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Peer Review File

Targeting a key protein-protein interaction surface on mitogen-activated protein kinases by a precision-guided warhead scaffold

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

NCOMMS-23-39198-T_449569
Póti et al target the so-called of MAPKs with a novel structurally complex chiral warhead. These sterically crowded cyclohexanone moieties are dubbed by the authors “frustrated” cyclohexanones.
The approach has the potential to be translated to other targets, the molecules are novel and will allow further modifications due the available exit vectors and attachment of electron withdrawing groups.
Currently the manuscript still lacks some important characterization concerning the covalent reaction, compounds and establishment of quantitative (quality) parameters of the covalent reversible mechanism. The authors have collected an impressive amount of data, which needs a revised and better digestible presentation.

Major points:
1) The authors start out the results section with compounds derived from D-peptide targeting the MAPK D-groove, including warheads 12 and 13 and a reversible control 14. These results defer somewhat from the main theme starting in Fig 1C. As the two approaches differ considerably, results concerning the covalent peptide approach leads to confusions. It would be better to start directly with group 3 compounds, and use the liberated space to explain the binding analytics (see below).

2) Without the presentation of quantitative data the molecule groups 1,2,4,5,6,7 should be eliminated. The screen in Fig S3 without assigned structures is not useful, the structures would have to be disclosed. Moreover, the nitroalkenes also show positive signals.

3) The thiol reactivity scheme 4<2<1… is very likely not correct and is undocumented. What would be needed is a determination of the actual reaction rates with a simple thiol, for example beta-mercaptoethanol. Ideally a kchem(on) and a kchem(off) should be established, which would then provide meaningful parameters to be compared with the kchem of covalent irreversible compounds.

4) The provided Kiapp values ± GSH are insufficient to document “non-reactivity” of the “frustrated” cyclohexanones. It is more likely that the “frustrated” cyclohexanones have rather high kchem(off) rates – and kinact(off) (see below). As Kiapp is a convolution all rate constants (see below) it is not the ideal parameter to describe covalent reactions. A Kiapp value as a function of time would improve this a bit, but fulfillment of query 5) would be ideal.

5) In Fig 1E, the use of k1, k2 equation for the off-target reaction is fine, but the same rate constants can not be used for MAPK targeting. Here an equation of I + E <=> I~E (reversible complex) <=> EI

(covalent complex) with k(on_enzyme) and a k(off_enzyme) and after reversible binding to the enzyme with kinact(on), kinact(off) is required.
6) Throughout the paper FP (for Kd) and FB (for Kiapp) results are presented (in Fig 1C for RU1). It should be indicated in the figure legends how the assays were performed, notably which peptide was used for the displacement assay (two are listed in methods). Without pointers the assessment of the data is a frustrating detective work.

7) In methods for the calculation of Kd and Kiapp should be elucidated in detail ("data to a competition binding equation" etc.). Quality control data of "home-made" reagents would be of advantage. In general, the methods section is somewhat obscured and does make reproduction difficult.

8) Fig 2: additional phys./chem. parameters of compounds are needed. At first sight, higher affinity seems to correlate with elevated cLogP. cLogP and ev. a correction of affinities for lipophilicity should be added.

9) In Fig 3 a small SAR is presented, mostly using racemic mixtures. An exception is RU68 (the S isomer of RU64). For RU64 the ERK2 C161A mutant shows a ca. 40x increase of Kiapp, documenting the importance of the protein's nucleophile. Inserted is in the following a ERK2+RU68 (3C) dialysis and an SPR experiment both demonstrating a reversible binding of RU68 to ERK2. To get some impression of the importance of the R,S configuration the reader has to OCR the SI file to finally find RU68/RU76 FB data for ERK2 in Fig S10.
In spite of the X-Ray crystal structures of many RU compounds, a SAR and interaction map does not emerge here.
In the 3D structure representations, it is not clear if the protein positioning is the same for RU68 and RU-67. The figure on the right obstructs the view on the covalent bound, which makes it difficult to validate the authors' claims. Interaction maps and orientation points in the structures should be provided.

10) The numbering of the compounds has no logic and data are presented in a confusing order. Renaming the compounds to reflect functional and structural features is necessary, and the order of appearance should be checked. Classical "schemes" as found in SAR studies in med. Chem. journals could help to give an overview.

11) PDB IDs should be listed in the figure legends.

12) Paragraph at L218: the statements made concerning reactivity towards Cys are not supported by quantitative data. See points 4/5) above. It is not clear what the substitution change, is it kchem(on) or kchem(off)?

13) In Fig 4 it is not clear why Ru67/75 and 68/76 etc are matched. Why not compare S and R? It is very interesting how the R,S configuration influences the orientation of the molecules in ERK2. But the reader has to little spacial information to make sense out of it.

14) The same is true for Fig 4B: the electron density map including His125 is not sufficient proof of a covalent bound. The adducts would have to be formed and analyzed.

15) “The RU60/RU187 enantiomeric pair”. Please replace lab codes by logical names.

16) The route to bivalent binders is interesting (RU128: RU188-PEG-IN-8). The linker appears to be a bit short to bridge the distance between C161 and the ATP-binding site. Moreover, the orientation of RU188 does not fit the exit vector used in in Ru128. More data would be needed, also the binding to the ATP-binding site should be documented. Here relatively simple TR-FRET experiments could be performed (commercial tracers available). One would expect that a covalent linkage would influence the time on target as measured by a TR-FRET tracer in the ATP-binding site.

17) 1H, 13C and MS spectra should be added. Annotations seem fine.

Reviewer #2 (Remarks to the Author):

This manuscript describes the discovery and development of a cyclohexanone-derived reversible covalent warhead able to disrupt the protein-protein interaction of MAPKs and their substrate. The study was initiated by specific covalent targeting of a conserved cysteine residue located in the D(ocking) groove of MAPKs using cleverly designed peptides bearing intrapeptide acryl amides as covalent warheads. After establishing a proof-of-concept for covalent binding to the targeted cysteines, the study continues by screening a library of selected covalent warheads resulting in the identification “frustrated” cyclohexenones as privileged structures for the specific targeting of D groove cysteines in different MAPKs. Using a previously developed concise and asymmetric synthetic approach, a library of cyclohexenone derivatives was prepared and tested in vitro for their covalent binding to MAPKs resulting in the discovery that these compounds were reversible covalent binders. This was corroborated extensive proteomics experiments, X-ray crystallography and an intracellular NanoBit assay. This work will be of significant interest to the drug discovery and MAPK research community since it describes an extensive study towards the discovery and development of a new type of reversible covalent warhead and its effectiveness as disruptor of protein-protein interactions in MAPK signaling. However, the manuscript contains several concerns that need to be addressed:

Major points:

1. While the development of the covalently binding D-groove peptides is indeed relevant, the authors did themselves a disfavor by including this paragraph to the manuscript since it added an additional layer of complexity. Moreover, the acquired findings are not discussed in further detail later on. Therefore, the covalent peptide part should be removed from the manuscript and a further developed into a separate story with additional data that can be published in a high impact journal

as well.

2. While the reported NMR values appear to be correct, it would be detrimental to include the NMR spectra of the synthesized molecules in the supporting information.

3. Although the authors show the activity of their compounds in the presence of sulfhydryl moieties, such as GSH, it would be crucial for the interpretation of the biological data to provide the intrinsic reactivity of the compounds towards GSH (or surrogates) under physiological conditions. Hence, the authors should assess the stability and half-life of their warheads using earlier reported assays (see: J. S. Martin et al. Bioorg. Med. Chem. 2019, 27, 2066-2074).

4. Extended Data Fig. 7 is missing.

5. Comparing the structural model provided in Fig. 5B and the exit vector for the linker (marked by the authors with an *), it appears that IN-8 would be positioned away from the ATP site instead towards it, and thus prevent a productive positioning of the IN-8 scaffold in the ATP pocket. Since the clear phosphorylation inhibition of RU128 indicates that the IN-8 part is indeed binding to the ATP site, the question arises as if the covalent part of the molecule is binding other residues (cysteines) on the protein surface that are within the reach of the linker instead of specifically targeting cys161. Hence, RU128 should be tested in a complementary assay that will prove binding to the targeted cysteine (e.g. NanoBiT PPI assay).

6. Fig 1C shows a list of warheads and a ranking according to their reactivity with thiols, but there is no reference provided for the shown ranking. It is very questionable that acrylamides have a higher reactivity with thiols than cyclohexenones or even chalcones. The authors should provide an adequate reference for this ranking or data proving these claims.

Minor points:

7. Due to the numerous discussed compounds and to facilitate the readability of the manuscript, a relabeling of the molecules with successive numbering is highly recommended. In this context, the enantiomers should be labelled with the accurate prefix, such as R-6 and S-6.

8. It should say Fig. 1C instead of Fig. 1D.

9. Fig 3E: The overlayed structures are hard to distinguish due to the similar colouring (both are blue).

Reviewer #3 (Remarks to the Author):

The study submitted for publication in Nature Communications by Póti et al. discusses the discovery of irreversible and reversible covalent binders targeting the MAPK D-groove, a substrate binding site common to all MAPKs that contains a conserved non-catalytic cysteine. While others had previously demonstrated the ligandability of this cysteine (refer to https://doi.org/10.1038/s41467-019-12996-8), ligands addressing this binding site remain scarce. Such ligands may offer advantages as they can selectively block a specific part of MAPK downstream signaling.

In the first part of the study, D-groove binding peptides were modified with internal acrylamide-based Michael acceptor groups to create irreversible covalent binders. However, these molecules exhibited relatively slow covalent binding kinetics and only slightly improved potency compared to non-covalent analogs.

In the second part, the authors adopted an "electrophile-first" approach, constructing a library of fragments that incorporated various Michael acceptor systems. Key compounds in this category featured a terpenoid-like structure with a central cyclohexenone system, along with an additional activating group at the carbonyl alpha position and bulky groups at the gamma position to generate "frustrated" electrophiles. These compounds were easily synthesized using a method previously described by the authors.

Screening these compounds against MAPKs through a fluorescence polarization assay revealed several binders with micromolar apparent affinities. Multiple experiments demonstrated the low reactivity of "frustrated" double-activated enones toward glutathione adduction and their ability to engage in reversible covalent interactions with the target, modifying the anticipated cysteine. Importantly, X-ray structures showed that some of these compounds exhibited a configuration-dependent modification. One enantiomer exclusively modified cysteine 161, while the other formed an adduct with the opposing histidine 125. It's worth noting that the ability of these compounds to modify histidine is not surprising, as previous studies by Jakob et al. had already observed similar behavior in cyclic, double-activated Michael acceptors, particularly in the context of cyanoenone compounds modifying a histidine in IDH1 (refer to https://doi.org/10.1021/acs.jmedchem.8b00305).

