nature portfolio

Peer Review File

Context-dependent translation inhibition as a cancer therapeutic modality

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript „Context-dependent translation inhibition as novel cancer therapeutic modality" describes the design, development and functions of small molecules that can inhibit protein synthesis in a peptide sequence-dependent manner, by binding to the PTC of the large ribosomal subunit. The authors tested two novel molecules based on anisomycin and identified stalling motifs within nascent peptides. CryoEM was used to identify the binding site and potential decisive structural changes. Finally, they also tested their novel components in different cancer cell lines and in xenografts in mouse models, and observed a potential therapeutic strategy to inhibit cancer growth. The submitted work presents a fairly complete description of the design, the in vivo and vitro biochemical characterization, structural studies and experiments regarding the potential of cancer therapy by these novel interdictors. In my opinion, this is a really interesting manuscript that is significant for the scientific community. It is well written, the methods are described in detail and the figures are nicely prepared. However, there is one aspect that I am confused about concerning the Myc inhibition.

The authors identified motifs that are associated with the inhibition by IDB-001 and performed the cryoEM studies with these potential stall sites. According to the story line of the manuscript, IDB-001 preferentially inhibits FTED motifs with acidic amino acids. Consequently, DSEEEEQEDE was identified as a potential stall site for 001 and this motif was then also used for the cryoEM structures. However, in Figure 5B, the Western blots indicate that 002 is more potent than 001, which is also confirmed by Figure S9. The concentration of 001 used for the Western Blots (5B) was 30 µM and for 002 only 10 µM. Figure S9 shows the 10 µM experiments for 001, where the inhibition is less efficient. This may be somewhat misleading as it is also not addressed in the manuscript. This also raises questions about the potential stalling motif (at least within Myc), which does not seem to be that good. Alternatively, there may be a peptide sequence within Myc that enables a stronger inhibition by IDB-002. Did you find Myc in the ribosome profiling data (analogous to H2BC17 or ANLN) for 001 or 002? I think this aspect needs some clarification!

Along these lines: how well did Anisomycin inhibit Myc expression?

At the beginning of the results, the authors describe the design of the novel compounds very superficially. Was it a purely rational design based on the structure of the ribosome? No molecular dynamic simulations or similar approaches to assist the design? No failed attempts? I have always had the impression that it is really difficult to tailor known molecules to improve/customize their function.

The in vitro experiments are based on reporter sequences carrying 6 repeats of the stalling sequences. Why 6? Is one or two not sufficient? Maybe it would also be interesting to include the potential stalling sequence of Myc and compare it with the identified motifs. How does in vitro compare to in vivo. It should be possible to create a reporter mRNA with the respective motifs and express them in cells (either directly by RNA transfection or expressed from a plasmid). This would also provide an idea of how strong the inhibition is at certain sites. Perhaps this could also provide an idea about the stalling elements of Myc (the point I raised earlier).

Maybe I missed it: do the compounds bind the PTC in the absence of stalling motifs or are they required for binding? This might allow us to speculate about the mode of action.

Do the cell lines tested (22RV1, HCC1143,…) express different levels of Myc, since 22RV1 or LS411 require higher levels

of the compound 001?

Very minor:

Page 1: I don´t know if this intentional but did you mean “selected protein targets” instead of “select protein targets”.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The manuscript “Context-dependent translation inhibition as a novel cancer therapeutic modality” by Paige D. Diamond et al. presents a study aiming at development of so-called interdictor molecules, context-specific inhibitors of protein synthesis as potential cancer drugs. Specifically, interdictors are small-molecule inhibitors of the human ribosome that target the beginning of the polypeptide exit tunnel at the peptidyl transferase center, with sequence specificity to the last few amino acids added to the nascent polypeptide chain. The authors use a combination of ribosome profiling, biochemistry and cryo-EM of the human ribosome to demonstrate the context specific inhibition with anisomycin (existing antibiotic) and three designed variants (IDB-001, IDB-002 and IDB-003). In addition, they test the potential of using IDB-003 for the treatment of MYC-related cancer in a mouse model.
General comments
The manuscript is based on a very interesting concept of using context-specific inhibition of the human ribosome to target “un-druggable” protein targets (e.g. intrinsically disordered proteins or protein regions) in their making on the ribosome. The bacterial ribosome is a main target for antibiotics, but there are currently only few approved drugs that target the human ribosome.
The manuscript is in general well written and the illustrations are clear. The authors make use of an impressive array of methods to characterize the effect of interdictor molecules in vitro and in vivo.
Major concerns
The four presented cryo-EM structures of nascent-chain complexes of human ribosomes with or without interdictors are all at high resolution. However, there are a number of severe issues with the deposited maps and models. The models need to be revised and the appropriate maps provided.
• There are multiple maps for each model and it is unclear which one is what. The unsharpened map that according to the manuscript text was used for modelling is lacking for three of the structures.
• The provided sharpened maps are over-sharpened in some important regions (lower resolution regions such as the nascent chain and the mRNA), making the interpretation challenging. According to the manuscript, these maps were used for automated placement of waters. As a result, over-sharpened regions have many unconvincing and unlikely waters, and many of them are clashing with the structure. In particular there are many waters way too close to IDB-001 or the nascent chain in 9O3X, which may explain the much higher clash score in this structure. This may also be the case in the other structures and in other regions. Other waters are placed in density that resembles a base (see next to A3908 and U4399). The waters need to be revised e.g. using the coot validate waters tool to remove waters with clashes or low signal.
• Details are lacking for the local refinement around “regions of interest”. Please specify which are these regions and how the corresponding masks were generated. Inspection of the locally refined maps does not show any clear improvement. For the nascent chain, this map looks worse. It is also not clear from the text to what extent these maps were used for interpretation.
• The mean B-factors are very high in all the structures. From looking at the maps provided the reason might be over-sharpening and/or including chains that have barely any density. Many regions do not have supporting density (look at the very low atom inclusion values of many chains in the model validation report), thus the high B-factor and low atom inclusion values.
• Some of the conformational changes described in Figure 4 are not correctly modeled, partly linked to misplacing of waters (detailed below).
• Please clarify the model building procedure. The inclusion of chains that lack density suggests that the model was only manually inspected in the regions of main interest, this has to be clarified.
The parts of the manuscript that is based on interpretation of the cryo-EM structure over-states what can be seen in the maps. The authors need to revise the text to clarify that the structures according to the attached maps do not show clear evidence of sequence-specific inhibition. They also need to provide clear evidence as figures with un-trimmed maps that in an un-biased way show what can be seen and not.
• In the results referring to Figure 3, the authors state that “side chain positions of the four residues in contact with the compound (in the 0, -1, -2, and -3 positions) could be assigned.”. Inspection of the provided maps does not support this, only the backbone is clearly assignable.
• Similarly, referring to Figure 4, the authors state that the identity of the nascent chain positions 0 to -3 could be well modeled. However, the density is very poor and only D-1 is clearly visible in the map.
• It is unclear if the conclusion of sequence specific stalling was drawn from the peptide sequence in the PTC or from the mRNA sequence. Please clarify how the authors decided what to model.
The cryo-EM processing workflow and cryo-EM map validation is presented for one of the structures in Supplementary Figures 7 and 8, but the corresponding figures for the other three structures are missing and need to be added. Also, the cryo-EM methods need to be clearly specified for each of the complexes. The ribosome concentration, number of frames, dose rate and total exposure time need to be specified for each imaged complex.

