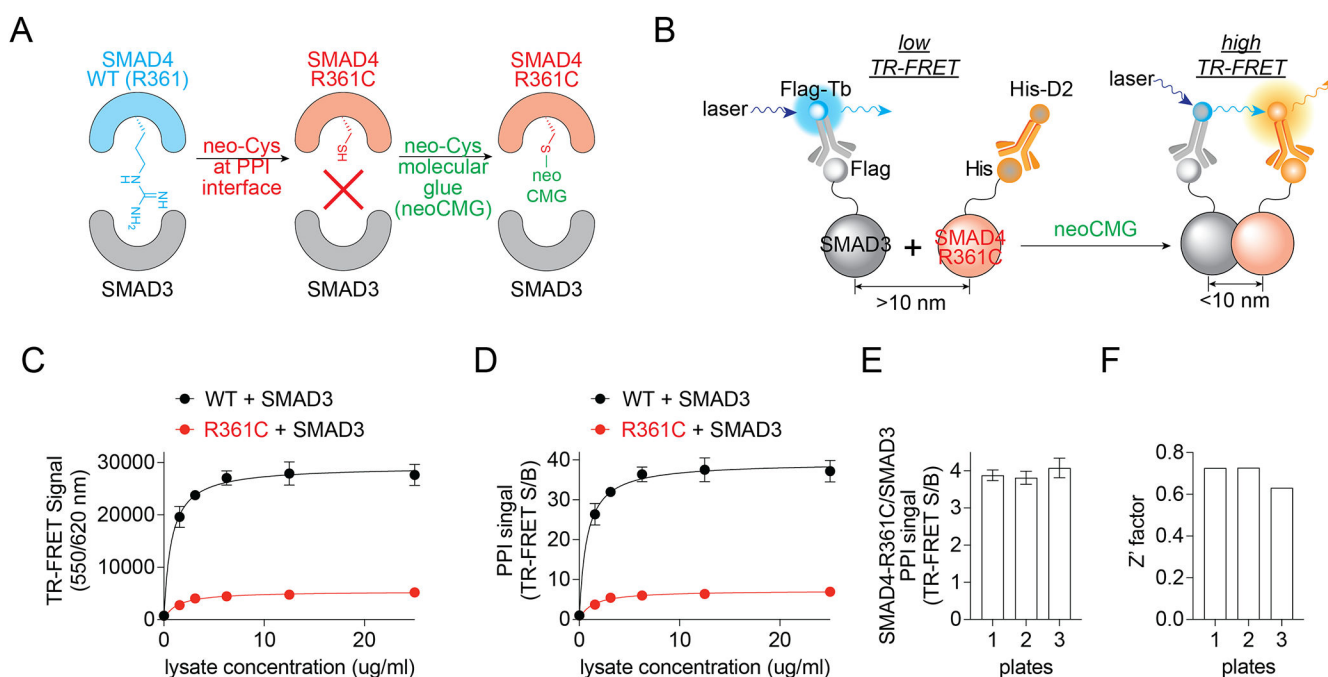
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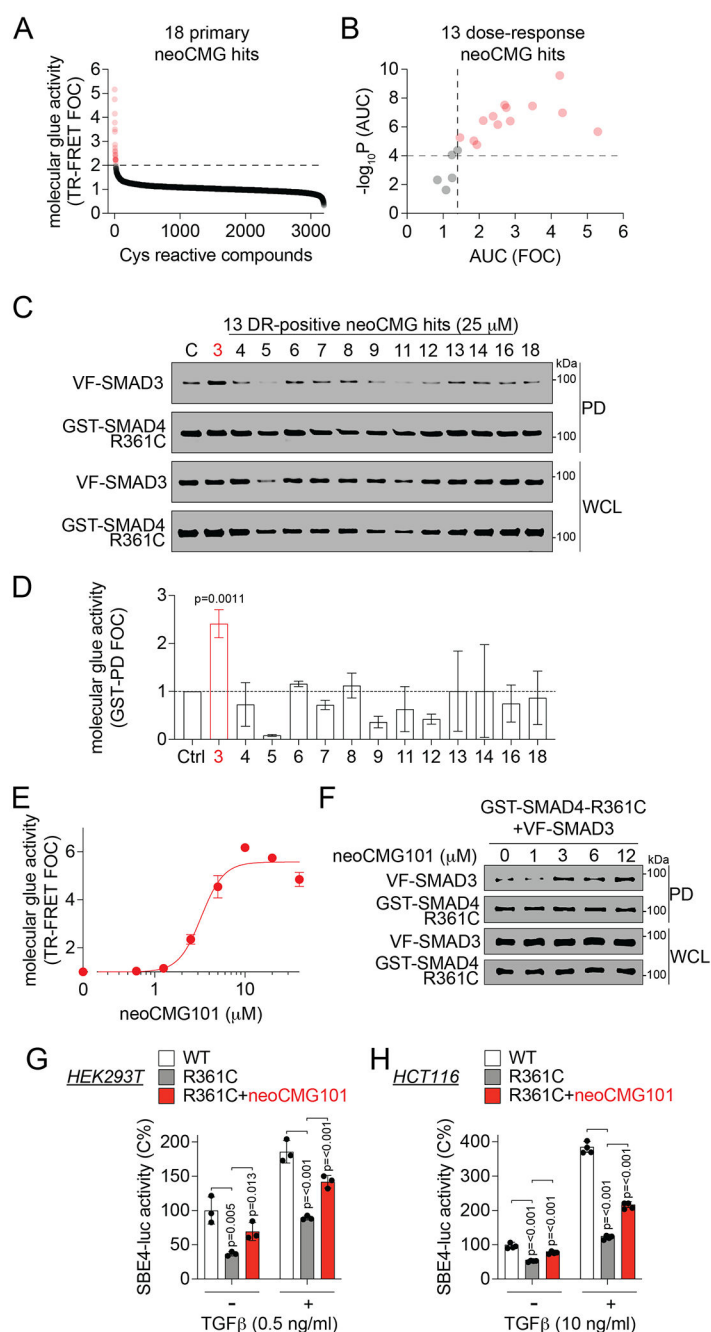