The authors further expanded their investigation by developing a structure-activity relationship (SAR) series and demonstrated that selected compounds had a distinctive impact on downstream signaling. However, it appears that the design of this SAR series was primarily driven by considerations of synthetic accessibility rather than rational design. In addition, they combined the reversible covalent D-groove binders with an orthosteric ligand to create bivalent compounds with enhanced affinity and reduced off-rate. It is worth noting that the extent to which this improvement can be attributed to the covalency of the compounds remains unclear, as it is a common feature of non-covalent bivalent inhibitors to exhibit increased potency and prolonged residence time compared to their monovalent counterparts.

The manuscript is accompanied by a second paper in which the authors apply their "frustrated" reversible covalent Michael acceptors to ATP-site targeted inhibitors derived from the known covalent JNK inhibitor JNK-IN-8.

One potential drawback of the presented electrophiles is their structural inclusion of two ester moieties, which could make them susceptible to hepatic and non-hepatic metabolism. While this may be advantageous in minimizing off-target effects of irreversible inhibitors forming long-lived adducts, such a feature may not provide the same benefit for rapidly reversible compounds. To demonstrate the applicability of their approach in an in vivo context, it is crucial to investigate and discuss the metabolic properties of the presented electrophiles. Additionally, a more comprehensive evaluation of the selectivity of the electrophilic compounds would enhance the study's value. Furthermore, the contributions of reversible pre-binding and the kinetics of covalent bonding and dissociation receive insufficient emphasis in both experimental design and discussion. The inclusion of further quantitative kinetics experiments is strongly recommended.

The manuscript is well-written, and the results are presented clearly. The citation of previous literature in the field is accurate. The experiments are appropriately described, and a substantial amount of data is provided in the supporting information. The experimental methods for biological evaluation, including fluorescence polarization assays, surface plasmon resonance, protein mass spectrometry, X-ray crystallography, in vitro kinase assays, and cell assays, are generally appropriate. However, additional characterization, as mentioned above, is strongly recommended. Furthermore, it appears that not all newly synthesized compounds have been fully characterized, and it should be ensured that at a minimum, 1H/13C NMR, high-resolution mass spectrometry (HRMS), and high-performance liquid chromatography (HPLC) purity data (where applicable) are provided for all new compounds.

The presented results are undoubtedly of interest to the chemical biology and medicinal chemistry communities, particularly to researchers focused on covalent inhibitors and MAPK inhibitors. Nevertheless, there are several weaknesses in the study, and it may not meet the level of significance required to appeal to a broader readership. Therefore, I recommend submitting the manuscript to a more specialized chemical biology journal after making the suggested revisions.

In addition to the aforementioned points, the authors should consider the following aspects:

1. The terminology regarding "cyclohexenone/pentenone" is misleading, as the reactivity of the compounds is driven by the presence of two electron-withdrawing groups. Please rephrase this to accurately reflect the mechanism.

2. It appears that the compounds exhibit rapid reversibility on the target. Ideally, reversible covalent compounds would have a slow dissociation rate on the target while rapidly dissociating from off-targets (see, for example, https://doi.org/10.1038/nchembio.1817). This aspect should be discussed in greater detail.

3. The authors should make a clearer distinction between "affinities" and "apparent affinities"

throughout the manuscript.

4. On line 214, the statement, "The cyclic structure of the new cysteine targeting covalent warhead is a key feature, since similar molecules with an acrylester or cyanoacrylester, which are open-chain (acyclic) Michael acceptor warheads, were far less efficient in binding to the MAPK D-groove," may not be valid given that Ki app differs by less than 10-fold, and the compounds have significantly different structures. Please revise this statement to accurately reflect the findings.

5. On line 323, the statement, "and some of these extensions led to molecules that had comparable in vitro binding affinity to that of the simpler designs," suggests that the modification does not provide a benefit when the molecular weight increases, and potency remains the same. Clarify this observation further.

6. The term "enthalpic anchors" is used in the context of the bivalent compounds. Consider using a more cautious phrasing since this behavior has not been unambiguously demonstrated.

Reviewer #4 (Remarks to the Author):

This manuscript by Poti et al. describes a new strategy to target inhibition of MAP kinases; by targeting a protein-protein interaction surface on MAPKs called the docking groove (D-groove) with peptidic inhibitors that block binding of substrates and activators. They show that the D-groove contains a conserved cysteine that could be targeted by reversible covalent modifiers. The group uses a number of structural, biophysical, and biochemical techniques to evaluate and develop this strategy. They first use existing crystal structures to design peptide and peptide-like inihibitors that bind the ERK2 D groove. They use Micheal acceptor chemistry to covalently target the D-groove cysteine. They then used a number of building blocks to target two hydrophobic pockets on the D-groove in order to optimize binding and create SAR. For some of the most contrasting compounds, binding was characterized by fluorescence polarization and SPR. Co-Crystal structures were determined of ERK2 with a handful of the different inhibitor compounds, validating the structural basis of inhibition. Cellular assays were used to demonstrate potency and selectivity for some of the best inhibitors. Overall, this is a very comprehensive and well-written manuscript (along with an accompanying manuscript describing more use in cellular context). I would recommend for publication upon scanning for/correcting for any typos. This manuscript is of high significance to both the specialist (structural/medicinal chemists targeting kinases) and also those interested in MAPK targets, which are involved in a myriad of diseases from cancers, to asthma, and more.

Please find our answers for the reviewers' comments *in italics*.

Reviewer #1 (Remarks to the Author):

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Póti et al target the so-called of MAPKs with a novel structurally complex chiral warhead. These sterically crowded cyclohexanone moieties are dubbed by the authors "frustrated" cyclohexanones. The approach has the potential to be translated to other targets, the molecules are novel and will allow further modifications due the available exit vectors and attachment of electron withdrawing groups. Currently the manuscript still lacks some important characterization concerning the covalent reaction, compounds and establishment of quantitative (quality) parameters of the covalent reversible mechanism. The authors have collected an impressive amount of data, which needs a revised and better digestible presentation.

We improved the presentation of the data by 1) simplifying the main figures (Fig. 1-6), 2) adding more Supplementary Information items (Fig 1-21 and Table 1-2), and 3) the compounds are presented/labeled more logically in the order of appearance in the text (Tables 1-4). We further characterized the covalent reactions and established the quantitative parameters of the reversible covalent mechanism (e.g., K_D´ and K_{chem} contribution to overall K_D, and kinetic measurements where this was feasible). The manuscript contains now substantially more quantitative data/analysis but we believe that restructuring of these according to the suggestions below made the story more digestible.

Major points:
1) The authors start out the results section with compounds derived from D-peptide targeting the MAPK D-groove, including warheads 12 and 13 and a reversible control 14. These results defer somewhat from the main theme starting in Fig 1C. As the two approaches differ considerably, results concerning the covalent peptide approach leads to confusions. It would be better to start directly with group 3 compounds, and use the liberated space to explain the binding analytics (see below).

We have greatly shortened the presentation of the intrachain acrylamide containing peptide and most of the data regarding this is moved into the Supplementary Information. The focus in Fig. 1 is on Group 3 compounds now.

2) Without the presentation of quantitative data the molecule groups 1,2,4,5,6,7 should be eliminated. The screen in Fig S3 without assigned structures is not useful, the structures would have to be disclosed. Moreover, the nitroalkenes also show positive signals.

We provided the structure of the compounds used in the primary screen in Supplementary Table 2 used in Fig S3 (now Supplementary Fig. 4). Nitroalkenes indeed show positive signal, however, this is attenuated in the presence of 10 mM GSH. Our motivation was to identify inhibitors that are resilient to high amounts of GSH.

3) The thiol reactivity scheme 4<2<1… is very likely not correct and is undocumented. What would be needed is a determination of the actual reaction rates with a simple thiol, for example beta-mercaptoethanol. Ideally a kchem(on) and a kchem(off) should be established, which would then provide meaningful parameters to be compared with the kchem of covalent irreversible compounds.

We accept that the reactivity scheme is not appropriate here and we removed this from the manuscript: it was not an important aspect of the discussion since we focus only on Group 3 compounds.

4) The provided Kiapp values ± GSH are insufficient to document "non-reactivity" of the "frustrated" cyclohexanones. It is more likely that the "frustrated" cyclohexanones have rather high kchem(off) rates – and kinact(off) (see below). As Kiapp is a convolution all rate constants (see below) it is not the ideal parameter to describe covalent reactions. A Kiapp value as a function of time would improve this a bit, but fulfillment of query 5) would be ideal.

Kiapp is a good proxy for comparing the binding of compounds capable of interfering with MAPK D-groove mediated protein-protein interactions and the D-peptide displacement FP assay provides a pragmatic solution to assess binding at this functionally critical MAPK region. We used the +GSH experiment to demonstrate that the "frustrated" cyclohexenones could be resilient to GSH (in vitro, under conditions emulating in-cell conditions in this respect). The Kiapp value does not change in time since the reactions are fast and reaches steady state before the measurements are taken (~5-10 minutes after pipeting all components together). There is a kinetic analysis presented on Fig. 1d showing that k_chem(on) is several orders of magnitude faster with GSH compared to irreversible inhibitors (see below). The on-rate of covalent bond formation with the protein surface thiol is also expected to be similarly fast or faster. This is supported by the fact that we did not see any difference in the Kiapp determined by the FP based assay immediately or after an hour of incubation (see Fig. 4a).

Friedman et al (1965) reported the k_chem(on) of the S^- group in mercaptopropionic acid at pH 8.1 and at 30 °C (in Table VI): for methyl acrylate this was found to be 0.11 $M^{-1}s^{-1}$ and 0.0046 $M^{-1}s^{-1}$ for acrylamide. The value indirectly determined for **2***-biotin in our kinetic binding measurements (70 $M^{-1}s^{-1}$) at RT and pH 7.4 is ~600- or ~15000-fold faster, respectively.*
Flanagan et al (2014) reported the $k_{pseudo1st}$ values of GSH-adduct formation determined at 10 mM GSH concentration with different acrylamides measured at 37 °C in PBS (pH 7.4). The fastest rate (Cpd 1, Table 2) for the corresponding k_chem(on) is 0.089 min^{-1} while for a simple acrylamide compound (Cpd 11) this rate was 0.0007 min^{-1}. Based on our measurements for **2**-biotin *(calculated from 70 $M^{-1}s^{-1}$) the corresponding $k_{pseudo1st}$ value at 10 mM GSH would be 42 min^{-1}, suggesting ~ 500-fold or 60000-fold faster reaction, respectively). Despite that direct comparison of these value is difficult (different measurement techniques and experimental conditions, irreversible vs reversible thiol reactions), it shows that the kinetic aspect of thiol-adduct formation of the new cyclohexenone warhead is very different from that of acrylamides/esters. Moreover, the result of ^{1}H NMR dilution experiment tested on the* ***3****R-BME adduct was also consistent with a very fast kchem(off) (see Supplementary Fig. 6b).*

Friedman et al, J. Am. Chem. Soc.1965, 87, 16, 3672–3682 (https://doi.org/10.1021/ja01094a025)
Flanagan et al, J. Med. Chem. 2014, 57, 10072−10079 (https://doi.org/10.1021/jm501412a)

5) In Fig 1E, the use of k1, k2 equation for the off-target reaction is fine, but the same rate constants can not be used for MAPK targeting. Here an equation of I + E <=> I~E (reversible complex) <=> EI (covalent complex) with k(on_enzyme) and a k(off_enzyme) and after reversible binding to the enzyme with kinact(on), kinact(off) is required.