Minor general concerns
• The error bars in graphs are not explained in the legends. Please add an explanation and provide information about the number of replicates.
• Several figures contain tables. Please make separate tables unless clearly motivated.

Specific comments to the text:
Page 2:
• Paragraph 2: Please re-write to be clear about what relates to bacterial versus human ribosomes. The sentence starting ”There is only one documented drug-like molecule binding to the NPET…” is too long and difficult to follow.
• Reference 18: This is about the exit tunnel of bacterial ribosomes, please add some reference that clarifies if the same is true for human ribosome.
• Paragraph 3: You state that “An alternate mechanism by which translation inhibition has been shown to impact cancer cell viability is mediated through stress response pathways that lead to apoptosis, as has been documented for anisomycin, further confirming translation inhibition as a therapeutic strategy even in the absence of biochemical selectivity.” It is not clear why it confirms it as a therapeutic strategy. Please clarify why this would not also affect “normal” cells.
Page 3: The final sentence is un-clear. Please clarify what “historically undruggable targets with high validation” means.
Page 4-5 and Figure 1:
• When comparing IDB-001 and ANS, IDB-001 shows enrichment of acidic residues but also positively charged residues. The latter one is suggested to be due to its weaker overall inhibition. If that is the case, the acidic residue enrichment is then less persuasive. This is particularly the case since the authors state that ANS strongly disfavors positively charged residues (when compared to DMSO), but IDB-001 also disfavors them. Further down the authors state “Notably, this enrichment disappears when IDB-001 is compared to DMSO” probably referring to positively charged residues, but it is unclear. Please improve the clarity of this paragraph.
• Figure 1B: it is difficult to differentiate the curves, in particular the blue one. Also, unclear if the shade indicates error bars.
• Figure 1H: Indication of codon positions under the figure would improve clarity.
Page 5-6 and Figure 2:
• Figure 2D shows an example of luminescence time traces. The rest of the traces for the other reporters should be added as a supplementary figure.
• The legends of Figure 2E-F legends should present how errors are calculated and how many data replicates were used.

Page 6-7 and Figure 3:
• Please clarify that “Similar to ANS30, the core binds in a pocket…” refers to both compounds.
• The statement that ”the methoxyphenyl forms p-stacking interactions with C4398 as it extends towards the tRNA (Figure 3B), all consistent with previous reports …” should preferably be supported with a supplementary figure showing a comparison with anisomycin.
• At the end of this section (page 7, middle of the page) the two last sentences are repetitive and should be revised.
• The inset in Figure 3A does not really add much. Possibly using the same style as Figures 3D-E but with the same camera view as the current inset could be more helpful.
• Please clarify what maps are shown in Figures 3D-E. Comparison with the provided maps suggests that these are trimmed around the model, which in such case is misleading.
• As commented above, there is no density for F-3. Figure 3F does not add any additional information to Figure 3E, and the surface display may confuse readers to believe that this is a map. Please remove. This side chain lacking density also seems to be the basis for the statement about the “hook-like conformation”. What is the evidence for this?
• Several statements are not supported by the maps, e.g. “Despite the inherently dynamic nature of the nascent chain in the exit tunnel, backbone and side chain positions of the four residues in contactwith the compound (in the 0, -1, -2, and -3 positions) could be assigned.” and the final sentence “These structures show small molecule interdictors can engage in sequence-selective interactions…”. These statements must be supported with clear evidence in maps from which the sequence is clear, or removed.
Page 7-8 and Figure 4:
• The authors suggest that the complementarity of the drug molecule rearranges the peptide chain to form new high-affinity interactions. This is a bit overstated considering that only D-1 has good density.
• The authors indicate that IDB-001 causes C4398 and C4399 to move by “several angstrom”. This should be specified more clearly. “angstrom” should be changed to “Å” or “Ångström”. Position 4399 is U, not C (typo). In addition, U4399 seems to have both conformations (the new described and the same as in the absence of the drug). Instead, the authors have modeled two waters in that density. This should be revised and shown in the figure.
• The authors discuss how A3908 is shifted towards the “bottom” conformer, closer to the compound. While this is supported by the map, there is actually density for both conformations in both of the structures (with and without the compound) but the authors have modeled waters in the alternative conformation. Please correct the model in agreement with the density for an alternative conformation of the base, not waters.
• The authors also discuss that U4452 alternates between two conformations in the absence of the compound. The map shows no density for this base, so the base must be dynamic rather than showing only two conformations. This does not affect the conclusion that the compound stabilizes one single conformation of this base.
• Figure 4A-B: Many waters do not have supporting density here, see comments above.
• Figure 4C-F: Please indicate in the legend how the structures were aligned.
• Figure 4D-F: Supplementary figures showing the density for these regions should be provided and fixed according to previous comments.
Page 9-10 and Figure 6:
• The authors do not specify where they think IDB-002 would stall ribosomes translating MYC, which then does not really

relate with the claimed specificity. Please clarify. Can the defined logo be found in the MYC gene?
• The authors indicate that IDB-003 shows a similar pattern of context-dependent inhibition as IDB-002 (Figure 1G and Supplementary Figure 10B). This is not supported by the data, as only the -1 position has a similar pattern, there is no clear specificity in the other positions.
• IDB-003 seems less specific than IDB-001 and -002, and in general, only showing specificity for small amino acids in position -1. This suggests that IDB-003 may affect most genes. Please clarify if and why you think that this inhibition relates to MYC.
• Figure 6: Please spell out SEM in the legend.
Page 11-13:
• There are many buzz-words in the discussion. Please reduce these vague statements, e.g. “a more advanced tool molecule”.
• The authors indicate that IDB-003 is highly effective at “well-tolerated oral doses”. Where is this shown? In the methods section it is indicated that all animals were monitored for changes in several parameters. Is there such data to support this claim?
• The authors indicate that “these data validate our approach of sequence-selective interdiction of protein synthesis as a powerful therapeutic strategy for MYC-driven cancers”. As mentioned above, this needs to be better supported.
• The authors mention again that the approach is sequence-selective to MYC, but this would need to be supported by data.
• The statement ”Similar considerations also explain the observed deenrichment of certain sequences that contain bulky aromatic residues, as they are likely to clash sterically with the molecule in the PTC (Figure 1I-J).” would need to be supported by a supplementary figure visualizing how such bulky residues would make clashes.
• The authors make a statement eluding to similarities between interdictors and PROTACS in ”substoichiometric or catalytic mode of action.” Please clarify this sentence; it is unclear which stoichiometry the authors refer to. Here it would actually be interesting to discuss the stoichiometry of inhibited and trailing ribosomes, interdictors and different nascent chains.
Methods:
• Page 16: The authors indicate the luminescence assay was performed in a luminescence-capable plate reader from BMG Labtech. Please specify the specific instrument used.
• Page 17: Please spell out IRES.
• Page 17: Why is the cryo-buffer in square brackets when the authors used parenthesis before?
• Page 19: The authors indicate two numbers for relative humidity, likely a typo.
• Page 19: A “2-day holiday” does not seem like a correct term to use here.
• Chemical synthesis section: The authors use calc’d to specify the m/z. Is this a typo?
Other comments:
• In the abstract the authors use “~” next to the reported resolution. This could be removed.
• Supplementary Figures are not mentioned in order in the text. There is a jump from Supplementary Figure 6 to 9 and 7 and 8 are mentioned much later only in the methods.
• Supplementary Figure 2 is very low resolution and difficult to judge.
• Supplementary Figure 8: The local resolution should be provided for the main map, not only the locally refined. The inset is misleading, as the resolution for the nascent chain is clearly not 1.9 when looking at this region of the map (looks more like 3.5 Å).