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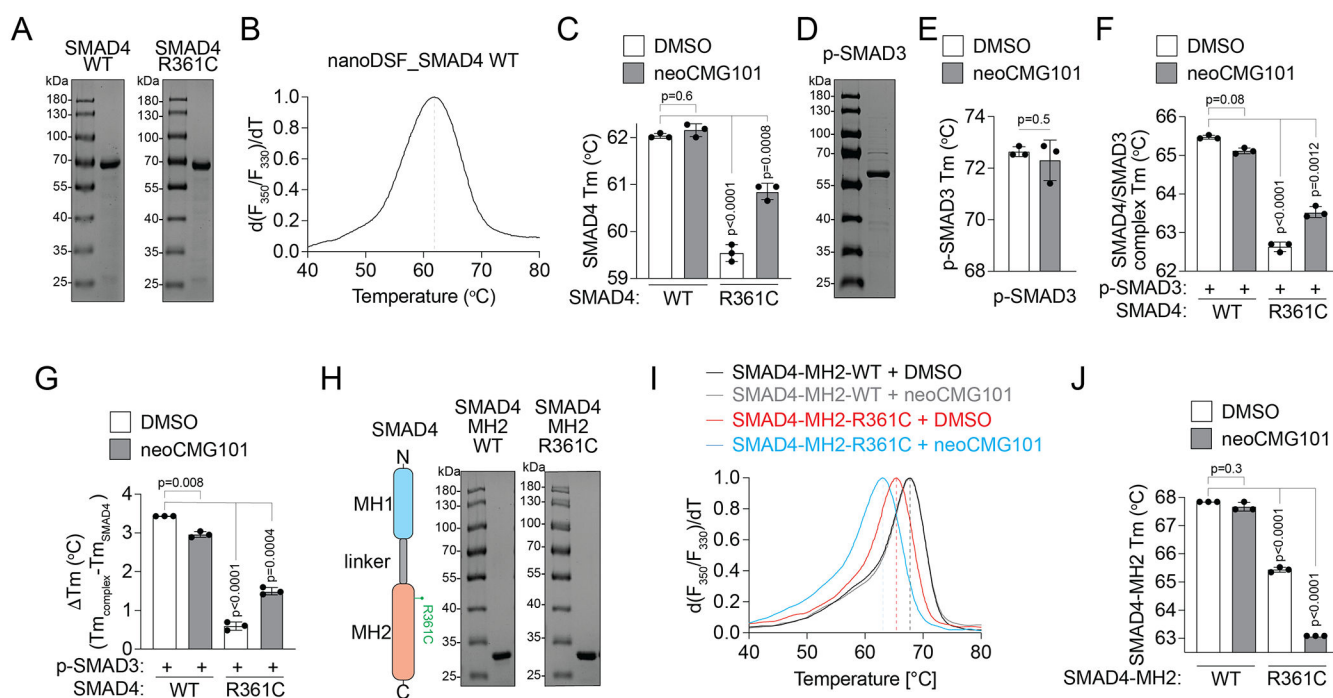
**Figure 1.**

