nature portfolio

Peer Review File

Reversible covalent c-Jun N-terminal kinase inhibitors targeting a specific cysteine by precision-guided Michael-acceptor warheads

Open Access This file is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. In the cases where the authors are anonymous, such as is the case for the reports of anonymous peer reviewers, author attribution should be to 'Anonymous Referee' followed by a clear attribution to the source work. The images or other third party material in this file are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Bálint et al. present a proof-of-concept study for the use of so-called “frustrated” cyclohexenones (see accompanying manuscript [related ms]) as new reversible covalent warheads. Using an ATP-binding scaffold derived from a previously described irreversible covalent inhibitor of JNK1/2/3, the authors prepared a library of different cyclohexanone warheads bearing reversible covalent inhibitors of JNK1/2/3 including a photocaged example. Next, the new inhibitors were evaluated in various biochemical and cellular experiments to promote the new reactive moieties as valuable alternatives to well established covalent warheads.
While the presented warheads are interesting, the manuscript’s novelty has been comprehensively covered by an accompanying manuscript. Also, this study does not manage to demonstrate the advantage and the translational potential of the new warheads due to missing detrimental experiments and poorly described results and procedures.

Point-by-point comments:

1. While the manuscript is written in a clear language and decent style, it is very difficult to follow the authors argumentations while discussing specific compounds, also due to their lab journal code labeling, but mainly due to somewhat non-transparent argumentations. Should be changed to a more functional labeling. Moreover, figures should contain the discussed structures to support that the readers navigate efficiently through the manuscript.

2. Some of the extreme bioactivity differences between IN-8 and its covalent versions most likely originate from the substantial physicochemical difference due the free aniline moiety. To compare covalent vs non covalent properties in a meaningful way, the authors should use the acetylated version of IN-8.

3. In Fig. 1B, the PhALC assay was performed in the presence of 10 mM GSH. However, to assess the influence of GSH on the inhibitory activity of the compounds, the assay should be performed without GSH as well. The data provided in Ext. Fig. 1 are not helpful either because a) the compounds were preincubated with GSH for a prolonged time which is not relevant to any of the performed experiments, and b) the data shows only two out of four compounds used in Fig 1B preventing a reasonable interpretation.

4. The authors claim that BDN837-IN-8 is resilient to GSH by showing an LC/MS analysis of a preincubated sample where no mass shift was detected. Conversely, JNK-IN-8 was completely converted to the GSH adduct. These results imply that BDN837-IN-8 does not react with sulfhydryls which is highly unlikely and contradictory to the other data provided in this manuscript. Since the authors miss to provide any information concerning the sample preparation in this experiment, its interpretation is does not make sense.

5. In Fig. 2, the PDB codes have to be provided in the figure legend.

6. Even though the authors are able to elucidate rate constants for several inhibitors using a two-step reversible kinetic binding model proposed by Mons et al., they miss to obtain the intrinsic reactivity of the new warheads that would allow a judgment for off-target reactivity. Also, in Fig. 3, the SPR graph for JNK-IN-8 contains the statement “k3 and k4 = 0” which is wrong because only k4 = 0 while k3 = kinact and has a positive value.

7. The inhibitory values from Fig. 4, which are summarized from Fig. S3-5, can not be evaluated for multiple reasons: 1) the standard deviations are missing for all data points, 2) some NanoBRET data is not available, especially for BDN-838 and RU144, the most potent compounds from the PhALC assay, 3) most of the p-c-Jun EC50 values were performed only once (n=1) rendering the results anecdotal.

8. In Fig S7B, the authors miss to provide inhibitor concentrations used in this particular assay.

9. Fig. S7C, no incubation time was provided.

10. Supplementary Table S4: The KINOMEScan (DiscoveryX) and the Wild Type Kinase Panel (Reaction Biology) are completely different assays and thus the results are not comparable.

11. The data and results from Extended Data Fig. 4 belong into the accompanying manuscript and are not useful in this discussion.

12. Fig 6: Again, the missing standard deviations in the table are preventing a reasonable interpretation of the data.

14. L125-126 and L131-132 contain the same sentence (repetition).

15. The tabulated NMR data appears to be correct but without corresponding spectra there is no structural proof. Also, some of the final compounds are missing an HRMS measurement and for all of them the authors should provide an HPLC purity. Furthermore, the syntheses of all enantiomeric isomers have been described for only one single isomer but it is not clear for which of both enantiomers the yields are given.

Reviewer #2 (Remarks to the Author):

The study by Balint et al. investigates the application of a novel electrophile to the development of reversible covalent JNK inhibitors. By way of motivating their study, the authors describe some of

the general challenges associated with irreversible inhibitors (off-target reactivity, haptenization and GSH depletion), highlighting that reversible covalent warheads (initially exemplified by cyanoacrylamides) can overcome some of these challenges but that the chemical toolkit for this approach is limited. This study builds on previous irreversible covalent acrylamide-derivatized ATP-competitive inhibitors (from Grey lab), targeting Cys116. Using their novel, highly-substituted cyclohexenone warhead (which is inferred to be intrinsically reversible covalent) they demonstrate potent cellular target engagement (Fig 1), prove Cys116 covalent bond formation by crystallography (Fig 2), characterize the binding kinetics by SPR (Fig 3), show some broad SAR trends (Fig 4), demonstrate on-target cellular activity (Fig 5) and investigate the potential for isoform selectivity (Fig 6). Some of the most compelling and exciting results include: (1) BD837-IN-8 (cyclohexenone) substantially outperforming RU155-IN-8 (cyanoacrylamide) in cellular target engagement, (2) BD837-IN-8 being resistant to extended treatment with high concentration GSH (Extended data fig 1), (3) BD837-IN-8 and RU159-isoPHEN (cyclohexenone) matching or bettering IN-8 (non-covalent), JNK-IN-8 (acrylamide, irreversible) and RU155-IN-8 (cyanoacrylamide) in the cellular AP-1 reporter and proliferation assays.
Overall, this study will be of interest to chemical biologists developing covalent probes and potentially provides tool compounds for studying JNK biology. However, further experimentation is required to characterize the reactivity, reversibility, and stability more clearly both in vitro and in cells (see Major Points below). In addition, further discussion of the nuances and limitations of the experiments and results is required throughout (see Minor points). I also found that this manuscript lacked clarity in the narrative at times and would benefit from some degree of restructuring (see Minor Point d). I anticipate that medicinal chemists may be somewhat reluctant to tryout this warhead because of concerns over its large MW and hydrophobicity relative to acrylamides etc - addressing this point might increase the real world impact of this study (see major point d and minor point e).