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
In this study, P.D. Diamond and colleagues used structure-based drug design to propose small molecules called interdictors, that selectively slow translation elongation of specific sequences in human ribosomes. They used ribosome profiling, cryo-EM analyses and in vitro translation assays to characterize and cross-validate the stalling sequences that are specific to their designed pharmacophores. They also show that translation of the pro-proliferation MYC gene can be specifically impeded by these interdictors in vitro (on purified ribosomes), in cellulo within Myc-dependent cancer cell line and finally in vivo, using human triple-negative breast cancer xenografted mice.
Overall, the study appears well designed, with many complementary techniques that convincibly validate the authors’ findings, both in vitro and in animal models. Their work represents yet another solid proof that ribosomes and translation are worth investigating as therapeutic targets, and the molecules they designed might prove extremely promising to fight against the vast panel of MYC-related cancers. Since I am not an expert in chemistry, I can not evaluate the drug design process herein presented. However I believe that this manuscript is worth publishing in Nature Communications, provided that the

following comments are addressed:
- The first part of the manuscript is about the extensive in vitro/in cellulo characterization of the two compounds IDB-001 and IDB-002, which seem to be efficient to stop the translation of specific sequences, including MYC's acidic stretch DSEEEQEDE. However, the in vivo validation on the xenografted mouse was performed using a third interdictor, IDB-003, which only appears late in the results section and has not undergone the same exhaustive validation as the other two compounds. This is confusing, and readers may wonder if IDB-001 and IDB-002 were tested on mouse models and found to be ineffective. If this is the case, I believe the authors should clearly justify their choice of the third compound IDB-003, and re-shuffle the corresponding results section so that the ribosome profiling and MYC expression assays (shown in figure S10) are presented earlier in the text. Otherwise, the current elliptical style slightly undermines the strength of the reported study.
- A CryoEM analysis of the third compound represents a significant amount of additional work and is probably out of the scope of this manuscript, thus, to reinforce their findings, could the authors also test the effect of IDB-003 on RSR, by WB experiments?

Minor comment:
The composition of the tetrapeptide/4-mers sequences should be described earlier (p.5) to improve clarity.

(Remarks on code availability)

Reviewer #5

(Remarks to the Author)
Diamond, Sauer, et al. report the exciting discovery of first in class molecules that can inhibit protein synthesis in a specific manner dependent upon the polypeptide sequence in the peptidyl-transferase center (PTC). The findings presented in this manuscript are an important step toward selectively targeting the translational machinery with the potential to specifically inhibit the growth of tumors, which could be programmed to combat other diseases. The authors conduct transcriptome-wide ribosome profiling to demonstrate the selective stalling of the ribosome, which, coupled with structural studies, illuminates the potential selective mechanism of action at the molecular level. They show that the compounds can inhibit cancer viability potentially through repressing of MYC translation and/or ribosomal stress response. Finally, they demonstrate in vivo efficacy of tumor growth inhibition with a third orally available compound. This study is of interest to the broad readership of Nature Communications. However, while this work is a valuable step towards harnessing the power of targeting selective translation, it requires additional experimentation to solidify the proposed claims mechanism of action and target specificity.

Major Points:

1. While the authors provide insight into the specificity of the stall sequences with interdictor treatment, there is a lack of validation at the proteome level. This is key to demonstrating that their compounds result in selective and specific changes at the protein level, which may mechanistically underpin the phenotype on cancer cell line viability. The authors should show global proteomic changes in their cell line models and analyze how those changes overlap with the observed stall sites transcriptome-wide.
2. The authors fail to show the kinetics and dynamics of interceptor therapy on endogenous targets. Together, the experiments detailed below are key for supporting their claim on p. 12 that the selectivity of the compounds for specific peptides in the PTC will be more effective against oncogenes with short half-lives over essential housekeeping genes.
a. Specifically, the authors should test the kinetics of inhibition of Myc or other endogenous targets (these will be better defined by the above proteomics experiments). Currently, the earliest time point of treatment is 2hrs at which time Myc protein level is already quite suppressed.
b. Also the authors should test the inhibition of endogenous protein expression across a drug titration as shown for RSR signaling in Fig 5. Are the short lived driver oncogenes inhibited at a lower concentration? This can help discriminate between the contributions of ribosomal stress response vs loss of driver oncogene expression for the compounds' efficacy.

Other points:

1. The authors should demonstrate the "on-target" effects of IDB-003 in the in vivo tumorigenesis study. That is, does Myc protein level decrease in the treated tumors?
2. The manuscript would benefit from additional discussion of potential rules or guidance on how to evolve the compounds to increase selectivity based on the structural data. Additionally, discussion of the relative contribution and interdependency of peptide binding and the A site inclusion would be appreciated and further illuminate the specificity of the molecules for their targets.
3. The authors should discuss the literature on the translation of Myc mRNA and how their work is relevant in this context.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My concerns and comments were addressed sufficiently!

All the best!

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The revised version of the manuscript "Context-dependent translation inhibition as a novel cancer therapeutic modality" by Diamond et al. has addressed all our comments on the previous version of the manuscript. The updated structures show reasonable validation statistics and are now adequately described in the text and the figures. Overall, the text is more stringent and clear.

With access to all the maps in a clearly organized folder, we identified an additional problem that needs to be addressed. Other than that, we find the revisions to be satisfactory.

A fast inspection makes us question whether the P-site tRNA modeled in the FTED RNC + IDB-001 structure (code 9O3X) correctly reflects what is mainly present in the complex. The anticodon density suggests that the residue 35 on the P-tRNA should be a purine (currently modeled as a pyrimidine), at the same time, residues 31 and 39 appear to be purine and pyrimidine respectively (currently modeled in the opposite way). We recommend the authors to take a look again at the tRNA density of this structure to confirm which tRNA it is mainly present in the density, and to adjust their interpretation of the tRNA and the nascent peptide accordingly. The other structures appear to be correct.

Minor comments:

The uploaded LSU focus map of 9O3V (MYC without IDB) appears to be shifted and does not fit to the model. The authors should correct this.

In the cryo-EM methods and perhaps in Table 1 and Supplementary Table 1, please clarify which classes from 3D classification are used for the final reconstructions and modelling. Based on the numbers that Supplementary Figure 6 shows for the MYC:IDB-001 RNC complex, this seems to be the POST state. In Supplementary Table 1, it is unclear what "best of" means for the POST states of IDB-001+FTED and IDB-002+FPAK - please clarify.