Development of the TR-FRET assay for neoCMG discovery. (A) Schematic illustration of the concept of neo-cysteine molecular glue (neoCMG), designed to target the SMAD4-R361C mutant and stabilize its interaction with SMAD3. (B) Schematic representation of the TR-FRET assay for monitoring SMAD4/SMAD3 protein-protein interactions (PPIs) and discovering small-molecule PPI inducers. Anti-Flag-Tb coupled with Flag-SMAD3 serves as the TR-FRET donor, while anti-His-D2 coupled with His-SMAD4-R361C serves as the acceptor. At the basal level, the R361C mutation disrupts SMAD4/SMAD3 interaction, resulting in low TR-FRET signals. Upon treatment with a neoCMG, the induced SMAD4-R361C/SMAD3 complex formation brings the two fluorophores into close proximity (<10 nm), generating a high TR-FRET signal. (C) Dose-dependent TR-FRET signals comparing SMAD4-WT and SMAD4-R361C mutants interacting with SMAD3. HEK293T cell lysates expressing His-SMAD4-WT or R361C with Flag-SMAD3, or empty vector controls, were serially diluted as indicated. Data are shown as mean $\pm$ SD from three independent experiments. (D) Dynamic assay window as defined by the signal-to-background ratio (S/B) of dose-dependent TR-FRET signals. HEK293T cell lysates expressing His-SMAD4-WT or R361C with Flag-SMAD3 were serially diluted for TR-FRET assays. Data are presented as mean $\pm$ SD from three independent experiments. (E) Bar graph showing the signal-to-background ratio (S/B) for the TR-FRET signal of His-SMAD4-R361C and Flag-SMAD3 across three 1,536-well plates from the primary screen. Data are expressed as mean values calculated from 16 replicates per plate in the primary screen. (F) Bar graph showing Z'-factor values for the TR-FRET signal of His-SMAD4-R361C and Flag-SMAD3 across three 1,536-well plates in the primary screen.