The earlier presentation on this figure was unfortunate and it is now updated. Originally we just simply wanted to emphasize the difference between two scenarios: 1) the double-activated Michael acceptor

warhead reacts with a free thiol or 2) it reacts with a thiol on the protein surface. The demonstration of the correct kinetic scheme, apart from emphasizing reversibility, was not part of the plan. We believe that this is now fixed by providing the complete kinetic schemes on Fig. 1d,e as suggested.

6) Throughout the paper FP (for Kd) and FB (for Kiapp) results are presented (in Fig 1C for RU1). It should be indicated in the figure legends how the assays were performed, notably which peptide was used for the displacement assay (two are listed in methods). Without pointers the assessment of the data is a frustrating detective work.

Unfortunately, this was indeed not specified but a new sentence in the methods "All measurements with ERK2 or p38α were done with the RHDF1 peptide, while the evJIP1 peptide was used with JNK1." clarifies this now.

7) In methods for the calculation of Kd and Kiapp should be elucidated in detail ("data to a competition binding equation" etc.). Quality control data of "home-made" reagents would be of advantage. In general, the methods section is somewhat obscured and does make reproduction difficult.

The specifics of Kd and Kiapp determination in the peptide displacement (FP) assay had been described in Garai et al., 2012 (ref 20) and in Wang, 1995 (ref 42)where the complexity of competitive binding titration equation is explained and a solution is presented, which we implemented in the fitting software.
The "home-made" reagents related to the PhALC assay, including the validation and benchmarking of this assay, is described now in a new paper, published during the review process of this manuscript (Póti et al, 2023; ref 27).

8) Fig 2: additional phys./chem. parameters of compounds are needed. At first sight, higher affinity seems to correlate with elevated cLogP. cLogP and ev. a correction of affinities for lipophilicity should be added.

Table 1,2, and 4 contains a logP column now and there indeed appears to be correlation with lipophilicity (Pearson's coefficients for ERK2, p38α, and JNK1 were -0.678, -0.590, -0.672), which could likely be explained by the fact that the compounds bind to a shallow solvent exposed hydrophobic surface patch (formed by the φ_L, φ_A, and φ_B pockets). This is now explicitly discussed in the text.

9) In Fig 3 a small SAR is presented, mostly using racemic mixtures. An exception is RU68 (the S isomer of RU64). For RU64 the ERK2 C161A mutant shows a ca. 40x increase of Kiapp, documenting the importance of the protein's nucleophile. Inserted is in the following a ERK2+RU68 (3C) dialysis and an SPR experiment both demonstrating a reversible binding of RU68 to ERK2. To get some impression of the importance of the R,S configuration the reader has to OCR the SI file to finally find RU68/RU76 FB data for ERK2 in Fig S10.
In spite of the X-Ray crystal structures of many RU compounds, a SAR and interaction map does not emerge here.
In the 3D structure representations, it is not clear if the protein positioning is the same for RU68 and RU-67. The figure on the right obstructs the view on the covalent bound, which makes it difficult to validate the authors' claims. Interaction maps and orientation points in the structures should be provided.

The handling of the stereoisomers is fixed now by a more logical numbering of the compounds and using the S or R specifications.
*A straightforward SAR interaction map cannot be established because of the alternative nucleophile choice depending on the stereochemistry of the compounds (see the example of **3**R or **3**S for example on Fig. 3a shown on the right panels).*
*The 3D structure representation on Fig. 2e is improved by explicitly stating that the two panels on the left are shown in the same view, moreover, orientation points (φ_L, φ_A, φ_B) are also shown on these two panels now as well as on the panel on the right. The latter shows the same structure as shown on the left panel (ERK2-**8**S), which is explicitly stated in the figure legend now, therefore the covalent bond is naturally there, too, even if it is somewhat obscured by the superimposed peptide. The view on this figure is optimized to show the similarities in how the hydrophobic grooves are occupied by a peptide and a small molecule (since all natural D-peptides occupy these hydrophobic grooves similarly to the covalently bound artificial peptide, but naturally the former lacks the covalent bond.)*

10) The numbering of the compounds has no logic and data are presented in a confusing order. Renaming the compounds to reflect functional and structural features is necessary, and the order of appearance should be checked. Classical "schemes" as found in SAR studies in med. Chem. journals could help to give an overview.

The numbering of the compounds is improved: it follows the order of appearance in the text.

11) PDB IDs should be listed in the figure legends.

PDB IDs for the novel structures are now shown in the figure legends, too, in addition to the table related to the crystallographic analysis.

12) Paragraph at L218: the statements made concerning reactivity towards Cys are not supported by quantitative data. See points 4/5) above. It is not clear what the substitution change, is it kchem(on) or kchem(off)?

The corresponding section was written as the following:
"In contrast to acrylester/acrylamide mediated irreversible adduct formation, covalent adduct formation by cyclohexenone compounds is reversible. The ester group next to C2 has a strong electron withdrawing effect making the unsaturated beta (C3) carbon atom more reactive towards the sulfhydryl group of the cysteine, but rendering the thiol adduct kinetically labile. An amide group at this position has less electron withdrawing capacity and thus it is expected to be less reactive to cysteine."

*For the kinetic statement "… but rendering the thiol adduct kinetically labile" we refer to the fact that acrylester/acrylamide esters are known to make irreversible thiol adducts while the cyclohexonone compounds make reversible ones (meaning that kchem(off) = 0 for irreversible binders but kchem(off) > 0 for reversible binders and thus the adduct of the latter is naturally more labile compared to irreversible binders.) The other part of these statements regarding reactivity (K_{chem}) was based on chemical intuition, namely an amide vs an ester at C2 will make a less reactive compound, due to its less electron withdrawing capacity on C3. This is now corroborated by experimental measurements where we used electronic circular dichroism (ECD) titrations to determine the K_{chem} of different cyclohexenone compounds with beta-mercaptoethanol (see Fig. 4d and compare **3**R vs **18**R).*

However, in order to avoid confusion, we changed the text in the revised version:
"In contrast to broadly applied acrylester/acrylamide warheads that form irreversible adducts with thiol groups, the covalent adduct formation by these cyclohexenone compounds is reversible. The reversible Michael adduct formation tendency can be attributed to both electronic (as in the case of cyanoacrylates) and counteracting steric crowding effect. The ester group adjacent to C2 has an explicitly strong electron withdrawing effect making the unsaturated beta (C3) carbon atom more reactive towards the sulfhydryl group of the cysteine, and replacement with an amide group or other less electron withdrawing group in the C2 position is expected to lower the reactivity in the Michael reaction due to its mitigated electron withdrawing capacity."

13) In Fig 4 it is not clear why Ru67/75 and 68/76 etc are matched. Why not compare S and R? It is very interesting how the R,S configuration influences the orientation of the molecules in ERK2. But the reader has to little spacial information to make sense out of it.

Unfortunately, the outline of this figure was confusing. We indeed intended to match the S and R stereoisomers. We improved the presentation of this figure and it should be more clear now that the corresponding panels are vertically positioned.

14) The same is true for Fig 4B: the electron density map including His125 is not sufficient proof of a covalent bound. The adducts would have to be formed and analyzed.

*The histidine-adduct was detected and analyzed by mass spectrometry with free histidine (Ac-His: N-acetyl-L-histidine, see Supplementary Fig. 15, and now we show that **6**R,R forms a covalent adduct with His-Test (N-acetyl-L-histidine methyl ester) by ^{1}H NMR (see Fig. 4c). The covalent adduct of ERK2-**6**R,R is detected by LC-MS as shown on Supplementary Fig. 11.*

15) "The RU60/RU187 enantiomeric pair". Please replace lab codes by logical names.

Lab codes are replaced by logical names, based on a numbering scheme in the order of appearance in the text.

16) The route to bivalent binders is interesting (RU128: RU188-PEG-IN-8). The linker appears to be a bit short to bridge the distance between C161 and the ATP-binding site. Moreover, the orientation of RU188 does not fit the exit vector used in in Ru128. More data would be needed, also the binding to the ATP-binding site should be documented. Here relatively simple TR-FRET experiments could be performed (commercial tracers available). One would expect that a covalent linkage would influence the time on target as measured by a TR-FRET tracer in the ATP-binding site.

Data and discussion on RU128 is removed from the new version. Figure 5 (the old Figure 3) now concentrates only on the peptide-warhead chimera which is an example of how to expand towards the CD-groove, which is more relevant for the MAPK D-groove manuscript. We did some further characterization on the role of the covalent bond in binding affinity and kinetics in the context of this chimera (see Fig. 5c,d). Inclusion of this new data addresses other comments requesting quantitative characterization on the role of the warhead mediated reversible covalent bond towards overall binding. Notwithstanding, we characterized RU128 more than earlier and addressed the role of the MAPK D-groove cysteine as well as that of the ATP-binding site. In addition to RU128, we have several versions of more complex molecules comprised of different ATP-binding moieties and warheads connected by

linkers different in length and chemical composition. The exit vectors from the D-groove or from the ATP-pocket are different in these and after additional experiments we intend to publish a separate story – to keep the focus only on MAPK D-groove occupancy in this manuscript – on the interesting features of these bivalent binders that involve extra regions beyond the MAPK D-groove.

17) 1H, 13C and MS spectra should be added. Annotations seem fine.

The spectra are added to Supplementary Note 3 describing the synthesis of the compounds.

Reviewer #2 (Remarks to the Author):

This manuscript describes the discovery and development of a cyclohexanone-derived reversible covalent warhead able to disrupt the protein-protein interaction of MAPKs and their substrate. The study was initiated by specific covalent targeting of a conserved cysteine residue located in the D(ocking) groove of MAPKs using cleverly designed peptides bearing intrapeptide acryl amides as covalent warheads. After establishing a proof-of-concept for covalent binding to the targeted cysteines, the study continues by screening a library of selected covalent warheads resulting in the identification "frustrated" cyclohexenones as privileged structures for the specific targeting of D groove cysteines in different MAPKs. Using a previously developed concise and asymmetric synthetic approach, a library of cyclohexenone derivatives was prepared and tested in vitro for their covalent binding to MAPKs resulting in the discovery that these compounds were reversible covalent binders. This was corroborated extensive proteomics experiments, X-ray crystallography and an intracellular NanoBit assay. This work will be of significant interest to the drug discovery and MAPK research community since it describes an extensive study towards the discovery and development of a new type of reversible covalent warhead and its effectiveness as disruptor of protein-protein interactions in MAPK signaling. However, the manuscript contains several concerns that need to be addressed:

Major points:

1. While the development of the covalently binding D-groove peptides is indeed relevant, the authors did themselves a disfavor by including this paragraph to the manuscript since it added an additional layer of complexity. Moreover, the acquired findings are not discussed in further detail later on. Therefore, the covalent peptide part should be removed from the manuscript and a further developed into a separate story with additional data that can be published in a high impact journal as well.