Major Points:
(a) There are two explanations for the result in Extended Data Fig 1, (1) the reactivity with GSH is rapid and totally reversible or (2) BD837-IN-8 doesn't react with GSH at all (or is exceptionally slow). Either result is interesting, but the authors must clarify this point. To accomplish this the authors should investigate whether BD837-IN-8 forms an adduct with GSH in aqueous buffer. This is probably best accomplished using NMR but alternative approaches could work too. If the reactivity with GSH is truly very fast on and off then such an adduct would likely be unstable to LCMS analysis – which would explain the result in Supp Fig 1. If possible, the authors should calculate a Kd for a representative cyclohexenone with GSH (should be possible by titration using NMR) such that it could be compared to cyanoacrylamides (Kd ~ 7 mM, PMID: 22466421).
(b) While the SPR analysis does provide support for the reversibility of the cyclohexenones with JNK, it can suffer from artifacts (see below minor point b). Further evidence of the reversibility with JNK in vitro is warranted. If the covalent adduct between BD837-IN-8 and JNK can be observed by intact protein mass spectrometry (which presumably depends on whether the covalent bond is stable to the LC conditions), then the authors should test whether denaturation of the protein with guanidinium/urea results in rapid bond dissociation. Alternatively, a simple dialysis experiment would be sufficient to investigate whether the kinase activity of JNK can be recovered after treatment with BD837-IN-8 but not with JNK-IN-8.

(c) Most importantly, the authors must address how the cyclohexenone effects cellular residence time upon washout. This should be tested for target engagement (for example using the NanoBRET assay) AND also using the phospho-cJun biomarker. One or more representative cyclohexenones should be compared to IN-8, RU155-IN-8 and JNK-IN-8.
(d) If possible, it would be very interesting to investigate the metabolic stability and physicochemical properties of BD837-IN-8 or RU159-isoPHEN in comparison to JNK-IN-8 and/or RU155-IN-8. Understanding the potential metabolic and physicochemical advantages or liabilities of this warhead in the context of a TCI would give other medicinal chemists more confidence to try it.

Minor Points:
(a) The crystal structures clearly show electron density between the covalent bond and Cys116 however these structures are challenging to model, especially at the modest resolutions obtained. The reaction generates two new stereocenters (ie 4 possible diastereomeric products). In the deposited PDBs, specific stereoisomers have been modelled (and the stereochemistry at both C3 and C2 are different for 837 and 838!). At the modest resolutions obtained, the stereochemical assignments are probably not unambiguous (and the observed electron density may be composite of multiple products). There is no discussion of this in the paper. Although I appreciate that the authors do not make any claims about the significance of the stereochemistry of the products, I still think they must comment on the confidence in the modelling as readers may not appreciate the inherent challenges here. Additional information on how the assignment was conducted should be included in the methods (for example did they try modelling all possible products and then decide based on which gave the best match to the electron density? Did they try any computational modelling or validation tools? Do all three subunits in the ASU give similar ligand densities and assignment confidences? The authors may also wish to comment on the ligand conformation (for example the cyclohexane ring is generally modelled here as boat-like rather than a chair) and try a conformation validation tool such as Mogul (https://www.ccdc.cam.ac.uk/solutions/software/mogul/) to support their modelling.
(b) The authors have carried out a large SPR study, comparing WT and mutant JNK in an attempt to derive the kinetic constants k1-4. This is a commendable effort. However I have some concerns about the validity of the results, especially relating to the determination of k4. In Supplementary Figure S2 the experimental data shows a decrease in signal-over-time for 20 nM JNK-IN-8 during the dissociation phase, despite it being an irreversible inhibitor (the extent of which is not so different from some of the reversible dissociation curves e.g. 837 and 838). This highlights the challenge of complex curve fitting to data with such a narrow signal window during the dissociation phase. The authors should either discuss the potential for error in this type of modelling and include a measure of the fit quality (equivalent to an r^2 value) or, better yet, conduct an experimental measurement of the error, for example by conducting replicates on key compounds. The authors should include the WT SPR sensorgrams and curve fitting in a supplementary figure as they did for the mutant in Supplementary Figure S2. Please also check for discrepancies between Supplementary table S2 and Figure 3, for example p(k2) for Ru155 in Figure 3 does not match the k2 listed in table S2.
(c) The authors make a comparison between the kinome profiling of 837 to JNK-IN-8, claiming that 837 shows better selectivity. However, because these compounds were profiled by different

companies using different assays, this may not be a fair comparison. For example, the selectivity will be strongly influenced by the ATP concentration used in the assay, as well as the incubation time and presence of reducing agents. The authors should highlight this in the main text or, better yet, reprofile JNK-IN-8 in the Reaction Biology panel.
(d) The narrative of this work is at times hard to follow and it would benefit from some degree of restructuring. Examples include showing crystal structures of 838-IN-8 and RU135-IN-8 in Figure 2 but not showing their chemical structures until Figure 3. Figure 4, which has broad SAR for many compounds, would probably make more sense if it came before the SPR analysis which seeks to understand the SAR of a selected subset in more detail. In Figure 5, it's not clear what question is being addressed by introducing a photocaged analogue (at present I found it to be a distraction from the main study)? The cell titration in Fig 5a seems like it should be a supplementary figure at best whereas extended data figure 2a would be a strong addition to Fig 5. I found Figure 3 awkward to interpret (especially because k1 and k3 are not explicitly shown), I actually was able to get a clearer understanding of the overall picture by looking at Supplementary Table 2 where you can compare all the rate constants very easily: I would consider incorporating Supp table 2 into this figure and dispensing with some of the graphs. I think that Extended Data Fig 1 is compelling and warrants being in a main figure (perhaps supported with additional data – see major point a).
(e) Some discussion of the physicochemical properties of this warhead is needed – including its large MW and hydrophobicity relative to acrylamides. In many cases these properties may be undesirable but in other cases, for example remote cysteines that exist in hydrophobic grooves, it could be beneficial.
(f) There is a large EC50 discrepancy between cellular target engagement (nanoBRET) and cellular p-c-Jun in Figure 4. E.g BD837-IN-8 is 45-times more potent than IN-8 by nanoBRET but only 3-times more potent in p-c-JUN. Can the authors comment on this? It will be interesting to see if BD837-IN-8 more clearly outperforms IN-8 at p-c-Jun inhibition in a cellular washout experiment (see major point c).

Point-by-point response to reviewers' comments related to "Reversible covalent c-Jun N-terminal kinase (JNK) inhibitors targeting a specific cysteine by precision-guided Michael-acceptor warheads" (NCOMMS-23-39210-T; "JNK story"; first submission)

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

Reviewer #1 (Remarks to the Author):

Bálint et al. present a proof-of-concept study for the use of so-called "frustrated" cyclohexenones (see accompanying manuscript [related ms]) as new reversible covalent warheads. Using an ATP-binding scaffold derived from a previously described irreversible covalent inhibitor of JNK1/2/3, the authors prepared a library of different cyclohexanone warheads bearing reversible covalent inhibitors of JNK1/2/3 including a photocaged example. Next, the new inhibitors were evaluated in various biochemical and cellular experiments to promote the new reactive moieties as valuable alternatives to well established covalent warheads.
While the presented warheads are interesting, the manuscript's novelty has been comprehensively covered by an accompanying manuscript. Also, this study does not manage to demonstrate the advantage and the translational potential of the new warheads due to missing detrimental experiments and poorly described results and procedures.