**Figure 2.**

Identification of neoCMG101 as a molecular glue hit for SMAD4-R361C/SMAD3 PPI. (A) The waterfall plot shows the identification of 18 primary neoCMG hits from the initial TR-FRET screen based on their fold-over-control (FOC) values for TR-FRET signal relative to the DMSO control. Data are presented as the percentage of the DMSO control from the primary screen. (B) The scatter plot illustrates the prioritization of 13 dose-response-positive neoCMG hits. Compounds were tested in a 2-fold serial dilution ranging from 40 μ M to 80 nM in the TR-FRET assay. TR-FRET signals are shown as the area under

the dose-response curve (AUC), and p-values were calculated for each compound relative to the DMSO control. Hits were prioritized based on cutoff values of AUC(FOC) > 1.4 and $p < 0.0001$. (C) Representative western blot images and (D) quantification show the molecular glue activity of neoCMG hits. GST pull-down (PD) assays were performed using cell lysates co-expressing GST-SMAD4-R361C and Venus-Flag-tagged (VF) SMAD3 treated with neoCMG hits at 25 μ M. Western blotting detected VF-SMAD3 in the PD fraction and whole-cell lysate (WCL), and quantified PPI signals are presented as FOC values relative to the DMSO control. Data in panel (D) are shown as mean $\pm$ SD from three independent experiments. (E) The dose-response curve of neoCMG101, corresponding to Compound #3 in panels (C) and (D), shows its effect on enhancing the TR-FRET signal for the SMAD4-R361C/SMAD3 PPI, with data presented as mean $\pm$ SD from three independent experiments. (F) Western blot analysis shows neoCMG101-induced dose-dependent stabilization of the SMAD4-R361C/SMAD3 PPI. Cell lysates co-expressing GST-SMAD4-R361C and VF-SMAD3 were treated with increasing concentrations of neoCMG101, and GST pull-down (PD) and whole-cell lysate (WCL) fractions were analyzed by western blotting to detect VF-SMAD3. (G-H) neoCMG101 restored SMAD4-R361C transcriptional activity in HEK293 (G) and HCT116 (H) cells. HEK293-SBE4-luciferase (luc) reporter stable cells (G) were transiently transfected with FLAG-SMAD3 and His-SMAD4 WT or R361C. HEK293 cells were treated with TGF β (0.5 ng/ml) and/or neoCMG101 at 25 μ M for 18h. Genetically engineered isogenic HCT116 WT and R361C colon cancer cells (H) were transiently transfected with Venus-flag-tagged TGFBR2, SBE4-luciferase, and Renilla luciferase plasmids. HCT116 cells were treated with TGF β (10 ng/ml) and/or neoCMG101 at 5 μ M for 18h. SBE4-luc activity were measured using Dual-Glo luciferase assay system. The data are normalized to the WT sample without TGF β treatment and are presented as mean $\pm$ SD from three (G) or four (H) independent biological replicates. Statistical significance was calculated using an unpaired two-tailed Student's t-test.

**Figure 3.**

Characterization of direct binding of neoCMG101 to SMAD4-R361C protein. (A) SDS-PAGE gel showing the purity of recombinant human SMAD4-WT and R361C full-length proteins. (B) Thermal denaturation curve of SMAD4-WT protein, with the melting temperature (Tm) determined using the nanoDSF assay. Tm values were calculated from the first derivative of the nanoDSF signal. Data are presented as the mean from three independent biological replicates. (C) Bar graph showing Tm values of SMAD4-WT and R361C, with or without neoCMG101 (50 μM) treatment. Data are presented as mean ± SD from three independent biological replicates. (D) SDS-PAGE gel showing the purity of recombinant human phosphomimetic SMAD3 (p-SMAD3), generated by introducing the S423E mutation to mimic phosphorylation. (E) Bar graph showing Tm values of p-SMAD3 with or without neoCMG101 (50 μM) treatment. Data are presented as mean ± SD from three independent biological replicates. (F) Bar graph showing Tm values of p-SMAD3/SMAD4-WT or R361C complexes with or without neoCMG101 (50 μM) treatment. Data are presented as mean ± SD from three independent biological replicates. (G) Bar graph showing the ΔTm values (Tm of the p-SMAD3/SMAD4 complex – Tm of SMAD4 alone) with or without neoCMG101 (50 μM) treatment. Data are presented as mean ± SD from three independent biological replicates. (H) Schematic illustration of SMAD4 domain structure and SDS-PAGE gel showing the purity of recombinant human SMAD4-WT and R361C MH2 domain truncation proteins (amino acids 319-552). (I) Thermal denaturation curves of SMAD4-WT and R361C MH2 domain truncation proteins, with or without neoCMG101 (50 μM). Tm values were determined using the nanoDSF assay. Data are presented as the mean from three independent biological replicates. (J) Bar graph showing Tm values of SMAD4-WT and R361C MH2 domain truncations, with or without neoCMG101 (50 μM) treatment. Data are presented as mean ± SD from three independent biological replicates.