In the methods, it is stated that "All data processing was carried out in Cryosparc v4.584 645 and is 646 schematically outlined in Supplementary Figure 7." This should probably be Supplementary Figure 6.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
With this revised version, P. Diamond and colleagues have appropriately addressed my previous comments. I believe their work should be published in Nature Communications, although the following sentences should be modified:

- Lines 331-332: “IDB-002 treatment showed a dose-response to ANS, while IDB-001 displayed modestly weaker activity (Figure 4A)”. I suggest rephrasing this sentence as follows: “IDB-002 treatment showed a dose-response similar to that of ANS, while IDB-001 displayed weaker activity (Figure 4A).” I am not sure that the term “modestly” is appropriate here, since there is a tenfold difference between the doses of IDB-001 and ANS/IDB-002 required to reach 50% cell viability.

- Lines 371-373 “Indeed, treatment of MCF7 cells with 1 μM IDB-003 led to rapid depletion of MYC and CCND1, with substantial loss evident by 1-2 hours and near-complete depletion by 4-8 hours (Figure 5D).” While this statement is accurate for CCND1 and CHX, the Western blot in Figure 5D does not show a significant decrease in MYC signal after 1–2 hours, nor near-complete depletion after 4–8 hours, when compared to the tubulin signal. However, this description does

hold true when using 10 μM IDB-003, as shown in Supplementary Figure 10D. Therefore, I recommend that the authors rephrase this sentence accordingly.

(Remarks on code availability)
There was no code to review.

Reviewer #5

(Remarks to the Author)
The authors argued that the validation of their ribosomal profiling by mass spectrometry or other means is out of the scope of this work, but I do not agree. It is still unclear how these compounds reduce cancer cell viability in culture and in vivo. Is this due to the reduction of translation of specific oncogenes or the activation of deadly stress? In fact, the title of the manuscript itself, “Context-dependent translation inhibition as a novel cancer therapeutic modality”, emphasis that the context-dependent translation is important as a cancer therapeutic. This indeed would be very exciting, but there is no data to substantiate such a claim without additional experiments to address this important distinction between translation-specific effects versus a general stress response.

It appears that the authors are unaware of the existing literature on the role of translation control and cancer, as well as the numerous efforts aimed at targeting translation specificity, which is more prevalent in cancer cells than in normal cells.

While targeting ribosome and translation control is an emerging field in cancer biology, and it is important to pursue, this work still falls short of being published in Nature Communications.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
All minor comments have been addressed in a satisfying manner.

However, the major comments on the FTED structure have not been addressed in a satisfying manner by moving this structure to the supplement and stating that "However, some ambiguity remained in the density-based distinction of three purine/pyrimidine bases in the aspartyl-tRNA of the FTED RNC, possibly because of impurities in the sample." The authors write in the answers that "We have chosen to be conservative in our interpretation of the exact base sequence, considering the biochemical setup of the experiment and the density of the mRNA in addition to the tRNA density itself", which does not at all make sense.

It has to be clarified that in this structure, the peptide sequence in the PTC and its interactions with the inhibitor (i.e. the focus of this study) are inferred from the tRNA species, not the other way around. While there may be some ambiguity, the cryo-EM map clearly shows that the major tRNA present in the complex is different from the one modelled; i.e. stalling does not mainly occur where they expect. This in turn means that the peptide modelled is not the dominating peptide present in the PTC of this complex. Thus, the current description is misleading. The authors have the choice to either re-model and re-interpret the structural data based on the density (i.e. purine-pyrimidine pattern) for the tRNA and mRNA to figure out where this complex has stalled and build the resulting peptide in the PTC or to remove this structure from the manuscript.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

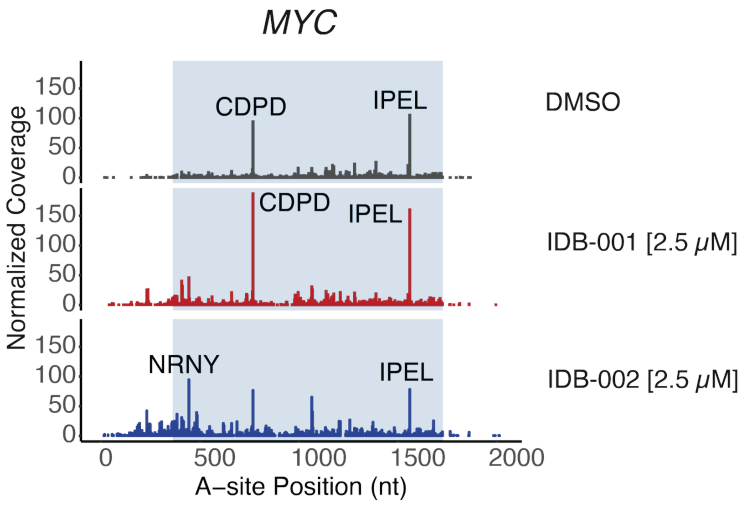
The manuscript „Context-dependent translation inhibition as novel cancer therapeutic modality" describes the design, development and functions of small molecules that can inhibit protein synthesis in a peptide sequence-dependent manner, by binding to the PTC of the large ribosomal subunit. The authors tested two novel molecules based on anisomycin and identified stalling motifs within nascent peptides. CryoEM was used to identify the binding site and potential decisive structural changes. Finally, they also tested their novel components in different cancer cell lines and in xenografts in mouse models, and observed a potential therapeutic strategy to inhibit cancer growth. The submitted work presents a fairly complete description of the design, the in vivo and vitro biochemical characterization, structural studies and experiments regarding the potential of cancer therapy by these novel interdictors. In my opinion, this is a really interesting manuscript that is significant for the scientific community. It is well written, the methods are described in detail and the figures are nicely prepared. However, there is one aspect that I am confused about concerning the Myc inhibition.

The authors identified motifs that are associated with the inhibition by IDB-001 and performed the cryoEM studies with these potential stall sites. According to the story line of the manuscript, IDB-001 preferentially inhibits FTED motifs with acidic amino acids. Consequently, DSEEEEQEDE was identified as a potential stall site for 001 and this motif was then also used for the cryoEM structures. However, in Figure 5B, the Western blots indicate that 002 is more potent than 001, which is also confirmed by Figure S9. The concentration of 001 used for the Western Blots (5B) was 30 µM and for 002 only 10 µM. Figure S9 shows the 10 µM experiments for 001, where the inhibition is less efficient. This may be somewhat misleading as it is also not addressed in the manuscript. This also raises questions about the potential stalling motif (at least within Myc), which does not seem to be that good. Alternatively, there may be a peptide sequence within Myc that enables a stronger inhibition by IDB-002. Did you find Myc in the ribosome profiling data (analogous to H2BC17 or ANLN) for 001 or 002? I think this aspect needs some clarification!

We thank the reviewer for this comment and have addressed these remarks in a few places in the text to clarify our messaging in the manuscript. In general, we have revised the text to more accurately describe context-dependent activity in relation to MYC. While the interdictors we present here have context-selective activity for select amino acids or amino acid motifs in the ribosome-bound polypeptide chain, our molecules are not single-mRNA selective (*i.e.,* one interdictor inhibits translation of one mRNA), and we realize this may not have been effectively communicated.