We would argue that the two manuscripts are complementary and their novelty is fully appreciated if these two stories were to appear together. The MAPK D-groove story shows the structural complexity of the new cyclic warheads and demonstrates the new features that emerge out of this. The JNK story shows how these warheads can be used as modular elements in composite drugs to target another selected cysteine and deals with specificity issues at a higher level and demonstrates the possibility of being able to fine-tune key binding properties of composite JNK drugs via simple synthetic modifications.

However, we acknowledge that some unfortunate mistakes in the preparation of the submitted material, bad wording in the text, and the lack of some key experiments might have lowered the enthusiasm on this manuscript. We believe that we addressed all comments, clarified the vague points by restructuring, rewording, and more importantly by adding new experimental data to both manuscripts that nicely complement each other regarding the use and translational potential of cyclic Michael acceptor based warheads.

Point-by-point comments:

1. While the manuscript is written in a clear language and decent style, it is very difficult to follow the authors argumentations while discussing specific compounds, also due to their lab journal code labeling, but mainly due to somewhat non-transparent argumentations. Should be changed to a more functional labeling. Moreover, figures should contain the discussed structures to support that the readers navigate efficiently through the manuscript.

The labeling of the compounds is now revised and the presentation of structures on figures is improved.

2. Some of the extreme bioactivity differences between IN-8 and its covalent versions most likely originate from the substantial physicochemical difference due the free aniline moiety. To compare

covalent vs non covalent properties in a meaningful way, the authors should use the acetylated version of IN-8.

There was on unfortunate mistake in old Figure 4 listing the cellular p-c-Jun EC50 values for IN-8 (instead of the correct value, 15800 nM or 15.8 μM as correctly shown in old Supplementary Fig. 5, the table in Figure 4 had showed a 10-fold lower value). In the light of the correct (10-fold higher) p-c-Jun EC50 value for IN-8, its difference compared to its NanoBRET value (~ 500 nM; ~ 30-fold difference) is not that surprising compared to the values measured for the other inhibitors.
Notwithstanding to the above, the acetylated version of IN-8 was produced (IN-8a), since it is indeed a better ATP-competitive control scaffold for the warhead containing compounds analyzed in this study, and we repeated some of the experiments with this new compound. The in vitro PhALC IC_{50} or binding characteristics in SPR were not different from the original molecule (IN-8) (see Table 1, Fig. 2b). We explicitly indicate if a concrete experiment was carried out using IN-8 or IN-8a. In the new version for the new experiments we used IN-8a as requested.
Please also note that the values in Table 1 are updated based on new experiments, as we increased the number of parallel measurements. Unexpectedly, IN-8a at the end turned out to be a weaker compound compared to IN-8, which further supports the importance of the covalent warhead in modulating the potency of the composite JNK inhibitors. (We have to note that IN-8a repeatedly behaved worse than IN-8 apart from when tested in "clean" biochemical measurements; for example in the cell-based NanoBRET assay with the K-5 tracer we could not detect efficient target engagement with NanoLuc JNK1, and this compound also performed poorly in HTRF p-c-Jun measurements.)

3. In Fig. 1B, the PhALC assay was performed in the presence of 10 mM GSH. However, to assess the influence of GSH on the inhibitory activity of the compounds, the assay should be performed without GSH as well. The data provided in Ext. Fig. 1 are not helpful either because a) the compounds were preincubated with GSH for a prolonged time which is not relevant to any of the performed experiments, and b) the data shows only two out of four compounds used in Fig 1B preventing a reasonable interpretation.

The motivation behind the PhALC assay experiments carried out in 10 mM GSH was that we wanted to show that all inhibitors can function in the presence of high amounts of off-target thiols, however if the warheads are exposed to an off-target thiol (under physiological pH) such as GSH, then the "potency" of acrylamide containing compounds could decrease since they make an irreversible covalent adduct with GSH, while this does not happen with reversible covalent compounds because their GSH adduct is also reversible (and since the other two compounds brought up in part b of the comment, namely IN-8 and RU155-IN-8 (now referred to as CA-IN-8), are also reversible binders, we did not show any analysis for these). In the early version of the manuscript we could not detect the reversible covalent GSH adducts of the new compounds. In the new version we shortened the LC step before the MS (by using a guard column only) and we can now detect the short-lived GSH adducts of the new compounds too (note that this product is very labile under physiological pH and we may have been able to detect this product because the dissociation of the adduct is greatly diminished due to highly acidic conditions of the LC-MS (pH ~ 2.5) where the deprotonation of the adduct is greatly lowered (see Supplementary Fig. 3).
Due to the reversible nature of the cyclohexenone-thiol(GSH) adduct (which is further characterized in the accompanying MAPK D-groove manuscript; K_{chem} ~ 1 mM), the reversible covalent composite JNK inhibitors are resilient to physiologically relevant, high amounts of off-target thiols, albeit they do form reversible GSH-adducts (and this was also corroborated by an analysis with another off-target adduct,

*BME–**1a**R-IN-8, in this manuscript by ^{1}H NMR measurements; see Supplementary Fig. 4). This latter, however, does not lower the effective concentration of the inhibitors, since these adducts are transient and their affinity is low (~mM); compared to the reversible covalent thiol adduct forming on the target (JNK) with a lot higher affinity (~ low nM) and with a lot smaller k_{off}. This is now better explained and discussed in the text. The data on Fig. 1f, which is described in more detail in the Methods section now, contains a comparison to a condition that lacked pre-incubation with 10 mM GSH as requested, namely the data acquired at 0 hr. This is the starting point for this GSH resilience experiment and it shows that at the beginning of this experiment both inhibitors are similarly "active" as expected.*

4. The authors claim that BDN837-IN-8 is resilient to GSH by showing an LC/MS analysis of a preincubated sample where no mass shift was detected. Conversely, JNK-IN-8 was completely converted to the GSH adduct. These results imply that BDN837-IN-8 does not react with sulfhydryls which is highly unlikely and contradictory to the other data provided in this manuscript. Since the authors miss to provide any information concerning the sample preparation in this experiment, its interpretation is does not make sense.

*We did not intend to imply that BD837-IN-8 (now referred to as **1a**R-IN-8) does not react with thiols. Our data suggest quite the opposite and it must have been due to some unfortunate wording why this was not properly clarified. This was improved in the text and additionally we also provide explicit proof for the existence of the GSH–BD837-IN-8 adduct by using a shortened LC-MS method, moreover, we detected and analyzed another off-target thiol adduct with BME by NMR under physiological pH (pH ~ 7.2) (see the answer for the earlier comment).*

5. In Fig. 2, the PDB codes have to be provided in the figure legend.

The new PDB IDs are now noted in the legend of Fig. 2.