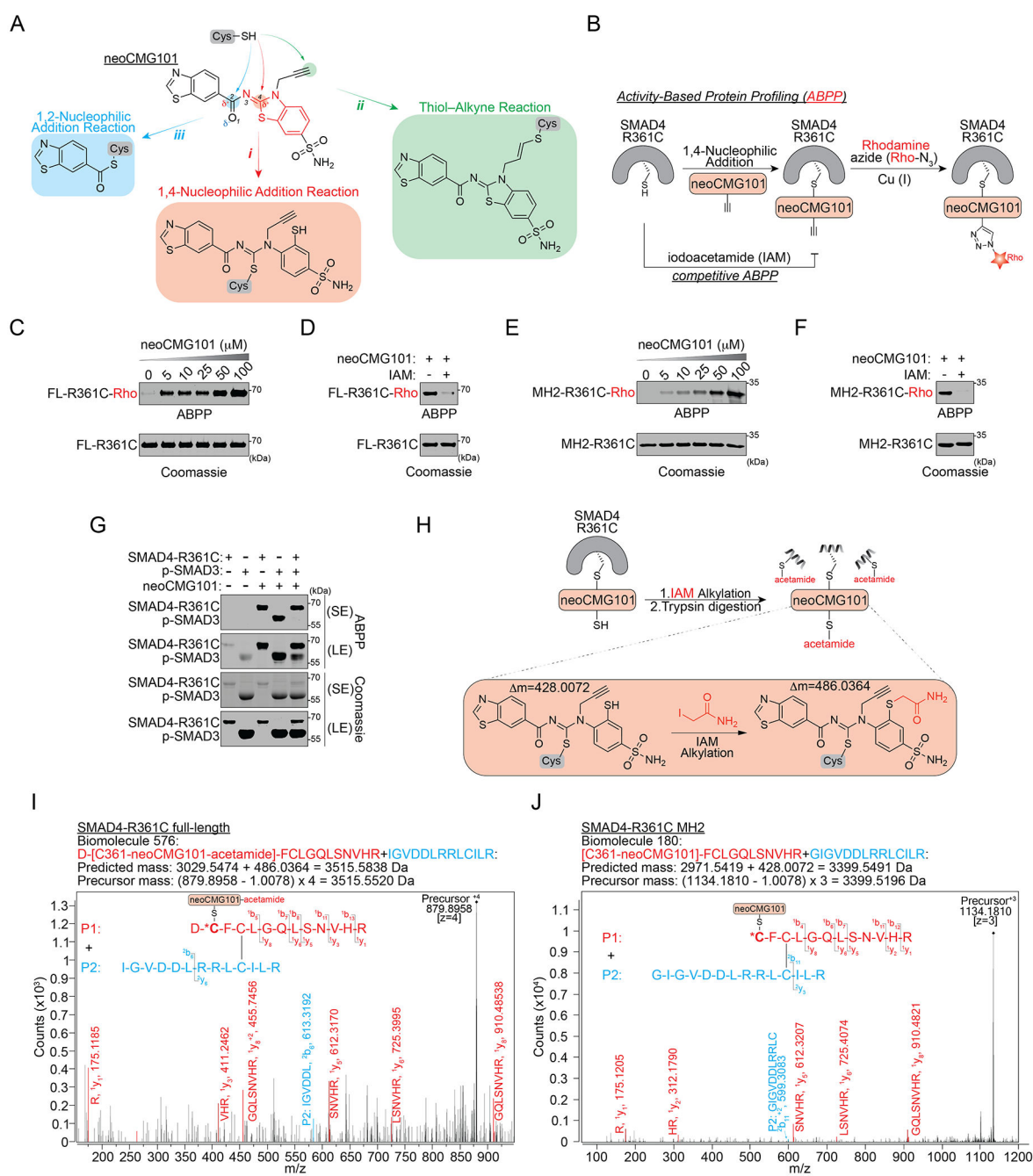
biological replicates. P-values were calculated using an unpaired, two-tailed Student's t-test without adjustments.