We believe this comment is addressed by multiple revisions to the text and figures, however we will summarize specific responses to this comment here as well. IDB-001 was designed to inhibit polypeptide sequences with acidic amino acids (D/E), and thus we thought this would be a great proof-of-concept reporter sequence to capture interdictor-peptide interactions in a ribosome nascent chain complex. This was accomplished and is highlighted in Figure 3 where we observe local structural rearrangements in this nascent polypeptide upon interdictor binding. However, as the reviewer noted, IDB-001 has relatively poor inhibition of cell viability compared to IDB-002 (Figure 4) and MYC depletion activity (Supplemental Figure 10) despite the D/E-selective activity we characterized (Figure 1F, 2E). This may be due to subtle differences in ribosome binding kinetics between the molecules, or as mentioned, stronger site(s) in the MYC open reading frame that might be interdicted by IDB-002 leading to a stronger impact on MYC translation. Since IDB-002 impacts the translation of more 4-mers than IDB-001

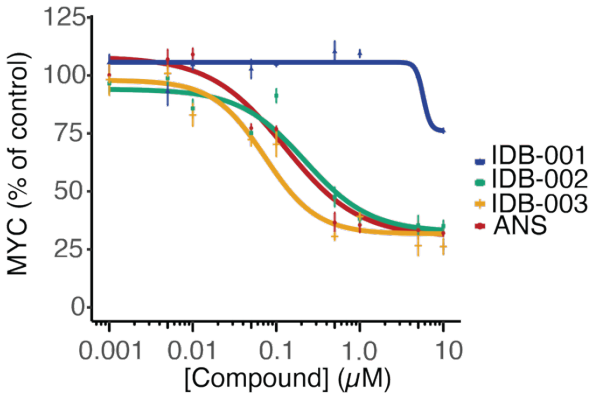
(Figure 2A-B) we anticipate IDB-002 treatment leads to more robust translation stalling by inhibiting more peptide bond formation events across the MYC open reading frame. Consistent with this, our ribosome profiling shows a greater accumulation of ribosomes near the N-terminus of MYC and in an upstream open reading frame for IDB-002 treatment compared to IDB-001 (shown below). We have revised our language around sequence-preferential activity to de-couple amino acid preferences we observe from the differential MYC depletion of the two molecules. More likely the enhanced activity for IBD-002 on MYC depletion is a result of stronger global activity.



Response Figure 1. Ribosome footprints (adjusted to A site) over the MYC locus for cells treated with either DMSO or 2.5 μM IDB-001 or IDB-002. Read counts were scaled based on library size. Blue color signifies the main open reading frame. Codons with high ribosome occupancy are labeled with corresponding nascent peptide sequence (positions -3 to 0).

Along these lines: how well did Anisomycin inhibit Myc expression?

We used HTRF to quantify endogenous MYC depletion in HCC1143 cells across a drug titration for interdictor or anisomycin and found that anisomycin reduces MYC to a similar extent to IDB-002 but is less potent than IDB-003. In contrast, IDB-001 shows little effect at matched concentrations for a short time interval (2 hours). Importantly, we added clarification on the scope of selectivity in the text as mentioned above. The sequence preferences we define operate at the level of short motifs (e.g. -3:-1 context in the PTC) that are shared by many transcripts. MYC still serves as a biologically relevant readout due to its short half-life and the addiction of certain cancers to high MYC expression, but not because it is uniquely targeted by the interdictors we present here.



Response Figure 2. HTRF assay for MYC levels in HCC1143 cells after 2 hours of treatment with interdictors, or anisomycin.

At the beginning of the results, the authors describe the design of the novel compounds very superficially. Was it a purely rational design based on the structure of the ribosome? No molecular dynamic simulations or similar approaches to assist the design? No failed attempts? I have always had the impression that it is really difficult to tailor known molecules to improve/customize their function.

We thank the reviewer for these thoughtful comments and have included some more description regarding the structure-based design principles in the intro and discussion. This paper was not intended to include the comprehensive design strategy. Instead, the focus of this manuscript is related to the structural and biological findings for IDB-001, IDB-002 and IDB-003 to provide a foundation for our learnings. We intend to publish a separate and more comprehensive paper that summarizes the details of our structure-based design, SAR observations, and computational approaches.

This paper focuses on insights obtained from our high-resolution cryo-EM structures which are the corner stone of our structure-based design efforts. Based on our analysis, the same structure-based drug design principles applied to traditional small molecule modalities are directly relevant to our rational approach to design and optimize interdictors as linker-less heterobifunctional small molecules. Our small molecules bind in the A-site near the nascent polypeptide. This is the narrowest portion of the PTC where the peptide does not have significant conformational freedom, forming predictable backbone and sidechain conformations. These structural observations have been further confirmed by molecular dynamics simulations where the average flexibility of the peptide along the length of the PTC is captured. In addition, the same complementary electronic (hydrogen bonding and hydrophobic) and steric interactions that drive the binding of small molecules to a receptor binding site are also important to the binding of the heterobifunctional small molecules to the rRNA and nascent peptide in the PTC. We apply free energy perturbation (FEP+ as implemented in the Schrodinger software suite) calculations to guide the design and optimization of our small molecules. We can rationalize SAR observations retrospectively, as well as, make successful prospective predictions for new designs. However, for this manuscript, our focus was specifically on IDB-001 and IDB-002, so we did not include additional computational details and SAR. Instead, we highlighted the core design principles for these two specific molecules which were rooted in literature-precedented and well-characterized interaction patterns between small-molecules and amino acids/RNA.

Additional references of interest on this topic:

1. Molecular recognition in the context of structure-based drug design and drug discovery (https://doi.org/10.3390/ijms20112783).
2. Engineering of hydrophobic and hydrogen bonding interactions improve potency (https://doi.org/10.1039/C7MD00381A)
3. Hydrogen bonds drive molecular recognition (https://doi.org/10.1021/ct400065j).
4. Fine-tuning both hydrogen bonding and hydrophobic interactions in the protease binding site, improved potency from millimolar to the nanomolar range (https://doi.org/10.1073/pnas.92.8.3298).

The in vitro experiments are based on reporter sequences carrying 6 repeats of the stalling sequences. Why 6? Is one or two not sufficient? Maybe it would also be interesting to include the potential stalling sequence of Myc and compare it with the identified motifs. How does in vitro compare to in vivo. It should be possible to create a reporter mRNA with the respective motifs and express them in cells (either directly by RNA transfection or expressed from a plasmid). This would also provide an idea of how strong the inhibition is at certain sites. Perhaps this could also provide an idea about the stalling elements of Myc (the point I raised earlier).

The HiBit tag itself is 11 AAs long, and while its sequence is predicted to be a very poor substrate for interdiction with the compounds presented here we wanted to ensure that the majority of the inhibitory signal we measured was derived from the sequence of interest rather than the tag. At the same time we did not want to make the reporters so long that differentiating between them would be difficult given the dynamic range of our assay. We settled on six repeats to achieve these two goals, though certainly slightly more or slightly fewer repeats would also have worked.

The primary goal of the reporter assay was to determine the largest extent of the sequence preferences of the two compounds in terms of biochemical drug inhibition parameters to aid in interpretation of the much richer ribosome profiling datasets. As such, we picked sequences with highly confident ribosome-profiling pause scores (*i.e.,* a high number of footprints at that position) and with very different pause scores for the two compounds IDB-001 and IDB-002. This assay can be expanded to any number of reporters but due to the simplifications needed to create a robust biochemical assay, in particularly the use of IRES-driven initiation, it will never perfectly capture the dynamics on complex targets such as Myc (see the answer to point 1 above). We have updated the main text to more clearly delineate the intended role of these measurements.