In spite of the suggestion, we kept this part of the story in this manuscript because it organically relates to the whole process how we went from a natural peptide to a unique set of small molecules containing a Michael acceptor via an intermediate step (namely through an artificial peptide rationally designed to be able to attack the nucleophilic residue in a certain position of the MAPK D-groove). However, we agree that this somewhat takes the focus away from the final compounds and therefore we greatly shortened the presentation of the intrachain acrylamide containing peptide and most of the data regarding this was moved into the Supplementary Information.

2. While the reported NMR values appear to be correct, it would be detrimental to include the NMR spectra of the synthesized molecules in the supporting information.

Unfortunately, the QC of the compounds was not represented in full, but this is corrected now and all NMR and MS are added at the end of Supplementary Note 3 describing the synthesis of the compounds.

3. Although the authors show the activity of their compounds in the presence of sulfhydryl moieties, such as GSH, it would be crucial for the interpretation of the biological data to provide the intrinsic reactivity of the compounds towards GSH (or surrogates) under physiological conditions. Hence, the authors should assess the stability and half-life of their warheads using earlier reported assays (see: J. S. Martin et al. Bioorg. Med. Chem. 2019, 27, 2066-2074).

The intrinsic reactivity of GSH (and BME) with some of the compounds was analyzed in PBS (pH 7.4) with different techniques (SPR and ITC) (see Fig 1d and Supplementary Fig. 5). The K_{chem} is ~1mM. Half-life measurements in a classical sense are not relevant here since the warhead's reversible thiol adduct formation is a dynamic process (kchem_off ~ 0.07 s^{-1} and kchem_on could be indirectly calculated to be ~ 70 $M^{-1}s^{-1}$; corresponding to 0.07 s^{-1}, or 4.2 min^{-1}, at 1 mM GSH concentration). Moreover, further adducts were also analyzed by ECD spectroscopy (in PBS) or by NMR (as close to physiological conditions as possible).

We looked into the literature for some guidance on what the k_chem(on) (also referred to as k_{inact}) for irreversible acrylester and acrylamide may be. Friedman et al (1965) reported the k_chem(on) of the S^- group in mecaptopropionic acid at pH 8.1 and at 30 °C (in Table VI): for methyl acrylate this was found to be 0.11 $M^{-1}s^{-1}$ and 0.0046 $M^{-1}s^{-1}$ for acrylamide. The value indirectly determined for **2**-biotin *in our kinetic binding measurements (70 $M^{-1}s^{-1}$) at RT and pH 7.4 is ~600- or ~15000-fold faster, respectively.*
Flanagan et al (2014) reported the $k_{pseudo1st}$ values of GSH-adduct formation determined at 10 mM GSH concentration with different acrylamides measured at 37 °C in PBS (pH 7.4). The fastest rate (Cpd 1, Table 2) for the corresponding k_chem(on) is 0.089 min^{-1} while for a simple acrylamide compound (Cpd 11) this rate was 0.0007 min^{-1}. Based on our measurements for ***2****-biotin (calculated from 70 $M^{-1}s^{-1}$) the corresponding $k_{pseudo1st}$ value at 10 mM GSH would be 42 min^{-1}, suggesting ~ 500-fold or 60000-fold faster reaction, respectively). Despite that direct comparison of these value is difficult (different measurement techniques and experimental conditions, irreversible vs reversible thiol reactions), it shows that the kinetic aspect of thiol-adduct formation of the new cyclohexenone warhead is very different from that of acrylamides/esters.*

Friedman et al, J. Am. Chem. Soc.1965, 87, 16, 3672–3682 (https://doi.org/10.1021/ja01094a025)
Flanagan et al, J. Med. Chem. 2014, 57, 10072−10079 (https://doi.org/10.1021/jm501412a)

4. Extended Data Fig. 7 is missing.

Unfortunately, this figure was left out from the submitted merged PDF file, and it was only present in the converted PDF file assembled from the individual figure submissions. Naturally, this is corrected, but the figures have a different numbering scheme now. This figure is Supplementary Fig. 21 in the revised version.

5. Comparing the structural model provided in Fig. 5B and the exit vector for the linker (marked by the authors with an *), it appears that IN-8 would be positioned away from the ATP site instead towards it, and thus prevent a productive positioning of the IN-8 scaffold in the ATP pocket. Since the clear phosphorylation inhibition of RU128 indicates that the IN-8 part is indeed binding to the ATP site, the question arises as if the covalent part of the molecule is binding other residues (cysteines) on the protein surface that are within the reach of the linker instead of specifically targeting cys161. Hence, RU128 should be tested in a complementary assay that will prove binding to the targeted cysteine (e.g. NanoBiT PPI assay).

Data and discussion on RU128 is removed from the new version. The old figure 3 became Figure 5 and now it concentrates only on the peptide-warhead chimera which is an example of how to expand towards the CD-groove, which is more relevant for the MAPK D-groove manuscript. We did some further characterization on the role of the covalent bond in binding affinity and kinetics in the context

of this chimera (see Fig. 5c,d). Inclusion of this new data addresses other comments requesting quantitative characterization on the role of the warhead mediated reversible covalent bond towards overall binding. Notwithstanding, we characterized RU128 more than earlier and addressed the role of the MAPK D-groove cysteine as well as that of the ATP-binding site. In addition to RU128, we have several versions of more complex molecules comprised of different ATP-binding moieties and warheads connected by linkers different in length and chemical composition. The exit vectors from the D-groove or from the ATP-pocket are different in these and after additional experiments we intend to publish a separate story – to keep the focus only on MAPK D-groove occupancy in this manuscript – on the interesting features of these bivalent binders that involve extra regions beyond the MAPK D-groove.

6. Fig 1C shows a list of warheads and a ranking according to their reactivity with thiols, but there is no reference provided for the shown ranking. It is very questionable that acrylamides have a higher reactivity with thiols than cyclohexenones or even chalcones. The authors should provide an adequate reference for this ranking or data proving these claims.

We accept that the reactivity scheme is not appropriate here and we removed this from the manuscript: it was not an important aspect of the discussion since we focus only on Group 3 compounds.

Minor points:

7. Due to the numerous discussed compounds and to facilitate the readability of the manuscript, a relabeling of the molecules with successive numbering is highly recommended. In this context, the enantiomers should be labelled with the accurate prefix, such as R-6 and S-6.

Compound numbering is updated according to the order of appearance in the text, so as the indication of S or R.

8. It should say Fig. 1C instead of Fig. 1D.

Yes, unfortunately, this call-out was wrong. It is fixed.

9. Fig 3E: The overlayed structures are hard to distinguish due to the similar colouring (both are blue).

The coloring is changed and the small molecule is shown in gray while the peptide in blue.

Reviewer #3 (Remarks to the Author):

The study submitted for publication in Nature Communications by Póti et al. discusses the discovery of irreversible and reversible covalent binders targeting the MAPK D-groove, a substrate binding site common to all MAPKs that contains a conserved non-catalytic cysteine. While others had previously demonstrated the ligandability of this cysteine (refer to https://doi.org/10.1038/s41467-019-12996-8), ligands addressing this binding site remain scarce. Such ligands may offer advantages as they can selectively block a specific part of MAPK downstream signaling.

In the first part of the study, D-groove binding peptides were modified with internal acrylamide-based Michael acceptor groups to create irreversible covalent binders. However, these molecules exhibited relatively slow covalent binding kinetics and only slightly improved potency compared to non-covalent analogs.

In the second part, the authors adopted an "electrophile-first" approach, constructing a library of fragments that incorporated various Michael acceptor systems. Key compounds in this category featured a terpenoid-like structure with a central cyclohexenone system, along with an additional activating group at the carbonyl alpha position and bulky groups at the gamma position to generate "frustrated" electrophiles. These compounds were easily synthesized using a method previously described by the authors.

Screening these compounds against MAPKs through a fluorescence polarization assay revealed several binders with micromolar apparent affinities. Multiple experiments demonstrated the low reactivity of "frustrated" double-activated enones toward glutathione adduction and their ability to engage in reversible covalent interactions with the target, modifying the anticipated cysteine. Importantly, X-ray structures showed that some of these compounds exhibited a configuration-dependent modification. One enantiomer exclusively modified cysteine 161, while the other formed an adduct with the opposing histidine 125. It's worth noting that the ability of these compounds to modify histidine is not surprising, as previous studies by Jakob et al. had already observed similar behavior in cyclic, double-activated Michael acceptors, particularly in the context of cyanoenone compounds modifying a histidine in IDH1 (refer to https://doi.org/10.1021/acs.jmedchem.8b00305).

The authors further expanded their investigation by developing a structure-activity relationship (SAR) series and demonstrated that selected compounds had a distinctive impact on downstream signaling. However, it appears that the design of this SAR series was primarily driven by considerations of synthetic accessibility rather than rational design. In addition, they combined the reversible covalent D-groove binders with an orthosteric ligand to create bivalent compounds with enhanced affinity and reduced off-rate. It is worth noting that the extent to which this improvement can be attributed to the covalency of the compounds remains unclear, as it is a common feature of non-covalent bivalent inhibitors to exhibit increased potency and prolonged residence time compared to their monovalent counterparts.

The manuscript is accompanied by a second paper in which the authors apply their "frustrated" reversible covalent Michael acceptors to ATP-site targeted inhibitors derived from the known covalent JNK inhibitor JNK-IN-8.

One potential drawback of the presented electrophiles is their structural inclusion of two ester moieties,

which could make them susceptible to hepatic and non-hepatic metabolism. While this may be advantageous in minimizing off-target effects of irreversible inhibitors forming long-lived adducts, such a feature may not provide the same benefit for rapidly reversible compounds. To demonstrate the applicability of their approach in an in vivo context, it is crucial to investigate and discuss the metabolic properties of the presented electrophiles. Additionally, a more comprehensive evaluation of the selectivity of the electrophilic compounds would enhance the study's value. Furthermore, the contributions of reversible pre-binding and the kinetics of covalent bonding and dissociation receive insufficient emphasis in both experimental design and discussion. The inclusion of further quantitative kinetics experiments is strongly recommended.

The manuscript is well-written, and the results are presented clearly. The citation of previous literature in the field is accurate. The experiments are appropriately described, and a substantial amount of data is provided in the supporting information. The experimental methods for biological evaluation, including fluorescence polarization assays, surface plasmon resonance, protein mass spectrometry, X-ray crystallography, in vitro kinase assays, and cell assays, are generally appropriate. However, additional characterization, as mentioned above, is strongly recommended. Furthermore, it appears that not all newly synthesized compounds have been fully characterized, and it should be ensured that at a minimum, 1H/13C NMR, high-resolution mass spectrometry (HRMS), and high-performance liquid chromatography (HPLC) purity data (where applicable) are provided for all new compounds.