6. Even though the authors are able to elucidate rate constants for several inhibitors using a two-step reversible kinetic binding model proposed by Mons et al., they miss to obtain the intrinsic reactivity of the new warheads that would allow a judgment for off-target reactivity. Also, in Fig. 3, the SPR graph for JNK-IN-8 contains the statement "k3 and k4 = 0" which is wrong because only k4 = 0 while k3 = kinact and has a positive value.

Naturally, for JNK-IN-8 "k3 and k4 = 0" is indeed totally incorrect, this was an oversight. Correctly: "k4 = 0". This is fixed.
*Intrinsic reactivity is addressed in the accompanying MAPK D-groove manuscript. K_{chem} is reported there for BME and GSH, which is in the millimolar range but it naturally depends on the electronic withdrawing strength of the substituent groups at C2 for example (see accompanying manuscript). Moreover, this value was also determined for the **1a**R-IN-8 + BME reaction (~ 1mM by NMR in this manuscript, showing good agreement with the EDC measurements for a simpler C2 anilide compound, **19**'S, presented in the accompanying manuscript). Additionally, off-target reactivity of the reversible covalent JNK inhibitors were directly addressed by the experiments containing 10 mM GSH (PhALC assay) and by kinome panel characterization.*

7. The inhibitory values from Fig. 4, which are summarized from Fig. S3-5, can not be evaluated for multiple reasons: 1) the standard deviations are missing for all data points, 2) some NanoBRET data is

not available, especially for BDN-838 and RU144, the most potent compounds from the PhALC assay, 3) most of the p-c-Jun EC50 values were performed only once (n=1) rendering the results anecdotal.

*This data is in Table 1 now and we indicated the errors in addition to the mean values. BD837-IN-8 (now **1a**R-IN-8) is similarly potent to other two compounds tested (JNK-IN-8, and RU144, which is called **1b**R-IN-8 and is shown on Fig. 6b now). All three have a similar EC50 in cellular p-c-Jun phosphorylation (~ 0.5 μM). NanoBRET data was indeed only used to compare one of our new inhibitors to benchmarks such as the IN-8 ATP pocket binding core, irreversible JNK-IN-8 or a reversible cyanoacrylamide compound (RU155-IN-8, called CA-IN-8 now). The p-c-Jun EC50 values were indeed determined based on one experiment for most of the inhibitors presented. However, we did not expect that any of the other inhibitors for which we just did one EC50 determination attempt would be any better than BD837-IN-8 (now called **1a**R-IN-8), since their in vitro characteristics were in agreement with their inferior cell-based effects based even on one experiment. However, to fully address this critique, we determined the western blot based EC50 values (p-c-Jun(Ser73)) anew for IN-8, since this was wrongly indicated as 1580 nM instead of 15800 nM earlier in this table, and for CA-IN-8 showing an unexpectedly weak EC50 value, 13000 nM; and the values for these inhibitors are updated in Table 1 and now fully match to the data shown on the corresponding supplementary figures).*
In addition we also set up a higher throughput cellular p-c-Jun phosphorylation assay through which the generation of parallels is less laborious. We used an HTRF approach capable of detecting p-c-Jun levels in cell lysates. This assay was used (N=3) for all inhibitors shown in Table 1 and overall the outcome shows a good agreement with the classical western blot based method used earlier, albeit the EC50 in absolute values quite differ for the same inhibitor determined by the two methods (4-8-fold). For the latter one reason may be that two distinct c-Jun phosphorylation states were detected (p-c-Jun(Ser73) or p-c-Jun(Ser63)) in these two methods and/or the classical western blot based procedure is less sensitive since its signal is based on the detection of phosphorylated species appearing in distinct bands (where some of them may be below the background because of low abundance) while the HTRF based method is more sensitive since it detects all species with better sensitivity in aggregate form. Despite this, the relative potency of the inhibitors determined by the two methods showed the same trends (Pearson coefficient is 0.68 based on nine data points).
Please also note that the values in Table 1 are updated based on new experiments, as we increased the number of parallel measurements.

8. In Fig S7B, the authors miss to provide inhibitor concentrations used in this particular assay.

The inhibitor concentration used in these experiments were 3 μM, which is now noted in the figure legend.

9. Fig. S7C, no incubation time was provided.

The measurement took place about 30 minutes after adding the competitor to the protein-reporter peptide mix (but the outcome did not change after the same binding mix was measured again after an additional one hour incubation time). The on-rate of reversible covalent bond formation is fast and the incubation time in carrying out these binding experiments is not expected to affect the outcome, since equilibrium is reached before we are technically able to carry out the measurements. Note that this is in contrast to acrylamide warhead based reactions which form a lot slower. There is some quantitative

discussion about this in the answer for a related comment in the accompanying manuscript. Notwithstanding, we explicitly state in the figure legend related to this experiment that incubation time was 30 minutes before taking the measurement.

10. Supplementary Table S4: The KINOMEScan (DiscoveryX) and the Wild Type Kinase Panel (Reaction Biology) are completely different assays and thus the results are not comparable.

We agree with this comment and this analysis is now put into the right context. Namely, we used it to choose a set of potential off-targets for testing them with ***1a****R-IN-8 and now show the results with a small off-target set (chosen based on JNK-IN-8 DiscoverX and* ***1a****R-IN-8 Reaction Biology data). Here the same DiscoverX assay platform was used to compare off-target binding (see Fig. 5b). The discussion on the specificity of* ***1a****R-IN-8 and other new JNK inhibitors is updated (see Fig. 5 and the response to the next comment).*

11. The data and results from Extended Data Fig. 4 belong into the accompanying manuscript and are not useful in this discussion.

We believe that topographic steric map and buried volume analyses, in spite of being based on theoretical calculations, are nice and useful additions to this manuscript, particularly at this part of the manuscript dealing with the general specificity of one of our cyclic warhead designs emerging from its unique cyclic structure. Naturally, it would also fit to the accompanying story, however, it is a better fit here. To keep this analysis simpler and more focused in the main part of the text, the comparison is made on Fig. 5c only for the simplest open-chain acrylamide and two cyclic warhead structures, while all the other relevant warheads are shown and are compared on Supplementary Fig. 12.

12. Fig 6: Again, the missing standard deviations in the table are preventing a reasonable interpretation of the data.

The relevant errors are indicated in Fig. 6c.

14. L125-126 and L131-132 contain the same sentence (repetition).

This is fixed now.

15. The tabulated NMR data appears to be correct but without corresponding spectra there is no structural proof. Also, some of the final compounds are missing an HRMS measurement and for all of them the authors should provide an HPLC purity. Furthermore, the syntheses of all enantiomeric isomers have been described for only one single isomer but it is not clear for which of both enantiomers the yields are given.

The NMR spectra, HPLC data, the missing HRMS measurements, and yields for distinct enantiomers of all compounds are included in Supplementary Note 4.