Our primary *in cellulo* readout is ribosome profiling, which measures stalls on endogenous mRNAs and avoids UTR and copy-number artifacts that might come from transgenic expression of reporters. Avoiding such artifacts has proven challenging with simple transfection-based assays, and we believe that the genetic engineering needed to create the required transgenic cell lines to overcome these challenges is outside the scope of this study and the intended role of the biochemical assays within it.

Maybe I missed it: do the compounds bind the PTC in the absence of stalling motifs or are they required for binding? This might allow us to speculate about the mode of action.

The compounds can bind the PTC without a nascent polypeptide chain via the same rRNA contacts that anisomycin leverages for binding. While the nascent-chain interactions are not required for binding, they further stabilize the interdictor-ribosome complex leading to inhibition of elongation kinetics. We have clarified this in the discussion and now describe the rRNA-anchoring effect of the ANS core.

Do the cell lines tested (22RV1, HCC1143,…) express different levels of Myc, since 22RV1 or LS411 require higher levels of the compound 001?

Relative to HCC1143, MYC expression across the cell lines is as follows: 22RV1 = 106%, MCF7 = 22%, LS411N = 79% (Ghandi, *et al.* 2019, Cancer Cell Line Encyclopedia). Based on MYC expression alone, we would hypothesize that MCF7 might be the 'weakest' cell line for interdictor treatment, but obviously that is not the trend we observe. However, the relationship between cell viability and interdictor EC_{50} is more complex, primarily owing to the fact that our cell killing activity is not derived exclusively from an impact to MYC, as we now show that other short half-life proteins are impacted in cells. The poly-pharmacology of depleting these short half-life oncogenes, as well as inducing ribosome surveillance pathways are likely to contribute to the cell growth inhibition phenotype we observe. We have modified the text regarding these results to remove any direct connections to MYC.

Very minor:

Page 1: I don´t know if this intentional but did you mean "selected protein targets" instead of "select protein targets".

We agree this was an error and have addressed this in the text.

Reviewer #2 (Remarks to the Author):

The manuscript “Context-dependent translation inhibition as a novel cancer therapeutic modality” by Paige D. Diamond et al. presents a study aiming at development of so-called interdictor molecules, context-specific inhibitors of protein synthesis as potential cancer drugs. Specifically, interdictors are small-molecule inhibitors of the human ribosome that target the beginning of the polypeptide exit tunnel at the peptidyl transferase center, with sequence specificity to the last few amino acids added to the nascent polypeptide chain. The authors use a combination of ribosome profiling, biochemistry and cryo-EM of the human ribosome to demonstrate the context specific inhibition with anisomycin (existing antibiotic) and three designed variants (IDB-001, IDB-002 and IDB-003). In addition, they test the potential of using IDB-003 for the treatment of MYC-related cancer in a mouse model.

General comments
The manuscript is based on a very interesting concept of using context-specific inhibition of the human ribosome to target “un-druggable” protein targets (e.g. intrinsically disordered proteins or protein regions) in their making on the ribosome. The bacterial ribosome is a main target for antibiotics, but there are currently only few approved drugs that target the human ribosome. The manuscript is in general well written and the illustrations are clear. The authors make use of an impressive array of methods to characterize the effect of interdictor molecules in vitro and in vivo.

Major concerns
The four presented cryo-EM structures of nascent-chain complexes of human ribosomes with or without interdictors are all at high resolution. However, there are a number of severe issues with the deposited maps and models. The models need to be revised and the appropriate maps provided.

We thank the reviewer for their rigorous evaluation of the cryo-EM maps and models. We have deposited revised versions of both into the review portal and with EMBD and PDB repositories. Below we have addressed all the specific comments as well.

• There are multiple maps for each model and it is unclear which one is what. The unsharpened map that according to the manuscript text was used for modelling is lacking for three of the structures.

For each of the four structures the following maps have now been provided: Unsharpened and sharpened consensus map, consensus half maps, consensus mask, unsharpened and sharpened local maps of the PTC and anticodon regions. For the review process, maps have been organized into clearly labeled folders.

• The provided sharpened maps are over-sharpened in some important regions (lower resolution regions such as the nascent chain and the mRNA), making the interpretation challenging. According to the manuscript, these maps were used for automated placement of waters. As a result, over-sharpened regions have many unconvincing and unlikely waters, and many of them are clashing with the structure. In particular there are many waters way too close to IDB-001 or the nascent chain in 9O3X, which may explain the much higher clash score in this structure. This may also be the case in the other structures and in other regions. Other waters are placed in density that resembles a base (see next to A3908 and U4399). The waters need to be revised e.g. using the coot validate waters tool to remove waters with clashes or low signal.

Using maps of different sharping levels is common practice in the field, and in this case these maps have only been used for water, ion and ligand placement where the unsharpened maps tend to smear out such features. However, we recognize that not all water molecules were well placed and therefore all four models have been rebuilt with an emphasis on physically plausible water molecule placement. To this end, stricter map value cutoffs for water placement have been employed. To improve confidence, only water density peaks that were consistent between the sharpened consensus map and the sharpened local map have been kept, with special emphasis on the large ribosomal subunit and the area close to the PTC and nascent chain. The new models have significantly reduced clash scores (see updated Table 1). Waters that have accidentally been placed into density for alternative conformations have been removed and alternative conformations for these bases have been added. As a result of the stricter model building procedure, some water molecules might be missing in the model where they could be sensibly inferred from the local chemistry (for example around Mg clusters) or where the local density is of lower quality.

• Details are lacking for the local refinement around “regions of interest”. Please specify which are these regions and how the corresponding masks were generated. Inspection of the locally refined maps does not show any clear improvement. For the nascent chain, this map looks worse. It is also not clear from the text to what extent these maps were used for interpretation.

The regions of interest are the PTC and the mRNA-anti-codon sites. Masks were generated by creating a roughly 50 Å diameter sphere centered on these regions and running the local refinement. The local refinements for the PTC are only slightly improved but noticeable upon careful inspection and comparison. The local refinements for the mRNA-anti-codon site are noticeably improved. The local maps were used to guide decision making where the consensus map was unclear. A clarifying sentence has been added to the methods.

• The mean B-factors are very high in all the structures. From looking at the maps provided the reason might be over-sharpening and/or including chains that have barely any density. Many regions do not have supporting density (look at the very low atom inclusion values of many chains in the model validation report), thus the high B-factor and low atom inclusion values.

The updated models have significantly reduced B-factors (see Table 1). The low atom inclusion is a result of the large size of the ribosome, it is not behaving as a rigid body to allow for homogenous resolution across the entire map. It is out of the scope of this work to provide high resolution maps for ribosome regions that are far away from the region of interest. Therefore, having a somewhat complete atomic model but low inclusion scores for some chains that are far away from the regions of interest is deemed an acceptable trade-off and is common practice in the field.

• Some of the conformational changes described in Figure 4 are not correctly modeled, partly linked to misplacing of waters (detailed below).

The issues are addressed in more detail below.

• Please clarify the model building procedure. The inclusion of chains that lack density suggests that the model was only manually inspected in the regions of main interest, this has to be clarified.