The presented results are undoubtedly of interest to the chemical biology and medicinal chemistry communities, particularly to researchers focused on covalent inhibitors and MAPK inhibitors. Nevertheless, there are several weaknesses in the study, and it may not meet the level of significance required to appeal to a broader readership. Therefore, I recommend submitting the manuscript to a more specialized chemical biology journal after making the suggested revisions.

*Metabolic stability of **3** (referred to as PK_test in the accompanying manuscript) was addressed using homogenized rat liver and blood plasma (see accompanying manuscript). Moreover, similar characterization was carried out with more complex composite JNK inhibitor compounds in the revised accompanying manuscript. This preliminary PK study with a handful of compounds already hinted on potential strategies on how to optimize/engineer PK properties, which will be pursued based on the results of a more extended and systematic analysis. The synthetic strategy that we developed to generate these cyclic Michael acceptor designs allows us to generate biochemically potent compounds with relative ease, and these compounds will be analyzed in due course in different metabolic stability tests (rat and human). As it might be expected, the ester groups in C2 and/or C4 could be substrates to esterases in the plasma, however this could be efficiently mitigated by structurally modifying the original hit scaffold and concomitantly retaining potency. We show in the accompanying manuscript for example that steric congestion at C4 makes the compounds much more stable in plasma. Moreover there are additional feasible alternatives to the hydrolizable tert-butyl or phenyl esters at C2 (even with stronger electron withdrawing capacity) such as sulphonic ester; or ester containing halosubstituated alkyl group to destabilize possible intermediates of hydrolysis by esterases or even the original hit ester compounds can be made less compatible to the reactive center of esterases by a known approach (for an example for phenyl esters see PMID: 36603103, DOI: 10.1021/jacs.2c12984). Please take note that one of the great advantages of the presented hit scaffold is the relative ease of synthetic elaboration. However, the hit-to-lead characterization aspect would be beyond the scope of this work in our opinion, and it is in the focus of a follow-up story.*

Binding in the MAPK D-groove was further characterized by more experiments to better address the complexity of reversible covalent binding (noncovalent + covalent mechanism), and this aspect is better discussed.

The characterization of all newly synthesized compounds is now complete (see Supplementary Note 3 on the chemical synthesis of all molecules).

In addition to the aforementioned points, the authors should consider the following aspects:

1. The terminology regarding "cyclohexenone/pentenone" is misleading, as the reactivity of the compounds is driven by the presence of two electron-withdrawing groups. Please rephrase this to accurately reflect the mechanism.

Cyclohexenone/pentenone compounds are now specified as "sterically crowded cyclohexenone/pentenone" compounds to emphasize the special features of the presented molecules which are indeed distinct from a general cyclohexenone/pentenone scaffold. We believe that this distinction is now appropriately discussed throughout the text.

2. It appears that the compounds exhibit rapid reversibility on the target. Ideally, reversible covalent compounds would have a slow dissociation rate on the target while rapidly dissociating from off-targets (see, for example, https://doi.org/10.1038/nchembio.1817). This aspect should be discussed in greater detail.

We believe that this is indeed the case. The compounds dissociate faster (~0.07 s^{-1}) from GSH or from a "free histidine/imidazole" compared to the target nucleophile in the MAPK D-groove. This was directly addressed by SPR measurements and also corroborated qualitatively by an NMR jump dilution experiment for the off-target adducts (see Fig. 1d,e and Supplementary Fig. 6b). Furthermore, the experiments on Fig. 4 and 5 address the distinct contributions of the noncovalent (K_D´) vs reversible covalent binding mechanisms on the target (K_{chem}´).

3. The authors should make a clearer distinction between "affinities" and "apparent affinities" throughout the manuscript.

We state in the legends of Fig. 1b that Ki_{app} (which is measured by the peptide displacement FP assays) is a proxy for the MAPK binding affinity of unlabeled compounds. Therefore this is indeed a measure of apparent binding affinity, therefore we took extra care in making distinctions between classical K_D ("affinity") and "apparent affinity" (Ki_{app}) wherever we referred to a value or comparison related to data obtained by the peptide displacement FP assay. Please also note that in this assay we calculate the Kiapp value of the unlabeled specimen by knowing the K_D value of the reporter peptide. Moreover, we also state the following in the main text:

"(Technically, this assay determines apparent K_i values for the small compounds, which may be used as a proxy for their K_D. Note that in the peptide displacement assay it is practical to assume a one-step binding model, thus the K_i determined based on the noncovalent binding competition equation must be referred to as apparent K_i, K_{iapp}. For competitors whose binding do not include a reversible covalent mechanism it is correct to refer to this value simply as K_i.)"

And we also demonstrate that the equilibrium is rapidly reached after mixing the components of this binding assay together even in the case of testing reversible covalent inhibitors (see Fig. 4a); therefore a pragmatic simplification of the two-step binding mechanism of the reversible covalent inhibitors is warranted.

4. On line 214, the statement, "The cyclic structure of the new cysteine targeting covalent warhead is a key feature, since similar molecules with an acrylester or cyanoacrylester, which are open-chain (acyclic) Michael acceptor warheads, were far less efficient in binding to the MAPK D-groove," may not be valid given that Ki app differs by less than 10-fold, and the compounds have significantly different structures. Please revise this statement to accurately reflect the findings.

We agree that this data is only an indication and should not be used as ultimate evidence, therefore the statement was weakened and we changed it to "is probably a key feature".

5. On line 323, the statement, "and some of these extensions led to molecules that had comparable in vitro binding affinity to that of the simpler designs," suggests that the modification does not provide a benefit when the molecular weight increases, and potency remains the same. Clarify this observation further.

We believe that the differences between these different designs will come to surface only in cells, in the context of a complex MAPK D-groove interactome. For compounds that are reversible covalent binders noncovalent and covalent contributions on steady state binding affinity and on kinetic rates may be quite different, nevertheless the complex relationship between these could ultimately result in similar apparent binding affinities in vitro. (A lower apparent global k_{off} may be compensated out by a slower apparent global k_{on}, and thus the apparent binding affinity of a compound may not be so different in vitro compared to another compound with faster global k_{on} and k_{off}, but a compound with lower global k_{off} could perform better in cells.)

6. The term "enthalpic anchors" is used in the context of the bivalent compounds. Consider using a more cautious phrasing since this behavior has not been unambiguously demonstrated.

The contribution of covalent binding to overall binding energy is now quantitatively characterized in the context of the peptide-warhead chimera suggesting such a role for the covalent bond in overall binding (see Fig. 5), despite this, to avoid confusion, the "enthalpic anchor" term is removed from the text.

Reviewer #4 (Remarks to the Author):

This manuscript by Poti et al. describes a new strategy to target inhibition of MAP kinases; by targeting a protein-protein interaction surface on MAPKs called the docking groove (D-groove) with peptidic inhibitors that block binding of substrates and activators. They show that the D-groove contains a conserved cysteine that could be targeted by reversible covalent modifiers. The group uses a number of structural, biophysical, and biochemical techniques to evaluate and develop this strategy. They first use existing crystal structures to design peptide and peptide-like inihibitors that bind the ERK2 D groove. They use Micheal acceptor chemistry to covalently target the D-groove cysteine. They then used a number of building blocks to target two hydrophobic pockets on the D-groove in order to optimize binding and create SAR. For some of the most contrasting compounds, binding was characterized by fluorescence polarization and SPR. Co-Crystal structures were determined of ERK2 with a handful of the different inhibitor compounds, validating the structural basis of inhibition. Cellular assays were used to demonstrate potency and selectivity for some of the best inhibitors. Overall, this is a very comprehensive and well-written manuscript (along with an accompanying manuscript describing more use in cellular context). I would recommend for publication upon scanning for/correcting for any typos. This manuscript is of high significance to both the specialist (structural/medicinal chemists targeting kinases) and also those interested in MAPK targets, which are involved in a myriad of diseases from cancers, to asthma, and more.

Naturally, we appreciate this positive assessment and we carefully scanned the manuscript for typos and fixed them in the revised version.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Poti et al. have improved the structure and argumentation of the manuscript considerable. It features an excellent introduction and improved, clean figures.

There are small, but important items that should be solved or better explained.

1) Figure 1d,e (Fig. 4b): the description of covalent reversible inhibitor equations is demanding. I cannot follow the argumentation on line 169-181, and the emerging formula for Kd= seems not to take into account an increased local concentration of the warhead when the inhibitor is bound to the protein and closer to the nucleophile. I am aware that there is not good framework for covalent reversible inhibitor equations at present, but the authors could maybe have a look at Borsari et al. 2022 JACS for reflections on proximity calculations. The limitations of the present calculations should be mentioned in some way.
2) Figure 2b: please spell out FP and FB on Y-axis (all figures). Add (R), (S) stereospecificity to formulas of 8R and 8S etc. (same in other figures).
3) Responses to queries are acceptable.

Reviewer #2 (Remarks to the Author):

The authors have addressed the majority of my concerns through a comprehensive revision. Furthermore, they have significantly enhanced the quality of the manuscript by incorporating pivotal experimental results that elucidate the chemical nature of these novel reversible covalent warheads. Consequently, after the correction of a few minor points (see below), I recommend this manuscript for publication.

Minor Points:
1. As the authors have chosen to retain the covalent peptide part as a prelude in the manuscript, it is essential that they provide structural characterization for all the discussed compounds, including the tested peptides.

2. In Supplementary Fig. 1a, the numbering of the warheads within the peptides contains the old numbers and should be changed.

3. While the provided NMR spectra have a high enough quality, there appears to be an issue with the numbering of the compounds in the figure legends starting with Supplementary Note 3 Fig. 28. Please carefully check the legends and correct the errors.

Reviewer #3 (Remarks to the Author):

The authors have made revisions to their manuscript, incorporating a wealth of additional data, particularly a thorough characterization of their compounds in terms of binding thermodynamics and kinetics on protein versus model nucleophiles. This has addressed some of my major concerns to an acceptable level, and several minor issues have also been resolved. While I am still not completely convinced whether the paper falls within the scope of the journal, I believe it exhibits a generally high quality and may be suitable for publication. However, there remain a couple of major issues that should be addressed prior to publication:

- The compounds still lack selectivity data. While it may not be feasible to conduct proteome-wide selectivity screens using chemoproteomics for such highly reversible binders, it would be beneficial to include some data on selectivity within the target family, such as from a small kinome panel. This is now considered a standard practice in kinase research and is easily accessible.

- Although the metabolic properties of the compound class are assessed in the accompanying manuscript, at least a brief discussion of this issue in the current manuscript is warranted.