Reviewer #2 (Remarks to the Author):

The study by Balint et al. investigates the application of a novel electrophile to the development of reversible covalent JNK inhibitors. By way of motivating their study, the authors describe some of the general challenges associated with irreversible inhibitors (off-target reactivity, haptenization and GSH depletion), highlighting that reversible covalent warheads (initially exemplified by cyanoacrylamides) can overcome some of these challenges but that the chemical toolkit for this approach is limited. This study builds on previous irreversible covalent acrylamide-derivatized ATP-competitive inhibitors (from Grey lab), targeting Cys116. Using their novel, highly-substituted cyclohexenone warhead (which is inferred to be intrinsically reversible covalent) they demonstrate potent cellular target engagement (Fig 1), prove Cys116 covalent bond formation by crystallography (Fig 2), characterize the binding kinetics by SPR (Fig 3), show some broad SAR trends (Fig 4), demonstrate on-target cellular activity (Fig 5) and investigate the potential for isoform selectivity (Fig 6). Some of the most compelling and exciting results include: (1) BD837-IN-8 (cyclohexenone) substantially outperforming RU155-IN-8 (cyanoacrylamide) in cellular target engagement, (2) BD837-IN-8 being resistant to extended treatment with high concentration GSH (Extended data fig 1), (3) BD837-IN-8 and RU159-isoPHEN (cyclohexenone) matching or bettering IN-8 (non-covalent), JNK-IN-8 (acrylamide, irreversible) and RU155-IN-8 (cyanoacrylamide) in the cellular AP-1 reporter and proliferation assays.
Overall, this study will be of interest to chemical biologists developing covalent probes and potentially provides tool compounds for studying JNK biology. However, further experimentation is required to characterize the reactivity, reversibility, and stability more clearly both in vitro and in cells (see Major Points below). In addition, further discussion of the nuances and limitations of the experiments and results is required throughout (see Minor points). I also found that this manuscript lacked clarity in the narrative at times and would benefit from some degree of restructuring (see Minor Point d). I anticipate that medicinal chemists may be somewhat reluctant to tryout this warhead because of concerns over its large MW and hydrophobicity relative to acrylamides etc - addressing this point might increase the real world impact of this study (see major point d and minor point e).

The manuscript went through major restructuring to improve the narrative, all the minor and major points were addressed and we believe that the insightful comments greatly helped to present a better story which focuses better on how cyclic Michael acceptors may be used as tunable reversible covalent anchors in composite ATP-competitive JNK inhibitors, or for other applications presented in the accompanying manuscript.

Major Points:
(a) There are two explanations for the result in Extended Data Fig 1, (1) the reactivity with GSH is rapid and totally reversible or (2) BD837-IN-8 doesn't react with GSH at all (or is exceptionally slow). Either result is interesting, but the authors must clarify this point. To accomplish this the authors should investigate whether BD837-IN-8 forms an adduct with GSH in aqueous buffer. This is probably best accomplished using NMR but alternative approaches could work too. If the reactivity with GSH is truly very fast on and off then such an adduct would likely be unstable to LCMS analysis – which would explain the result in Supp Fig 1. If possible, the authors should calculate a Kd for a representative cyclohexenone with GSH (should be possible by titration using NMR) such that it could be compared to cyanoacrylamides (Kd ~ 7 mM, PMID: 22466421).

We believe that reactivity with GSH is very dynamic and is totally reversible. In the early version of the manuscript we could not detect the reversible covalent GSH adducts of the new compounds. In the new

version we shortened the LC step before the MS (by using a guard column only) and we can now detect the short-lived GSH adduct too (see Supplementary Fig. 3). Due to the reversible nature of the cyclohexenone-thiol(GSH) adduct (which is further characterized in the accompanying MAPK D-groove manuscript; K_{chem} *~ 1 mM; and also analyzed for the* ***1a****R-IN-8–thiol(BME) adduct by* ^{1}H *NMR), the reversible covalent composite JNK inhibitors are resilient to physiologically relevant, high amounts of off-target thiols, albeit they do form reversible GSH-adducts. This latter, however, does not lower the effective concentration of the inhibitors, since these adducts are transient and their affinity is low (~mM); compared to the reversible covalent thiol adduct that forms on the target (JNK) with a lot higher affinity (~ low nM) and with a lot smaller* k_{off}*. This is now better explained and discussed in the text.*

(b) While the SPR analysis does provide support for the reversibility of the cyclohexenones with JNK, it can suffer from artifacts (see below minor point b). Further evidence of the reversibility with JNK in vitro is warranted. If the covalent adduct between BD837-IN-8 and JNK can be observed by intact protein mass spectrometry (which presumably depends on whether the covalent bond is stable to the LC conditions), then the authors should test whether denaturation of the protein with guanidinium/urea results in rapid bond dissociation. Alternatively, a simple dialysis experiment would be sufficient to investigate whether the kinase activity of JNK can be recovered after treatment with BD837-IN-8 but not with JNK-IN-8.

We carried out the suggested dialysis experiment and found that JNK samples incubated with reversible covalent inhibitors indeed recover their activity in a long dialysis experiment (lasting for several days), in contrast to a sample that had been incubated with the irreversible JNK-IN-8 inhibitor (see Fig. 1g). Note that this experiment also tested CA-IN-8 (the reversible covalent cyanoacrylamide reference compound) and samples pre-incubated with this compound reached full recovery after 5 days (under this competition-free experimental set-up) but the new ***1a****R-IN-8 reversible covalent compound appeared more efficient since samples pre-treated with this compound recovered only up 50% after 5 days; which was in agreement with the results of SPR based biochemical* k_{off} *measurements.*
Despite lots of efforts, we could not detect the JNK-reversible inhibitor adduct by intact LC-MS because we believe that JNK1 is denatured (but not precipitated) under the low pH condition of the LC-MS procedure and "releases" the inhibitor; which is also consistent with the reversible covalent binding mechanism since if it were otherwise we should have been able to see an increased intact mass. (Note that we could easily detect the adduct of MAPK D-groove cysteine reactive (reversible) molecules with related cyclohexenone based structures with ERK2 as shown in the accompanying manuscript because this MAPK is far more resilient to low pH and the dissociation of the adduct from ERK2 is presumably lowered under low pH conditions).

(c) Most importantly, the authors must address how the cyclohexenone effects cellular residence time upon washout. This should be tested for target engagement (for example using the NanoBRET assay) AND also using the phospho-cJun biomarker. One or more representative cyclohexenones should be compared to IN-8, RU155-IN-8 and JNK-IN-8.