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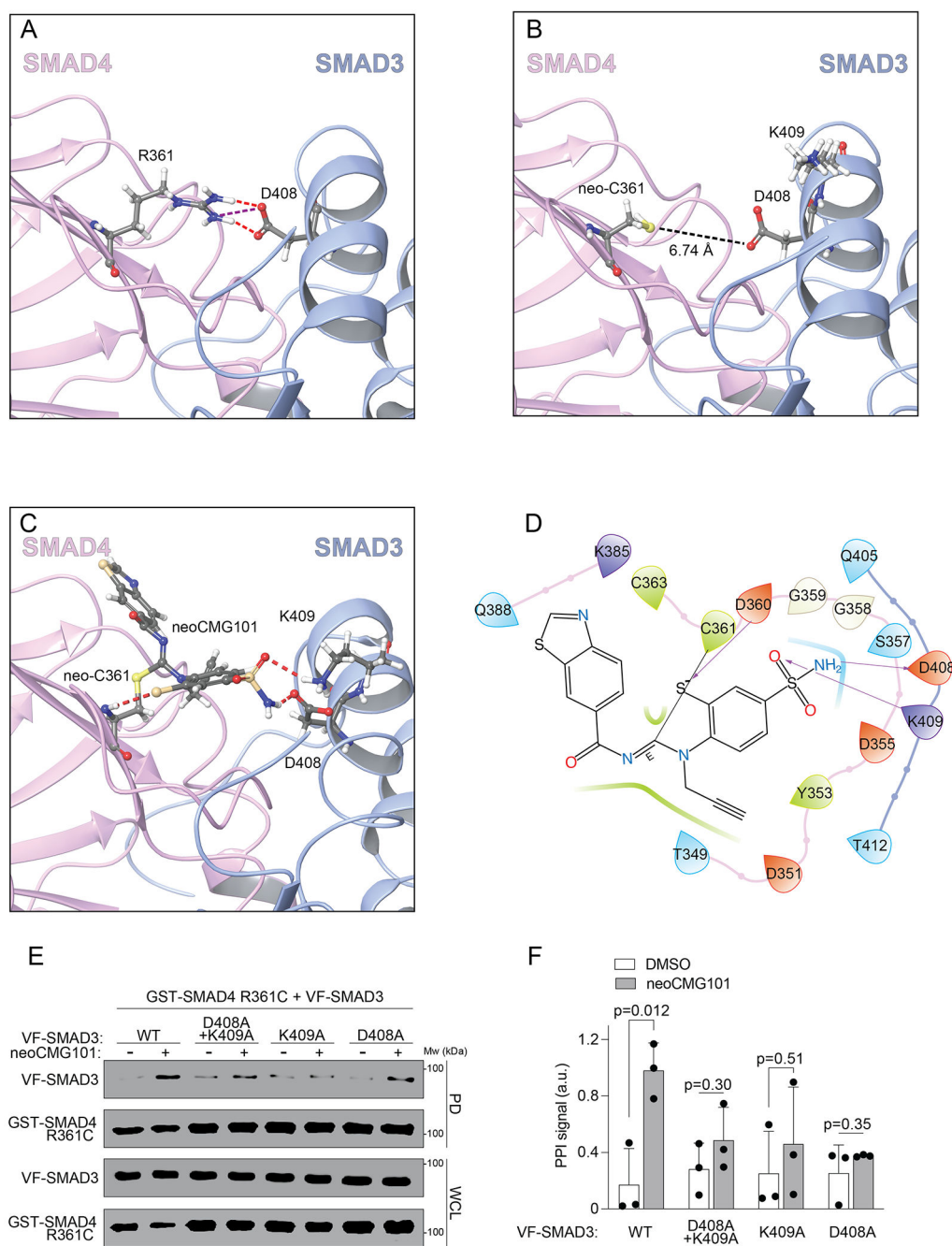
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**Figure 4.**