Additional clarifications about model building have been added to the methods and copied here: “For the IDB-001 QEDE RNC, all residues of each chain were manually checked, keeping residues in peripheral regions with low density to allow for a somewhat complete consensus model. Newly built residues include an ARG containing loop in the 60S ribosomal protein L10 that contacts the CCA of the tRNA, the tRNA itself and the mRNA for each complex. The obtained model was placed into the other three maps and locally adjusted in the regions of interest before minimizing the entire model in Isolde v1.8. B-factor sharpened maps were used for placing water molecules, ions and ligands for each complex individually. Final macromolecular refinement was carried out in Phenix v1.21.2, using the sharpened maps and restraints generated in elBOW for modified nucleotides and compounds in addition to reference restraints generated from the Isolde-minimized model.”

• The parts of the manuscript that is based on interpretation of the cryo-EM structure over-states what can be seen in the maps. The authors need to revise the text to clarify that the structures according to the attached maps do not show clear evidence of sequence-specific inhibition.

The cryo-EM work by itself cannot prove or disprove context-dependent inhibition. These ribosomes have been programmed by omitting a stop codon and are therefore not 'inhibited' by the drug on its own. The cryo-EM work is a robust biophysical method that complements the other experiments described in the paper, and useful to understand the most likely mode of interaction of the drug and the nascent chain. Other biophysical methods we use to show context-sensitive inhibition include a biochemical *in vitro* transcription-translation assay (IVTT) and ribosome profiling (Figures 1 & 2). The observations from cryo-EM support these findings, most specifically through the structural rearrangements observed for IDB-001 binding with the QEDE peptide. We acknowledge the limitations of these data and have updated the language of the cryo-EM section to clarify this, but we believe that in aggregate the data presented here make a case for context-dependent translation inhibition.

• They also need to provide clear evidence as figures with un-trimmed maps that in an un-biased way show what can be seen and not.

We have updated Figure 3 to include untrimmed density around the displayed residues (panels A, D, E, F, G). Density for not displayed residues in the image foreground has been removed to enhance clarity.

• In the results referring to Figure 3, the authors state that “side chain positions of the four residues in contact with the compound (in the 0, -1, -2, and -3 positions) could be assigned.”. Inspection of the provided maps does not support this, only the backbone is clearly assignable.

The language of the text has been adjusted to better reflect that not all residues/side chains can be unambiguously assigned.

• Similarly, referring to Figure 4, the authors state that the identity of the nascent chain positions 0 to -3 could be well modeled. However, the density is very poor and only D-1 is clearly visible in the map.

The language of the text has been adjusted to better reflect that not all residues/side chains can be unambiguously assigned, except D_{-1}.

• It is unclear if the conclusion of sequence specific stalling was drawn from the peptide

sequence in the PTC or from the mRNA sequence. Please clarify how the authors decided what to model.

The mRNA and nascent polypeptide sequences were inferred from the non-stop mRNA construct used to generate the stalled RNC sample and validated against the tRNA and mRNA densities for each construct. This explanation has also been added to the main text: "Assignment of the backbone and side chain positions of the inherently dynamic nascent chain in the exit tunnel was based on both the nascent chain density itself, the density of the tRNA, the mRNA-anticodon site and the sequence of the input mRNA."

• The cryo-EM processing workflow and cryo-EM map validation is presented for one of the structures in Supplementary Figures 7 and 8, but the corresponding figures for the other three structures are missing and need to be added.

All four datasets were processed in the same way. Therefore, in addition to the representative processing workflow presented in the Supplementary Figure 6 for one of the three structures, we have supplied the resulting classes and particle distributions among those classes for all four complexes in a new Supplementary Table 1. We have also expanded Supplementary Figures 7 & 9 to include the unsharpened consensus map, FSC, conical FSC, and local resolution for all four complexes.

• Also, the cryo-EM methods need to be clearly specified for each of the complexes. The ribosome concentration, number of frames, dose rate and total exposure time need to be specified for each imaged complex.

All requested information has been added to Table 1.

Minor general concerns

• The error bars in graphs are not explained in the legends. Please add an explanation and provide information about the number of replicates.

We have provided such explanations where appropriate.

• Several figures contain tables. Please make separate tables unless clearly motivated.

Tables have been separated where appropriate.

Specific comments to the text:

Page 2:

• Paragraph 2: Please re-write to be clear about what relates to bacterial versus human ribosomes. The sentence starting "There is only one documented drug-like molecule binding to the NPET…" is too long and difficult to follow.

We thank the reviewer for this comment and realize it was written in a confusing way. We have revised this part of the introduction and reduced some of the extraneous detail for ease to the reader.

• Reference 18: This is about the exit tunnel of bacterial ribosomes, please add some reference that clarifies if the same is true for human ribosome.

We have clarified this point in the text that this reference is for bacterial ribosomes and included a reference in 80S ribosomes.

• Paragraph 3: You state that “An alternate mechanism by which translation inhibition has been shown to impact cancer cell viability is mediated through stress response pathways that lead to apoptosis, as has been documented for anisomycin, further confirming translation inhibition as a therapeutic strategy even in the absence of biochemical selectivity.” It is not clear why it confirms it as a therapeutic strategy. Please clarify why this would not also affect “normal” cells.

We thank the reviewer for pointing this out. We’ve clarified that RSR-driven apoptosis is not inherently tumor-specific, but many cancers are more vulnerable because they run at high translation flux (and therefore have more potential for collisions), depend on short-lived oncogenic drivers like Myc or Cyclin D, and have in some cases acquired adaptations or mutations that limit their capacity to down-regulate protein synthesis.

Page 3: The final sentence is un-clear. Please clarify what “historically undruggable targets with high validation” means.

The text has been updated to reflect the meaning more clearly, these are targets that have strong supporting evidence for high dependence in cancer, i.e. they are required for survival of cancer cells to a much higher degree than healthy cells but have historically not been successfully targeted by drugs.

Page 4-5 and Figure 1:
• When comparing IDB-001 and ANS, IDB-001 shows enrichment of acidic residues but also positively charged residues. The latter one is suggested to be due to its weaker overall inhibition. If that is the case, the acidic residue enrichment is then less persuasive. This is particularly the case since the authors state that ANS strongly disfavors positively charged residues (when compared to DMSO), but IDB-001 also disfavors them. Further down the authors state “Notably, this enrichment disappears when IDB-001 is compared to DMSO” probably referring to positively charged residues, but it is unclear. Please improve the clarity of this paragraph.

We thank the reviewer for pointing this out and agree this paragraph can benefit from additional clarification. Our intent was to isolate sequence preferences attributable to the IDB-001 substituent by comparing IDB-001 to ANS. That comparison, however, is influenced by ANS’s strong depletion of R/K at -1. As a result, R/K appear “enriched” for IDB-001 only relative to ANS (essentially, we are diving by a very small number which makes the R/K appear enriched. When we benchmark IDB-001 against DMSO, the R/K signal does not persist. In contrast, the D/E enrichment at -1 is robust in all analyses (vs. ANS or DMSO). We have rewritten the paragraph to better illustrate this point.

• Figure 1B: it is difficult to differentiate the curves, in particular the blue one. Also, unclear if the shade indicates error bars.

We have changed DMSO line to gray to help distinguish it from the blue one.

• Figure 1H: Indication of codon positions under the figure would improve clarity.
Page 5-6 and Figure 2:

We apologize for any confusion here. We have revised the figure legend to reflect that we are showing the number of enriched stall sites corresponding to transcriptome locations (rather than particular nascent peptide sequences).

• Figure 2D shows an example of luminescence time traces. The rest of the traces for the other reporters should be added as a supplementary figure.