The cellular residence time upon washout was addressed as suggested by the NanoBRET assay and by the AP-1 promoter assay, a proxy for the phospho-c-Jun biomarker (see Fig, 4c, d). The results of these experiments are in good agreement with the biochemical k_{off}*-s and irreversibility vs reversibility characterized earlier. The cellular effects of IN-8a (the noncovalent ATP-competitive inhibitor), irreversible JNK-IN-8,* ***1a****R-IN-8 (reversible covalent cyclohexenone compound), and* ***6****S,S-IN-8*

(another version of the new cyclohexenone warhead with the lowest biochemical k_{off}) were compared in a washout experiment using the AP-1 promoter HEK293 cell line (Fig. 4c) and in JNK1 target engagement monitored by the NanoBRET assay in HEK293T cells; where the latter also included CA-IN-8 (RU155-IN-8), the reversible covalent cyanoacrylamide reference compound (Fig. 4d).

(d) If possible, it would be very interesting to investigate the metabolic stability and physicochemical properties of BD837-IN-8 or RU159-isoPHEN in comparison to JNK-IN-8 and/or RU155-IN-8. Understanding the potential metabolic and physicochemical advantages or liabilities of this warhead in the context of a TCI would give other medicinal chemists more confidence to try it.

We added a new section to the Results entitled "Translational potential of JNK composite inhibitors with a new reversible covalent warhead" including a preliminary PK study on different compounds (see the second paragraph in this section and Table 4). Briefly, the metabolic stability of some selected compounds were tested in rat primary hepatocytes and in rat blood plasma. Hepatic stability was surprisingly robust while the stability of some of the compounds in plasma was low presumably due to high esterase activity. However, even this limited study revealed interesting PK properties about activated cyclohexenone scaffolds as well as on the composite JNK inhibitors. We are carrying out a more systematic PK study on a much greater panel of these new compounds using human samples (liver and plasma) combined with straightforward synthetic optimization designed particularly to improve PK properties, which will be reported in due course.

Minor Points:

(a) The crystal structures clearly show electron density between the covalent bond and Cys116 however these structures are challenging to model, especially at the modest resolutions obtained. The reaction generates two new stereocenters (ie 4 possible diastereomeric products). In the deposited PDBs, specific stereoisomers have been modelled (and the stereochemistry at both C3 and C2 are different for 837 and 838!). At the modest resolutions obtained, the stereochemical assignments are probably not unambiguous (and the observed electron density may be composite of multiple products). There is no discussion of this in the paper. Although I appreciate that the authors do not make any claims about the significance of the stereochemistry of the products, I still think they must comment on the confidence in the modelling as readers may not appreciate the inherent challenges here. Additional information on how the assignment was conducted should be included in the methods (for example did they try modelling all possible products and then decide based on which gave the best match to the electron density? Did they try any computational modelling or validation tools? Do all three subunits in the ASU give similar ligand densities and assignment confidences? The authors may also wish to comment on the ligand conformation (for example the cyclohexane ring is generally modelled here as boat-like rather than a chair) and try a conformation validation tool such as Mogul (https://www.ccdc.cam.ac.uk/solutions/software/mogul/) to support their modelling.

The model building of the crystallographic covalent adduct, as well as the pitfalls of this at this resolution, is explicitly discussed in the Methods section now. We did not intend to elaborate too much on the structure of the adduct in this study because this will naturally be ambiguous due to the lack of higher resolution data. However, we have several other crystal structures of related cyclohexenone scaffold containing compounds where data well below 2Å is available, albeit the C-S adduct forms in these in a different protein surface context. Naturally, we are a lot more confident about the concrete conformation of the adduct in these cases. For this JNK inhibitor study, in spite of lower resolution, the

pose of the compounds regarding the functional groups at C4 was clear even from the beginning of the refinement for ***1a***R*-IN-8 (BD837-IN-8), since the relative orientation of the methyl versus carboxymethyl groups could be decided based on unbiased maps even at the early stages of the refinement process. Due to somewhat poorer quality of the electron density map for* ***1a****S-IN-8 (BD838-IN-8) or for* **1a'***R-IN-8 (RU135-IN-8), the binding poses had to be decided by generating the different alternatives and analyzing the peaks in the Fo-Fc density maps. The two different stereoisomers emerging due to forming the C-S bond at C3 were drawn up in JLigand and whichever gave a better fit into the density was retained and the refinement was then finalized with the better fitting stereoisomer at C2 (since the covalent bond generates a new center at C2 as well). Density features were suitable to decide on the more likely stereoisomer at the A or B complexes. However, the asymmetric unit contains three different JNK-inhibitor adducts (A,B,C) and C was worse and had significantly higher B factors. Unfortunately, the density for this chain was not good enough to decide on the concrete stereochemistry at C2 and C3 without any bias. The stereoisomer from chain A and B were used to fit the weaker adduct density for C, therefore the final configurations in C need to be handled with caution.*
The concrete conformation of the cyclohexenone ring is a very interesting aspect of the new cyclic warheads, because this potentially allows better control over the positioning of pendant substituents in space. We are currently working on the relevance of the chair versus twisted boat conformation of the cyclohexenone scaffold, which we consistently see in the crystal structures of our different protein adducts. This is, however, will be the focus of another study that we are currently pursuing via higher resolution experimental structure solution combined with theoretical calculations on the conformation of cyclohexenone/cyclopentenone based scaffolds with different pendant substituents.

(b) The authors have carried out a large SPR study, comparing WT and mutant JNK in an attempt to derive the kinetic constants k1-4. This is a commendable effort. However I have some concerns about the validity of the results, especially relating to the determination of k4. In Supplementary Figure S2 the experimental data shows a decrease in signal-over-time for 20 nM JNK-IN-8 during the dissociation phase, despite it being an irreversible inhibitor (the extent of which is not so different from some of the reversible dissociation curves e.g. 837 and 838). This highlights the challenge of complex curve fitting to data with such a narrow signal window during the dissociation phase. The authors should either discuss the potential for error in this type of modelling and include a measure of the fit quality (equivalent to an r^2 value) or, better yet, conduct an experimental measurement of the error, for example by conducting replicates on key compounds. The authors should include the WT SPR sensorgrams and curve fitting in a supplementary figure as they did for the mutant in Supplementary Figure S2. Please also check for discrepancies between Supplementary table S2 and Figure 3, for example p(k2) for Ru155 in Figure 3 does not match the k2 listed in table S2.