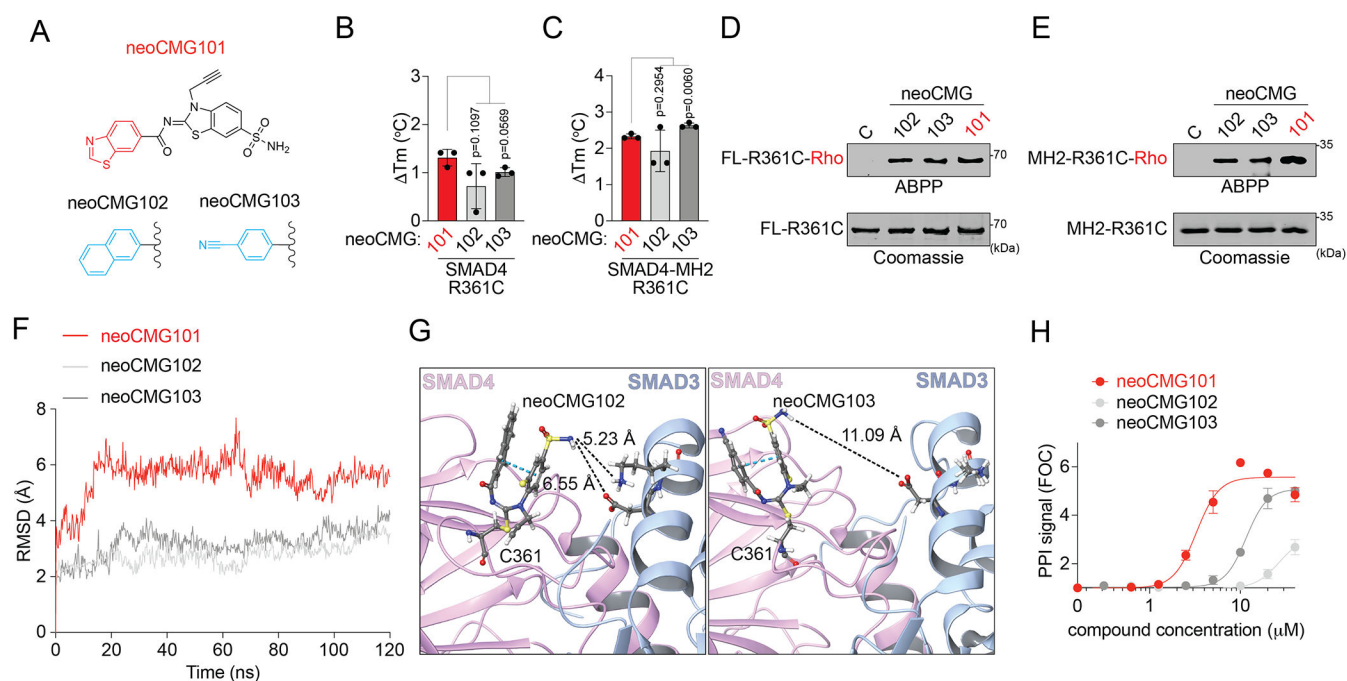
Identification of neoCMG101 covalent modification biochemical mechanisms and modification sites. (A) Schematic illustration of the hypothesized biochemical mechanisms of neoCMG101-induced covalent modification of cysteine residues (Cys) and the resulting Cys-adducts. Potential covalent warheads and their reactive centers are highlighted in different colors. (B) Schematic overview of the activity-based protein profiling (ABPP) and competitive ABPP assays. The unique free alkyne group of neoCMG101 enables fluorescent labeling of the SMAD4-R361C-neoCMG101 adduct via an azide-alkyne