- I recommend further adjustment of the warhead terminology. While "sterically crowded cyclohexenone/pentenone" is not incorrect, it does not fully reflect the fact that two electron-withdrawing groups in conjugation with the double bond (and not just a simple enone motif) are a major determinant of the compounds reactivity.

- As anticipated in my previous review, the compounds exhibit fast dissociation rates. A half-life (t1/2) of 9 minutes is very short for a reversible covalent ligand (some cyanoacrylamides achieve t1/2 > 1 week) and even faster than dissociation of many non-covalent ligands. This compromises some of the advantages of covalent binders, such as prolonged action, PK/PD decoupling, and potential residence time-driven selectivity. While I do not consider this to be a dealbreaker, it should be thoroughly discussed.

- The binding characteristics, especially the kinetic aspect of reversible covalency, should be explained in a few additional sentences, ideally with a scheme in the introduction.

Additional minor points:

- On page 8, please specify that "Kchem ´" refers to chemical reactivity on the target.
- On page 11, correct "enantioriched" to "enantiomerically enriched."
- On page 12, mention that the statement about the energy of C-S(Cys) and/or C-N(His) adduct formation versus non-covalent binding is based on the assumption that Cys/His do not contribute to non-covalent binding affinity.

- On page 12, instead of "off-target thiol," consider "model thiol" or "surrogate thiol" as it refers to GSH, not a protein.
- On page 15, whereas "reactivity" usually refers to kinetics, in this section, the authors refer to equilibrium constants (Kchem). Using a more precise terminology is suggested.
- On page 15, consider if there are other interactions involved besides the hydrophobic component.
- On page 15, correct "mehcanism" to "mechanism."

Please find our response written in *italics.*

Reviewer #1 (Remarks to the Author):

Poti et al. have improved the structure and argumentation of the manuscript considerable. It features an excellent introduction and improved, clean figures.

There are small, but important items that should be solved or better explained.

1) Figure 1d,e (Fig. 4b): the description of covalent reversible inhibitor equations is demanding. I cannot follow the argumentation on line 169-181, and the emerging formula for Kd= seems not to take into account an increased local concentration of the warhead when the inhibitor is bound to the protein and closer to the nucleophile. I am aware that there is not good framework for covalent reversible inhibitor equations at present, but the authors could maybe have a look at Borsari et al. 2022 JACS for reflections on proximity calculations. The limitations of the present calculations should be mentioned in some way.

The scheme of a reversible covalent binder (I) + protein (P) reaction is shown on Fig. 1e. For a free thiol K_{chem} is equal to K_D by definition. For a thiol on the protein surface the reversible covalent binding reaction can be best interpreted with a 2-step process: it has a noncovalent (leading to I:P; characterized by k1 and k2 kinetic constants; $K_D´$) and a reversible covalent ($I\text{-}P^{cov}$; characterized by k3 and k4 kinetic constants; $K_{chem}´$) component and their contribution to the K_D, which is the overall equilibrium dissociation constant, can be obtained the following way:
*$K_D = [P]*[I] / ([I:P] + [I\text{-}P^{cov}])$ since both components listed in the denominator would represent the inhibited forms of P, and the numerator contains the original interacting species. A division by [I:P] then gives the following formula: ([P] * [I] / [I:P]) / (1 + ([I-P^{cov}] / [I:P])) and by definition [P] * [I] / [I:P] equals to $K_D´$ and [I-P^{cov}] / [I:P] is equal to $K_{chem}´$, giving the final equation $K_D = K_D´ / (1 + (1/K_{chem}´))$ that we used to describe the binding of a reversible covalent inhibitor to a protein surface thiol. The "beauty" of this approach is that it is not necessary to know the individual kinetic constants to address the noncovalent versus covalent contribution in inhibitor binding, and note that this is in contrast to irreversible inhibitors (e.g., k_{inact} or k_{chem}).*
Note that $K_{chem}´$ is not the same as K_{chem} (see Fig 1e), and comparison of these two steady-state values gives insights into the quantitative relationship between these two key parameters, which is particularly useful for reversible covalent inhibitor design (and this is discussed in more detail in the accompanying JNK manuscript).
We agree that increased local concentration plays a role in increasing k_{chem_on} (k3) but for reversible inhibitors k_{chem_off} is also very much relevant (since k4 is not equal to 0), and thus in addition to increased local concentration effects for addressing the kinetics of the reversible covalent component, k4 would also need to be addressed (and k4, describing the elimination of the thiol, is very much affected by the sterical crowding effects that we call the "frustration" of the covalent adduct).
However, as we said earlier, consideration of all these factors is not necessary for interpreting the scheme shown on Fig. 1e. We also agree that a kinetics focused framework for reversible covalent inhibitors would also be useful and this is something that we are currently pursuing with theoretical chemical calculations combined with experimental measurements (but because k4 > 0, the latter needs to be approached differently compared to irreversible covalent inhibitors.)

In order to improve the presentation of the final formula on Fig. 1e, we added new information to this panel and the figure legend was updated. We also explicitly noted the limitation of the presented 2-step binding mechanism, however, we believe that it is a reasonable assumption and is a good approximation for a complex problem.

The following sentence was added to the main text after the indicated section:

"Based on the scheme above the formation of the I-P^{cov} complex was assumed to follow a 2-step process. This provides a pragmatic solution for a complex problem with some inherent limitations related to parameter calculation underlying the noncovalent versus covalent components. Undoubtedly, the concrete mechanistic details related to covalent bond formation and thiol elimination related to k_3 (or k_{chem_on}) and k_4 (or k_{chem_off}) kinetic rates, respectively, for example are not directly addressed. Fortunately, the steady-state K_D´ and K_{chem}´, which are important parameters for reversible inhibitor design, could be experimentally directly addressed or at least approximated (see later)."

2) Figure 2b: please spell out FP and FB on Y-axis (all figures). Add (R), (S) stereospecificity to formulas of 8R and 8S etc. (same in other figures).

*FP (fluorescence polarization) and FB (fraction bound) are now spelled out in every relevant figure legends now. It is explicitly stated in the figure legends now that "**8**R or **8**S refers to the (R) and (S) stereosimers of **8**, respectively, and all other stereoisomers of a given compound is similarly labeled with this simpler labeling scheme henceforth".*

3) Responses to queries are acceptable.

Reviewer #2 (Remarks to the Author):

The authors have addressed the majority of my concerns through a comprehensive revision. Furthermore, they have significantly enhanced the quality of the manuscript by incorporating pivotal experimental results that elucidate the chemical nature of these novel reversible covalent warheads. Consequently, after the correction of a few minor points (see below), I recommend this manuscript for publication.

Minor Points:
1. As the authors have chosen to retain the covalent peptide part as a prelude in the manuscript, it is essential that they provide structural characterization for all the discussed compounds, including the tested peptides.

Preparation of unnatural amino acids (36-38; which are published compounds) are described at page 65 in Supplementary Note 3. HRMS analysis of the peptides are now included in Supplementary Note 3.

2. In Supplementary Fig. 1a, the numbering of the warheads within the peptides contains the old numbers and should be changed.

Yes, this was an oversight. The numbers are updated.

3. While the provided NMR spectra have a high enough quality, there appears to be an issue with the numbering of the compounds in the figure legends starting with Supplementary Note 3 Fig. 28. Please carefully check the legends and correct the errors.

Yes, this was an oversight. The NMR spectra for molecules S11-13 and S16-23 were off. This is now corrected in the updated Supplementary Note 3 (see p145-p148).

Reviewer #3 (Remarks to the Author):

The authors have made revisions to their manuscript, incorporating a wealth of additional data, particularly a thorough characterization of their compounds in terms of binding thermodynamics and kinetics on protein versus model nucleophiles. This has addressed some of my major concerns to an acceptable level, and several minor issues have also been resolved. While I am still not completely convinced whether the paper falls within the scope of the journal, I believe it exhibits a generally high quality and may be suitable for publication. However, there remain a couple of major issues that should be addressed prior to publication:

- The compounds still lack selectivity data. While it may not be feasible to conduct proteome-wide selectivity screens using chemoproteomics for such highly reversible binders, it would be beneficial to include some data on selectivity within the target family, such as from a small kinome panel. This is now considered a standard practice in kinase research and is easily accessible.

*Standard kinase panels address general kinase activity and in our opinion are not good to address the selectivity of these MAPK D-groove binding molecules. The MAPK D-groove is a unique PPI hotspot and is absent in other kinases. We show that the examined inhibitors can specifically target the D-groove cysteine (C162) in p38 while the adjacent cysteine (C116) is not targeted (**8**R, see Supplementary Fig. 8). Moreover, we also found that **28**S did not block MAPK mediated phosphorylation of an F-groove docking site containing artificial substrate (F-SENSOR) in the PhALC assay, in contrast to D-SENSOR, thus kinase active site mediated processes remain intact in the presence of the inhibitor (and this was shown in Extended Fig. 5B from the original submission; additionally we formerly also tested **28**S in a RSK2 specific PhALC assay (see Poti et al, 2023) and we found that this compound does not have any effect on RSK2 activity as expected, not shown). Moreover, in the accompanying JNK manuscript we addressed the selectivity of ATP-competitive JNK inhibitors containing double-activated, sterically crowded cyclohexenone/pentenone warheads in kinome panels, albeit the directing group was naturally different in those compounds; and as expected new ATP-competitive JNK inhibitors presented in that study did not interfere with MAPK D-groove mediated binding (see Supplementary Fig. 11B in that manuscript).*
We agree that none of the above directly address proteome-wide selectivity of simpler sterically crowded, double-activated cyclohexenone/pentenone containing compounds. Their reversible covalent binding indeed poses a challenge for classical chemoproteomics, however, we are currently developing an approach through which this could be tackled and would allow proteome-wide characterization of reversible covalent binders easily.

- Although the metabolic properties of the compound class are assessed in the accompanying manuscript, at least a brief discussion of this issue in the current manuscript is warranted.

An explicit reference to the PK data described in the accompanying JNK manuscript is now given, including the following text put into Discussion section of the current manuscript:

*"The metabolic properties of some of the compounds with different cyclohexenone scaffolds were examined in a preliminary pharmacokinetic study (using rat hepatocyte culture and blood plasma; see accompanying manuscript). Hepatic clearance of **3** was found to be intermediate but plasma stability due to high esterase activity was low, since this compound contained esters at C2 and C4. Gratifyingly, experiments with other more complex compounds showed that steric congestion at C4 makes the*

compounds much more stable in plasma. Fortunately, one of the great advantages of the presented double-activated cyclohexenone/pentenone scaffolds is the relative ease of their synthetic elaboration, and this is a great asset in engineering their metabolic properties in the future."

- I recommend further adjustment of the warhead terminology. While "sterically crowded cyclohexenone/pentenone" is not incorrect, it does not fully reflect the fact that two electron-withdrawing groups in conjugation with the double bond (and not just a simple enone motif) are a major determinant of the compounds reactivity.