The k2 value for RU155 was indeed incorrect in the table, it had one "0" less after the decimal separator. Moreover, as we were checking all the values in the table we noticed that we made another mistake regarding the k2 value for BD837-IN-8, therefore we redid the analysis for this inhibitor, which slightly changed the outcome since this inhibitor gave now very similar values to its enantiomer, which we believe is reasonable, as opposed to be stronger. All this is now fixed and Table 3 shows the correct values now. The representation of the SPR data is also updated: it is simplified on Fig. 3 as suggested since the list of the k1-4 values became part of the main text (Table 3).
We would argue that SPR data analysis can be made reasonably robust after careful sample preparation and applying standardized SPR protocols for the measurements, however, we also discuss the results of an error analysis on the determined parameters for four JNK inhibitors: JNK-IN-8 (reference compound), CA-IN-8 (formerly RU155-IN-8, reference compound with an incorrectly

indicated k_3 value earlier), ***1a****R-IN-8 (formerly BD837-IN-8, a simple potent new inhibitor whose values were incorrectly noted down earlier) and* ***6****S,S-IN-8 (formerly RU212-IN-8, a more complex and potent inhibitor with the lowest k4 among the new compounds). We carried out several parallel analysis for k1 or k2 and for k3 and k4 related measurements to demonstrate that despite the complexity of these measurements/calculations the analysis is fairly robust. Based on the new parallel measurements we estimate the precision of these measurements about ~ 50 % (provided that the technical pitfalls of the SPR measurements are properly handled for a comparative study; for example, surface immobilization level with the ligand (JNK1) should be reasonable to avoid mass transfer effects – for which a lower level is more optimal for kinetic measurements, however to obtain a good signal-to-noise ratio with these small molecules a higher value would be better, moreover, for the measurements executed with different inhibitors the concentration of the analyte should be chosen around its equilibrium K_D – ideally a bit below and a bit above).*
The experimental data for 20 nM JNK-IN-8 during the dissociation phase indeed shows some decrease in signal-over-time (which however is also discernible for the lower concentrations too). This inhibitor makes an irreversible bond to JNK, and the formation of the final covalent adduct is likely to be slow. We believe that during the course of the injection not all bound JNK-IN-8 inhibitor forms the irreversible adduct. Another unrelated technical problem is that at high ligand saturation level the ligand is more prone to "bleed" and this is difficult to handle. However, this we deemed only marginal compared to the overall signal, and k4 was set to 0 during the fit of the JNK-IN-8 binding curves. We repeated this JNK-IN-8 binding experiment at lower ligand saturation and we obtained a more ideal k4=0 type behavior and show the results of this experiment as a new parallel experiment in Supplementary Fig. 5 (and we show that even due to this non-ideal behavior the k3 parameter does not change to a great extent and the precision is still acceptable in our opinion; naturally this is because the k4 was set to 0, though). The limitations of this complex analysis is better discussed and the caveats are explicitly stated in the text now (see Methods).

(c) The authors make a comparison between the kinome profiling of 837 to JNK-IN-8, claiming that 837 shows better selectivity. However, because these compounds were profiled by different companies using different assays, this may not be a fair comparison. For example, the selectivity will be strongly influenced by the ATP concentration used in the assay, as well as the incubation time and presence of reducing agents. The authors should highlight this in the main text or, better yet, reprofile JNK-IN-8 in the Reaction Biology panel.

We did not have the opportunity to reprofile JNK-IN-8 in the Reaction Biology panel, but addressed this point the following way. As pointed out, we agree that direct comparison between results obtained with the DiscoverX's binding based and Reaction Biology's activity based platform is not adequate. However, we used the DiscoverX data on JNK-IN-8 from Zhang et al 2012 (as well as our Reaction Biology data with BD837-IN-8) to choose a set of likely off-targets for the IN-8 ATP-competitive scaffold and tested this set (6 kinases) with the same (DiscoverX) platform to allow a direct, albeit undoubtedly limited, comparison (see Fig. 5b). We restructured and rephrased this analysis on specificity according to this new data.

(d) The narrative of this work is at times hard to follow and it would benefit from some degree of restructuring. Examples include showing crystal structures of 838-IN-8 and RU135-IN-8 in Figure 2 but not showing their chemical structures until Figure 3. Figure 4, which has broad SAR for many compounds, would probably make more sense if it came before the SPR analysis which seeks to understand the SAR of a selected subset in more detail. In Figure 5, it's not clear what question is being

addressed by introducing a photocaged analogue (at present I found it to be a distraction from the main study)? The cell titration in Fig 5a seems like it should be a supplementary figure at best whereas extended data figure 2a would be a strong addition to Fig 5. I found Figure 3 awkward to interpret (especially because k1 and k3 are not explicitly shown), I actually was able to get a clearer understanding of the overall picture by looking at Supplementary Table 2 where you can compare all the rate constants very easily: I would consider incorporating Supp table 2 into this figure and dispensing with some of the graphs. I think that Extended Data Fig 1 is compelling and warrants being in a main figure (perhaps supported with additional data – see major point a).

*We restructured the whole manuscript according to the suggestions above. The structures of the compounds appear now in Table 1 with the broad SAR analysis, which precedes Figure 2. The experiment with the photocaged analogue is moved to the beginning (Fig. 1e) and the relevance of it is better highlighted: it shows that the inhibitory capacity of **1a**R-IN-8 directly depends on ATP-pocket engagement in cells, as it is expected from an ATP-competitive inhibitor, and is not due to some unspecific effect of the warhead moiety. The cell titration in former Fig 5a is moved to a supplementary figure, while extended figure 2 is part of new Fig. 1. Figure 3 on the SPR binding analysis is reorganized and its corresponding table, which was a supplementary table earlier, became Table 3. Extended Data Fig 1 is part of Fig. 1 now and is supported by additional new data (see the answer for major point a). Note that the summary on the k1-k4 values obtained based on the SPR analysis became a main text table (Table 3; while the earlier figure related to this analysis got simplified, Fig. 3).*

(e) Some discussion of the physicochemical properties of this warhead is needed – including its large MW and hydrophobicity relative to acrylamides. In many cases these properties may be undesirable but in other cases, for example remote cysteines that exist in hydrophobic grooves, it could be beneficial.

In comparison to acrylamides, the new warhead is indeed greatly different and we argued that its distinct cyclic structure compared to more flexible open-chain acrylamide based warheads could have advantages in terms of specificity (which is now better highlighted by moving some of the results of the theoretical steric map and buried volume analysis to be part of one of the main figures, see Fig. 5c). The molecular mass of the bare irreversible acryalamide warhead is 70 Da. The minimal mass of the cyclopentenone/cyclohexenone-based warheads (with two methyl substituent groups at C4) is about 2.5 times greater than this (and the double-activated C2/C4 acrylamide/acrylesters are ~ 3-fold bigger). In medicinal chemistry applications the higher molecular mass (although the size of the bare acrylamide or cyanoacrylamide warheads are also often increased to achieve more favorable PK properties) is disadvantageous in general. In spite of this, we believe that there could be specific applications where this may be balanced out by unique properties emerging due to the cyclic structure: these warhead moieties would naturally better fit to shallow hydrophopic grooves as this is shown in the accompanying MAPK D-groove manuscript. In addition to this, in this JNK manuscript we show and argue that simpler synthetic elaboration, resilience against off-target GSH, and possibly higher intrinsic specificity because of extra 3D features are some of the unique properties why medicinal chemistry applications might benefit from this new warhead architecture.