click reaction. (C) Gel images showing dose-dependent fluorescence labeling of the His-SMAD4-R361C full-length protein by neoCMG101 in the ABPP assay. Coomassie staining confirms equal protein loading. (D) Gel images showing inhibition of neoCMG101 (50 μ M) -induced fluorescence labeling of the His-SMAD4-R361C full-length protein by iodoacetamide (IAM, 10 mM) in the competitive ABPP assay. Coomassie staining confirms equal protein loading. (E) Gel images showing dose-dependent fluorescence labeling of the His-SMAD4-R361C MH2 domain truncation protein by neoCMG101 in the ABPP assay. Coomassie staining confirms equal protein loading. (F) Gel images showing inhibition of neoCMG101 (50 μ M) -induced fluorescence labeling of the His-SMAD4-R361C MH2 domain truncation protein by IAM in the competitive ABPP assay. Coomassie staining confirms equal protein loading. (G) Gel images showing dose-dependent fluorescence labeling of His-SMAD4-R361C, phosphomimic SMAD3 (p-SMAD3), or their 1:2 molar ratio mixture by neoCMG101 in the ABPP assay; (SE: short exposure, LE: long exposure). Coomassie staining confirms equal protein loading for His-SMAD4-R361C and p-SMAD3 full-length proteins. (H) Schematic of the Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) sample preparation process. Native conditions were used during sample preparation to preserve neoCMG101 modifications. Expected peptide mass shifts (Δm) from neoCMG101 or neoCMG101-acetamide modifications are indicated. (I-J) Representative LC-MS/MS spectra showing identified peptides containing neo-C361 from His-SMAD4-R361C full-length (I) and MH2 domain truncation (J) proteins. Predicted and detected precursor masses confirm that neo-C361 is covalently modified by neoCMG101 as expected. Representative gel images are shown from three independent biological replicates.

**Figure 5.**

Identification of the structural basis of neoCMG101's molecular glue activity. (A) Crystal structure (PDB: 1U7F) of the SMAD4-WT/SMAD3 complex showing the critical salt bridge and hydrogen bond between R361 of SMAD4 and D408 of SMAD3 at the protein-protein interaction (PPI) interface. (B) Predicted 3D structure illustrating the disrupted interface caused by the neo-C361 residue in SMAD4-R361C, which eliminates the stabilizing salt bridge and hydrogen bond with D408 of SMAD3 and increases the inter-residue distance to 6.74 Å. (C-D) Predicted 3D (C) and 2D ligand interaction (D) structures showing the

covalent modification of neo-C361 by neoCMG101. The sulfonamide group of neoCMG101 re-establishes electrostatic interaction with D408 and introduces additional hydrogen bond interaction with K409 on SMAD3, stabilizing the complex. Structures shown in panels (B) and (C) represent conformations extracted from the stabilized time frame of molecular dynamics simulation studies. (E) Representative western blot images and (F) bar graph from gel quantification showing the molecular glue activity of neoCMG101 upon SMAD3 mutations. GST pull-down (PD) assays were performed using cell lysates co-expressing GST-SMAD4-R361C and Venus-Flag-tagged (VF) SMAD3 WT or mutant as indicated treated with neoCMG hits at 25 μ M. Western blotting detected VF-SMAD3 in the PD fraction and whole-cell lysate (WCL), and quantified PPI signals are presented as the ratio of band intensity of VF-SMAD3 over GST-SMAD4 R361C in the PD fraction. Data in panel (F) are presented as mean $\pm$ SD from three independent experiments.

**Figure 6.**

Characterization of neoCMG101 analogs for initial structure-activity relationship (SAR) interrogation. (A) Chemical structures of neoCMG101 and its analogs, neoCMG102 and neoCMG103. (B-C) Bar graphs showing changes in the melting temperature (ΔT_m) of His-SMAD4-R361C full-length (B) or MH2 domain truncation (C) proteins in the presence of neoCMG101, 102, or 103 (50 μ M). Melting temperatures were measured using NanoDSF assays, with data presented as mean $\pm$ SD from three independent experiments. (D-E) Representative gel images from ABPP assays showing fluorescence labeling of His-SMAD4-R361C full-length (D) or MH2 domain truncation (E) proteins induced by neoCMG101, 102, or 103. Coomassie gels display total protein input. (F) Root Mean Square Deviation (RMSD) curves showing the average positional deviation of binding site residues at the SMAD4-R361C/neoCMG101/SMAD3 PPI interface. (G) Predicted 3D structure of the SMAD4-R361C/SMAD3 PPI interface in the presence of neoCMG102 and neoCMG103, which adopts a folded conformation that hinders interaction with SMAD3 residues. (H) Dose-dependent curves of neoCMG101 and its analogs, 102 and 103, showing TR-FRET signals for the SMAD4-R361C/SMAD3 PPI. Data are presented as mean $\pm$ SD from three independent experiments.