We changed "sterically crowded cyclohexenone/pentenone" to "double-activated sterically crowded cyclohexenone/pentenone" wherever this was relevant in the text, since the reviewer was right about the extra unique features of these compounds regarding the importance of the electron-withdrawing groups.

- As anticipated in my previous review, the compounds exhibit fast dissociation rates. A half-life (t1/2) of 9 minutes is very short for a reversible covalent ligand (some cyanoacrylamides achieve t1/2 > 1 week) and even faster than dissociation of many noncovalent ligands. This compromises some of the advantages of covalent binders, such as prolonged action, PK/PD decoupling, and potential residence time-driven selectivity. While I do not consider this to be a dealbreaker, it should be thoroughly discussed.

We would like to note that the ~9 minutes half-life (shown in the experiments from Fig. 5d) was estimated in the presence of a high-affinity binder (unlabeled competitor pepMNK1) which was present in close to three orders of magnitude higher concentration compared to the labeled peptide chimera (40 μM vs 50 nM). The dissociation of the mentioned noncovalent ligands or some cynoacrylamides with half-life > 1 week was likely determined under very different conditions compared to our experiment on Fig. 5d (which was specifically designed to demonstrate the contrast between two scenarios where covalent binding can or cannot happen).

We believe that the original statement about this experiment captures well that this is not a classical t1/2 half-life experiment and 9 minutes should not be considered as a simple half-life value, which is normally obtained by classical dialysis experiments under noncompetitive conditions.
"These experiments showed that in this "competitive environment" the peptide chimera lasted far longer if the MAPK D-groove cysteine were intact ($t_{1/2}$ ~ 9 minutes; calculated with an exponential decay equation), compared to the C162A mutant where the complex was found to be fully dissociated already in the first time point (the lag time of the measurement is about 30 seconds)."

In one paper from the Taunton lab (Serafimova et al, 2012; see accompnying JNK manuscript) the half-life of cyanoacrylate (compound 14) and cyanoacrylamide (compound 15) containing RSK2-CTD inhibitors was examined by dialysis experiments and these were found to be less than a day or ~ 3 days, respectively (see Fig 2c in Serafimova et al, 2015), however, the same reversible covalent inhibitors could compete only with greatly reduced dissociation half-lifes against a high-affinity binder (FMK): with 42 and 240 minutes, respectively.
*Figure 2C shows the result of a classical dialysis experiment, where the ERK2-**8**R complex was dialysed for 24 hours and the LC-MS analysis shows that the relative amount of the adduct indeed dropped but its decrease was far less than expected for t1/2 being in the range of minutes, and it was rather indicative for a half-life in the range of several hours (and please note that this was only for a*

*small compound binding with micromolar binding affinity and not for the peptide chimera that binds with nanomolar affinity.) To demonstrate this point further we did a similar dialysis experiment using the p38α–peptide chimera (**28**S-pepMNK1_C) complex and found that the ratio of the adduct and the intact protein (p38α) did not change greatly measured even after 12 hours of dialysis (see Figure 1 below), which is consistent with our argumentation above.*

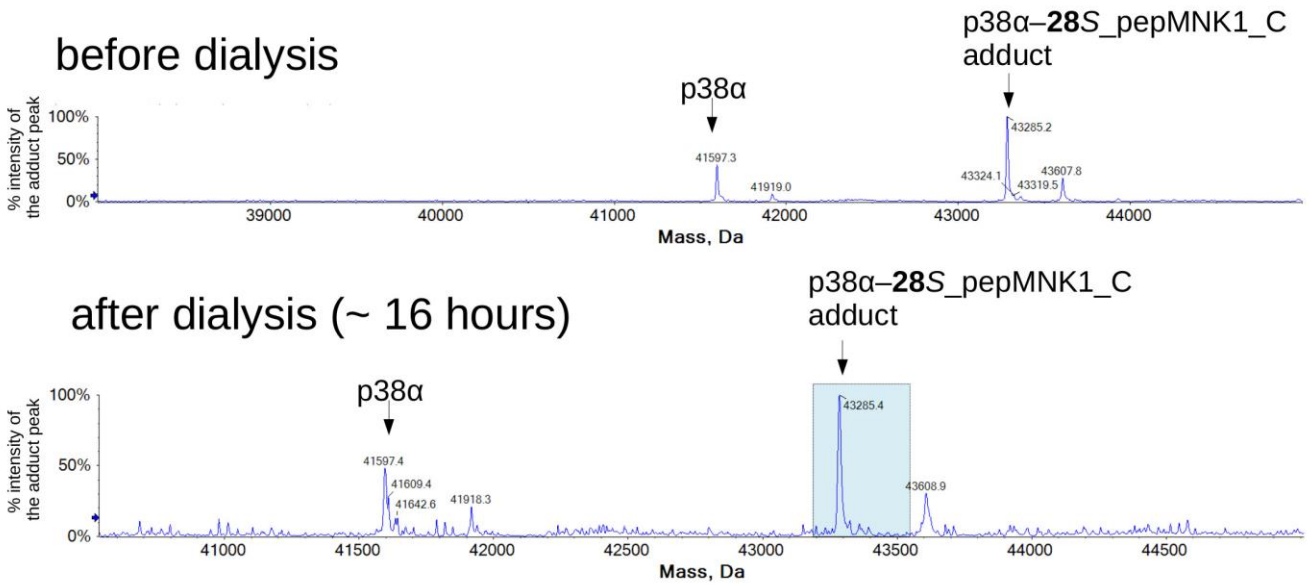


*Figure 1. LC-MS results of the dialysis experiment with p38α + peptide chimera (**28**S-pepMNK1_C). 5 µM p38α was incubated with 7.5 µM peptide chimera for one hour in 1 x PBS (pH ~ 7.4). 1ml of the sample was loaded into a dialysis tube (MWCO: 10 kDa) and was dialyzed for ~16 hours in 500 mL of 1 x PBS (pH ~ 7.4). Samples, before dialysis and after, were subjected to LC-MS analysis using a short LC step (a short security cartridge, Phenomenex C18, as described in the accompanying JNK manuscript for the characterization of reversible covalent adducts).*

- The binding characteristics, especially the kinetic aspect of reversible covalency, should be explained in a few additional sentences, ideally with a scheme in the introduction.

The kinetic aspect of reversible covalency is introduced and discussed in Fig. 1e, which is now also updated.

Additional minor points:

- On page 8, please specify that "Kchem´" refers to chemical reactivity on the target.

We explicitly state at the first appearance on "Kchem´" that this refers to "chemical reactivity on the target".

- On page 11, correct "enantioriched" to "enantiomerically enriched."

Corrected.

- On page 12, mention that the statement about the energy of C-S(Cys) and/or C-N(His) adduct formation versus non-covalent binding is based on the assumption that Cys/His do not contribute to non-covalent binding affinity.

Done. "(provided that Cys/His do not contribute to noncovalent binding)" is inserted at the relevant place in the text.

- On page 12, instead of "off-target thiol," consider "model thiol" or "surrogate thiol" as it refers to GSH, not a protein.

"off-target thiol" is changed to "model thiol"

- On page 15, whereas "reactivity" usually refers to kinetics, in this section, the authors refer to equilibrium constants (Kchem). Using a more precise terminology is suggested.

"reactivity" is changed to "adduct formation"

- On page 15, consider if there are other interactions involved besides the hydrophobic component.

"hydrophobic" is changed to "noncovalent, likely hydrophobic,"

- On page 15, correct "mehcanism" to "mechanism."

Corrected.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Rev#1 Comment to point 1)

The authors comments are acceptable. In a precise analysis (which is not available currently) "[I-Pcov] / [I:P] is equal to Kchem ´" is a descriptive parameter, as the two I:P complexes show a stochastic behavior. The statement "Undoubtedly, the concrete mechanistic details related to covalent bond formation and thiol elimination related to k3 (or kchem_on) and k4 (or kchem_off) kinetic rates, respectively, for example are not directly addressed." sufficiently explains that further progress in covalent/reversible inhibitor kinetic descriptions are required in the future.

The replies to the reviewer would be in part a useful addition (in supplementary information) for readers with less background in (equilibrium) kinetics.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my concerns to a sufficient extent. However, two important issues remain to be resolved:

1) Selectivity: I understand the authors arguments that these compounds have a special binding mode which is not possible for many kinases. This does, however, not preclude that the compounds may bind to other targets in a different manner. Therefore, I strongly recommend including a meaningful experiment to assess selectivity of key analogs on a broader scale.

2) Dissociation half-life: as dissociation is a 1st order process, it should generally not be impacted by competitors. Of course, mechanistic details may be more complex and I trust in the authors data. However, the fact that reversible-covalent BTK inhibitors did show significantly longer half-lives, even in competition experiments (see 10.1038/nCHEMBIO.1817) cannot be ignored. Even in a highly competitive environment, a t1/2 of 9min is by no means spectacular and the authors should properly account for this fact in their discussion.

Please find our response written in *italics.*

Rev#1 Comment to point 1)

The authors comments are acceptable. In a precise analysis (which is not available currently) "[I-Pcov] / [I:P] is equal to Kchem´" is a descriptive parameter, as the two I:P complexes show a stochastic behavior. The statement "Undoubtedly, the concrete mechanistic details related to covalent bond formation and thiol elimination related to k3 (or kchem_on) and k4 (or kchem_off) kinetic rates, respectively, for example are not directly addressed." sufficiently explains that further progress in covalent/reversible inhibitor kinetic descriptions are required in the future.

The replies to the reviewer would be in part a useful addition (in supplementary information) for readers with less background in (equilibrium) kinetics.

We are grateful for the comments through which the presentation of the reversible covalent binding concept could be improved in the manuscript.
The derivation of the formula describing the K_D for reversible covalent binding is discussed in the legend of Fig. 1e, covering the essence of what was written in our response about equilibrium kinetics.