(f) There is a large EC50 discrepancy between cellular target engagement (nanoBRET) and cellular p-c-Jun in Figure 4. E.g BD837-IN-8 is 45-times more potent than IN-8 by nanoBRET but only 3-times more potent in p-c-JUN. Can the authors comment on this? It will be interesting to see if BD837-IN-8 more clearly outperforms IN-8 at p-c-Jun inhibition in a cellular washout experiment (see major point c).

*This was another mistake that the reviewer correctly spotted. The EC50 of IN-8 was not 1580 nM in the cellular p-c-Jun western blot based assay but it was measured to be 15800 nM (namely 10-fold worse, as this had been shown correctly in the old Supplementary Fig. 5) and had unfortunately been wrongly jotted down in the table in old Figure 4 (now Table 1). This would make the EC50 value differences between the NanoBRET and p-c-Jun assay ~ 30-fold which is comparable what we found for **1a**R-IN-8. Note that the values reported in Table 1 are now updated per request of another reviewer: more parallel measurement were carried out and the p-c-Jun values (Ser73) somewhat changed because of this, moreover, another detection method for p-c-Jun phosphorylation (Ser63) was also implemented (HTRF).*

*Yes, BD837-IN-8 (now called **1a**R-IN-8) indeed outperforms IN-8a (and IN-8, not shown) or even a reversible open-chain warhead containing compound (CA-IN-8) in cellular washout experiments (see Fig. 4c,d).*

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this revised manuscript, Bálint et al. describe the application of a novel, reversible covalent warhead class, reported in an accompanying manuscript, to specifically target JNK isoforms. The reorganized structure and the addition of valuable and insightful experiments have markedly elevated the quality of the updated manuscript. The authors effectively showcase the translational potential of the new warheads and underscore their significance in the development of specific reversible covalent inhibitors.
Accordingly, after resolving of some minor issues with the NMR data, I recommend this study for publication.

While the NMR annotation and spectra of the reported compounds appear to be correct, there are many minor issues in their reporting and presentation, such as:
• compound 5 1H-NMR: peak pickings missing
• compound 5 13C-NMR: 202.1 ppm instead of 200.1 ppm
• compound S20 13C-NMR: signal at 172.6 ppm missing in the tabulation
• compound S26: 1H- and 13C-spectra do not correspond to the tabulated signals
• compound 1a''R-IN-8 1H-NMR: peak pickings missing
Please carefully and thoroughly check the reported NMR values and spectra and correct all mistakes.

Reviewer #2 (Remarks to the Author):

The authors have added additional data to address specific concerns raised by myself and reviewer 1 and have slightly restructured parts of the paper. I do find the additional data to be convincing and the manuscript is now more robust in its characterization of the reversibile modification. Sadly, however, the presentation of the results is still very poor. The narrative is unclear throughout - with different pieces of data on different compounds and few clear conclusion. Figure 3 in particular is remarkably inconsequential. Despite the work being interesting, I can not recommend this manuscript for publication in this form in Nature Communications.

For any future submissions of this manuscript to any journal, I recommend that the authors refine the narrative and presentation significantly, focussing figures on a limited set of compounds and data that support a set of well defined conclusions.

Please find our response written in *italics.*

Reviewer #1 (Remarks to the Author):

In this revised manuscript, Bálint et al. describe the application of a novel, reversible covalent warhead class, reported in an accompanying manuscript, to specifically target JNK isoforms. The reorganized structure and the addition of valuable and insightful experiments have markedly elevated the quality of the updated manuscript. The authors effectively showcase the translational potential of the new warheads and underscore their significance in the development of specific reversible covalent inhibitors.
Accordingly, after resolving of some minor issues with the NMR data, I recommend this study for publication.

Naturally, we are happy about these comments and are grateful to the Reviewer for patience and useful comments through which the manuscript could get improved.

While the NMR annotation and spectra of the reported compounds appear to be correct, there are many minor issues in their reporting and presentation, such as:
• compound 5 1H-NMR: peak pickings missing
• compound 5 13C-NMR: 202.1 ppm instead of 200.1 ppm
• compound S20 13C-NMR: signal at 172.6 ppm missing in the tabulation
• compound S26: 1H- and 13C-spectra do not correspond to the tabulated signals
• compound 1a"R-IN-8 1H-NMR: peak pickings missing
Please carefully and thoroughly check the reported NMR values and spectra and correct all mistakes.

Unfortunately, something went wrong during the final edit of the NMR section of the Supplementary Material. All the indicated mistakes have been fixed and the reported NMR annotation and spectra were checked for mistakes (and other reported NMR values were also carefully checked; and all other remaining small mistakes were corrected; e.g., "compound 5" was changed to its correct "compound S5" reference.). A new version of the Supplementary Material with the fixed NMR annotations and spectra are uploaded.

Reviewer #2 (Remarks to the Author):

The authors have added additional data to address specific concerns raised by myself and reviewer 1 and have slightly restructured parts of the paper. I do find the additional data to be convincing and the manuscript is now more robust in its characterization of the reversibile modification. Sadly, however, the presentation of the results is still very poor. The narrative is unclear throughout - with different pieces of data on different compounds and few clear conclusion. Figure 3 in particular is remarkably inconsequential. Despite the work being interesting, I can not recommend this manuscript for publication in this form in Nature Communications.

For any future submissions of this manuscript to any journal, I recommend that the authors refine the narrative and presentation significantly, focussing figures on a limited set of compounds and data that support a set of well defined conclusions.

We hoped that we had improved the narrative and tried to follow the specific comments regarding this from the earlier round. This manuscript meant to cover a great scope of composite JNK inhibitors to demonstrate the tunability of the reversible covalent mechanism, therefore it had to be highly comparative in nature and the full study indeed includes about two dozens of JNK inhibitors.

Per specific comment regarding Figure 3, we simplified this figure and show the structure of only those compounds that we experimentally addressed on this main figure (while SPR data for the rest can be found in Supplementary Fig. 5, and the structure of the full set is already shown in Table 1). A new version of Fig. 3 is uploaded.

We are thankful for the reviewer for comments and giving a chance to improve the manuscript, and we are hoping that with some additional clarification on the chosen narrative the Reviewer would find our approach for presenting the work now overall acceptable (see the text added at the end of the Introduction or below):

"First we compare JNK inhibitors with a new cyclohexenone/pentenone warhead scaffold with other formerly known open-chain Michael acceptor containing compounds and demonstrate that composite inhibitors with a cyclic warhead may have beneficial properties. We show that the new inhibitors form a reversible covalent adduct with the target cysteine on JNKs and that inhibitor residence time can be fine-tuned by adjusting the electronic properties and/or steric crowding around the Michael acceptor. In addition to the specificity of the so-called directing group responsible mainly for classical noncovalent binding in the ATP-pocket, the cyclic warhead endows composite drugs with increased kinase and JNK isoform specificity. Finally, we showcase the translational potential of the new reversible covalent warheads and underscore their significance in the development of specific reversible covalent inhibitors."

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all the points raised and have revised the manuscript accordingly. Therefore, I am happy to recommend this manuscript for publication.