Reviewer #3 (Remarks to the Author):

The authors have addressed most of my concerns to a sufficient extent. However, two important issues remain to be resolved:

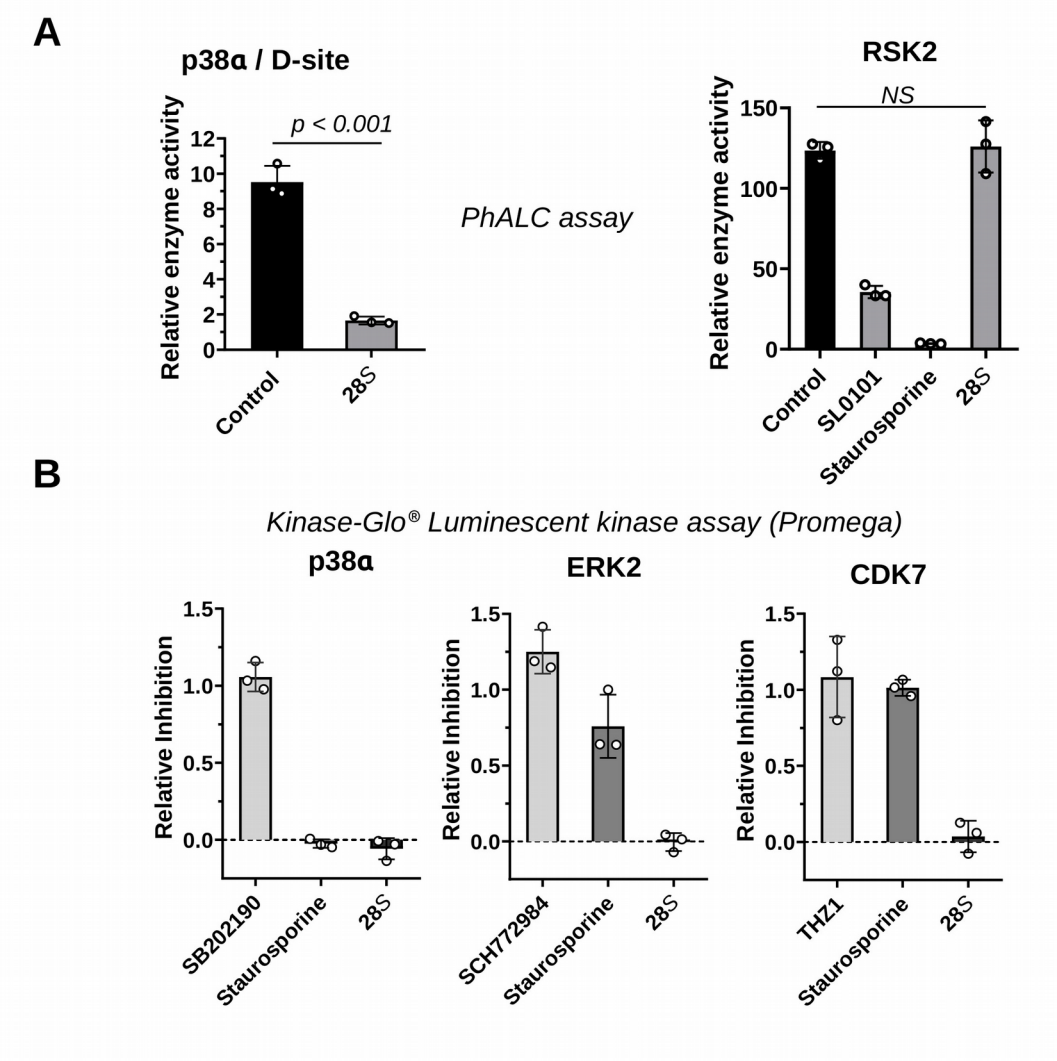
We are grateful for the critical comments of the reviewer allowing us to further improve the manuscript. We better highlighted the contrast between irreversible versus reversible covalent binding from a methodological point-of-view for off-target identification as well as the impact of competitors on the dissociation process. The concrete changes that were introduced into the revised text are highlighted in yellow, however a much more detailed discussion including additional experiments, for example focusing on potential off-targets containing "sensitive" cysteines from experimentally tractable kinases, are provided below.

1) Selectivity: I understand the authors arguments that these compounds have a special binding mode which is not possible for many kinases. This does, however, not preclude that the compounds may bind to other targets in a different manner. Therefore, I strongly recommend including a meaningful experiment to assess selectivity of key analogs on a broader scale.

Analysis of reversible covalent binders on a broader scale would require a novel methodology based on a novel concept. Contrary to irreversible binders, where the covalent adduct stays stable even after tryptic digestion, and thus gives a mass spec detectable fingerprint, reversible covalent binders fall apart readily and this classical methodology would likely give false positives (and note that the requirement for intact 3D protein structure and equilibrium conditions during the full analytical process poses a technical challenge for nanomolar binders too; see the accompanying manuscript with JNK inhibitors). Notwithstanding to this, we agree with the reviewer that the compounds potentially may bind to off-targets and selectivity of these MAPK binding molecules will need to be further analyzed at proteome level. We provided several instances where potential off-target effects were

investigated by choosing a few concrete, relevant examples (see our earlier response and three new experiments where this was experimentally further addressed).

Proteome-wide selectivity needs to be and will be addressed, however this requires methodology development far beyond the state-of-the art, which is being pursued but it is a longer term goal. For now, we provide three further examples where off-target effects were investigated: 1) RSK, which is a downstream component of ERK signaling pathways and had been targeted via its ATP-pocket cysteine by a covalent warhead (10.1126/science1108367), 2) an ATP-pocket cysteine in ERK2 (C166) formally shown to be covalently targeted by hypothemycin (a natural polyketide) (10.1016/j.jsb.2008.05.002), and 3) cycline-dependent kinase 7 (CDK7), which is a close relative of MAPKs (10.1038/nature13393). These proteins contain an accessible and sensitive cysteine that had been formally targeted by covalent inhibitors. As shown in the figure below, RSK activity, p38α or ERK2 docking independent functionality, or CDK7 activity were not affected by the presence of high amounts of cyclohexenone warhead containing compounds, as expected.



Panel (A): The left panel shows the results of in vitro PhALC assay experiments with a D-SENSOR construct whose phosphorylation is dependent on intact p38 docking (see ref 27; Póti et al, 2023). (Control shows the relative activity for the reaction with active kinase normalized to the signal of the

*test without kinase added; **28**S: 10 μM inhibitor was added that efficiently blocked docking and thus inhibited phosphorylation assisted luciferase complementation (PhALC); relative enzyme activity: the luminescence kinetic slope was normalized to that of the reaction which did not have active enzyme added.) The right panel shows a PhALC experiment with RSK-SENSOR (capable of detecting RSK2-mediated phosphorylation; see ref 27; Póti et al, 2023). (Control shows the relative activity for the uninhibited reaction; this was normalized to the reaction which did not have any active kinase added; SL0101, staurosporine, and **28**S show the results with an RSK-specific or nonselective ATP-competitive inhibitor, or with the MAPK docking inhibitor respectively, all used in 10 μM concentration). Notice, that the ATP-competitive inhibitors both inhibit RSK2 activity, while **28**S does not have this effect, as expected. (p-value: two-sided, unpaired t-test, n=3; NS: not significant).*
*Panel (B): Results of Kinase-Glo Luminescent kinase assay with p38α, ERK2 and CDK7. Note that this assay monitors kinase activity by monitoring ATP co-factor decrease and the graphs show relative inhibition compared to the test containing no kinase. The experiments were carried out using specific inhibitors (e.g., SB202190, SCH772984, or THZ1 for p38α, ERK2 and CDK7, respectively), the non-specific ATP-competitive staurosporine as well as the MAPK D-groove binding **28**S compound. All compounds were used in 10 μM concentration. Note that **28**S did not inhibit the intrinsic kinase activity of the kinases, while the specific inhibitors or staurosporine, depending on its specificity profile, were effective. (The more detailed description of the Kinase-Glo experiments is the following: The Kinase-Glo Max assay kit (Promega, Madison, WI; catalog no. V6071) was used to screen compounds for inhibition of CDK7/cyclin H/MNAT1, p38α, and ERK2. The enzymes CDK7/cyclin H/MNAT1 (catalog no. VA7402) were purchased from Promega, and the active MAP kinases (p38α and ERK2) were produced in-house as previously described. The kinase reaction system contained 25 ng/μL CDK7/cyclin H kinase, 10 μM of compounds, and 167 μg/μL kinase substrate (Native Swine Myelin Basic Protein) (Promega, Madison, WI) with 20 μM ATP (Promega, Madison, WI) in the case of CDK7 and 50 μM in the case of MAPKs. The reaction in each well was started immediately by adding ATP, followed by incubation for one hour at 25 °C. Then, 6 μL of Kinase-Glo Max reagent was added to each well, followed by 10 minutes of incubation. The luminescence signal was measured using a BioTek Cytation 3 microplate reader. After the signal was stable, the data were collected for five minutes. Data averages were normalized against control measurements both with and without kinase. Subsequently, the normalized data were plotted utilizing the GraphPad Prism 8.0.1.)*

We believe that these three new examples lend support to high level selectivity towards the MAPK D-groove, however this data was not included in the revised version and it is disclosed only in this response letter, since we believe that it is fairly specific information and the general reader may not benefit from this data too much; albeit it could be important for experts. Notwithstanding to this, and to highlight the difficulty of a proteome-wide off-target analysis, the following text was added to the penultimate paragraph of the Discussion to strike a balance.

"We showed that new D-groove binding compounds can selectively target the D-groove cysteine in a MAPK (see Supplementary Fig. 8), moreover, other compounds with different (ATP-competitive) directing groups with the same covalent warheads did not target the D-groove cysteine as expected (see accompanying manuscript). Unfortunately, proteome-wide selectivity of sterically crowded, double-activated cyclohexenone/pentenone containing compounds can not be easily tested because their reversible covalent binding poses a challenge for classical chemoproteomics. There is a need to develop an equilibrium binding based approach allowing unbiased off-target identification for reversible covalent binders."

2) Dissociation half-life: as dissociation is a 1st order process, it should generally not be impacted by competitors. Of course, mechanistic details may be more complex and I trust in the authors data. However, the fact that reversible-covalent BTK inhibitors did show significantly longer half-lives, even in competition experiments (see 10.1038/nCHEMBIO.1817) cannot be ignored. Even in a highly competitive environment, a t1/2 of 9min is by no means spectacular and the authors should properly account for this fact in their discussion.

Reversible covalent inhibitors binding via a 2-step process as outlined in our model are expected to be sensitive to the presence of competitors since the covalent and the noncovalent contributions are synergistic (and thus their dissociation is only formally 1st order but conceptually the process is more complex). These could be the reason why the half-life in our study and in 10.1038/nCHEMBIO.1817 are greatly different under classical versus competitive conditions, as we pointed this out in our earlier response. We agree with the reviewer that the half life of the MAPK inhibitors are lower compared to reversible ATP competitive BTK inhibitors (10.1038/nCHEMBIO.1817), however in the latter case the whole molecule is buried in a deep crevice which is easily druggable, while the MAPK D-groove is a shallow protein-protein interaction surface where even natural peptides bind with very fast dynamics. Currently, we do not now how much time a compound needs to "spend" in the MAPK D-groove to be effective (but our live cell NanoBit experiments show that they are effective as small compounds and interfere with MAPK signaling relevant D-groove dependent interactions). Furthermore, this experiment with the peptide chimera was only meant to show that covalent binding does increase residence time as expected.

To comply with the request, we included the following clause (shown in bold font) in the sentence discussing the results of this experiment (and also weakened the statement as shown):
"***Despite that this half-life increase is not high in this artificial peptide chimera context***, it still highlights an important aspect of the reversible covalent mechanism: it ~~is efficient in making~~ makes the protein-inhibitor complex ~~long-lived~~ longer-lived even under conditions where there are high amounts of noncovalent (natural) binders/competitors present."

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have added additional explanations that clarify the limitations of their study. My comments have been taken into account in an acceptable manner and I support the publication of the manuscript in its current form.