Due to multiple performed titrations across 5 reporters and 2 compounds there are in total some 300+ luminescence time traces which are hard to fit into a legible figure. In our best attempt to address this, we have updated Supplementary Figure 4 with additional data from all reporters to show the range of the assay results both in terms of translation kinetics and extent.

• The legends of Figure 2E-F legends should present how errors are calculated and how many data replicates were used.

This information has been added to the figure legend, the error bars represent standard error of the mean derived from the non-linear fit to the titration data.

Page 6-7 and Figure 3:
• Please clarify that “Similar to ANS30, the core binds in a pocket…” refers to both compounds.

The section has been changed to “As intended in our design, modification and addition of functional substituents to the anisomycin core did not disrupt binding, and each compound retains the stabilizing interactions of ANS with the rRNA (Figure 3B)[30]. The core of each compound binds in a pocket...”

• The statement that ”the methoxyphenyl forms p-stacking interactions with C4398 as it extends towards the tRNA (Figure 3B), all consistent with previous reports …” should preferably be supported with a supplementary figure showing a comparison with anisomycin.

Figure 3 has been updated and now shows an overlay between anisomycin and IDB-001 in panel B, demonstrating how both compounds form pi stacking interactions with C4398.

• At the end of this section (page 7, middle of the page) the two last sentences are repetitive and should be revised.

This has been revised in the manuscript.

• The inset in Figure 3A does not really add much. Possibly using the same style as Figures 3D-E but with the same camera view as the current inset could be more helpful.

The updated Figure 3 has a new inset in panel A that has the same style as the other panels while retaining the camera view.

• Please clarify what maps are shown in Figures 3D-E. Comparison with the provided maps suggests that these are trimmed around the model, which in such case is misleading.

The panels have been updated with untrimmed maps.

• As commented above, there is no density for F-3. Figure 3F does not add any additional

information to Figure 3E, and the surface display may confuse readers to believe that this is a map. Please remove. This side chain lacking density also seems to be the basis for the statement about the “hook-like conformation”. What is the evidence for this?

We agree with the reviewer’s comment and have removed previous panel 3F and the corresponding text to avoid confusion.

• Several statements are not supported by the maps, e.g. “Despite the inherently dynamic nature of the nascent chain in the exit tunnel, backbone and side chain positions of the four residues in contact with the compound (in the 0, -1, -2, and -3 positions) could be assigned.” and the final sentence “These structures show small molecule interdictors can engage in sequence-selective interactions…”. These statements must be supported with clear evidence in maps from which the sequence is clear, or removed.

The language of these statements has been adjusted to better reflect the data presented.

Page 7-8 and Figure 4:
• The authors suggest that the complementarity of the drug molecule rearranges the peptide chain to form new high-affinity interactions. This is a bit overstated considering that only D-1 has good density.

We have adjusted the language to emphasize that while the structural change in the nascent chain is small, it is significant. Because salt bridges represent some of the strongest chemical interactions, we believe that using the wording ‘high-affinity’ is justified. The sentence has been changed to: “This suggests that small-molecule interdictors can induce small but impactful structural rearrangements within the nascent peptide to form new stabilizing high affinity interactions.”

• The authors indicate that IDB-001 causes C4398 and C4399 to move by “several angstrom”. This should be specified more clearly.

The sentence has been revised in the text. In addition, Figure 3H now shows this movement more clearly

• “angstrom” should be changed to “Å” or “Ångström”.

We have corrected the instances in the manuscript.

• Position 4399 is U, not C (typo).

We thank the referee for catching this type, it has been fixed.

• In addition, U4399 seems to have both conformations (the new described and the same as in the absence of the drug). Instead, the authors have modeled two waters in that density. This should be revised and shown in the figure.

Thank you for noticing this mistake. The water molecules have been removed and alternative conformations have been added to each model where appropriate, including U4399 (Figure 3H).

• The authors discuss how A3908 is shifted towards the “bottom” conformer, closer to the compound. While this is supported by the map, there is actually density for both conformations in both of the structures (with and without the compound) but the authors have modeled waters in the alternative conformation. Please correct the model in agreement with the density for an alternative conformation of the base, not waters.

The model has been corrected to include the alternative conformation of A3908 (Figure 3J)

• The authors also discuss that U4452 alternates between two conformations in the absence of the compound. The map shows no density for this base, so the base must be dynamic rather than showing only two conformations. This does not affect the conclusion that the compound stabilizes one single conformation of this base.

The unsharpened map for the model in question shows clear density for both conformations. In the updated atomic models, alternative conformations for U4452 have been added (Figure 3I).

• Figure 4A-B: Many waters do not have supporting density here, see comments above.

As mentioned earlier, waters have been rebuilt based on stricter cutoffs and therefore the models contains much fewer water molecules now.

• Figure 4C-F: Please indicate in the legend how the structures were aligned.

Structures were aligned on their 28S rRNA using the matchmaker algorithm in ChimeraX. A sentence has been added to the legend.

• Figure 4D-F: Supplementary figures showing the density for these regions should be provided and fixed according to previous comments.

The density has been provided in the updated panels for Figure 3 (that is also now merged with the previous Figure 4).

Page 9-10 and Figure 6:
• The authors do not specify where they think IDB-002 would stall ribosomes translating MYC, which then does not really relate with the claimed specificity. Please clarify. Can the defined logo be found in the MYC gene?

We thank the reviewer for this comment and have addressed these remarks in a few points in the manuscript to clarify our messaging regarding context-dependent activity and MYC. While the interdictors we present here have context-selective activity for amino acids or amino acid motifs at particular locations in the ribosome-bound polypeptide chain, our molecules are not single-mRNA selective (*i.e.,* one interdictor inhibits translation of one mRNA), and we realize this may not have been effectively communicated.

Regarding this specific comment, IDB-002 stalls ribosomes engaged with many peptide motifs (as depicted in the weblogo from Figure 1) but with preference for small hydrophobic residues and proline in the -1 position. This stalling activity, while contextually dependent, still leads to global rearrangement of ribosomes as identified by ribosome profiling (Figure 1B-C), and is not limited only to MYC mRNA. Since IDB-002 was observed to impact the translation of

more 4-mers than IDB-001 (Figure 2A-B) we anticipate it to just lead to more robust translation stalling in aggregate by inhibiting more peptide bond formation events across the MYC open reading frame. Consistent with this, our ribosome profiling shows a larger pile up of ribosomes near the N-terminus of MYC and in an upstream open reading frame for IDB-002 treatment compared to IDB-001 (shown below). We added a small note about this to the Results section.

• The authors indicate that IDB-003 shows a similar pattern of context-dependent inhibition as IDB-002 (Figure 1G and Supplementary Figure 10B). This is not supported by the data, as only the -1 position has a similar pattern, there is no clear specificity in the other positions.

We have clarified this point in the text, that the context-dependent inhibition is similar between IDB-003 and -002 (Figure 1G and 5B), especially in the -1 position where we have designed our molecules to interact. We only performed ribosome profiling at 1.27 μM with IDB-003, and this concentration led to global elongation effects and a number of ribosome pause sites that is most similar to IDB-002 at 10 μM. Therefore, we believe comparison to support our claim of similarity in the sequence preferences between IDB-002 and IDB-003 is at these two concentrations (see below).

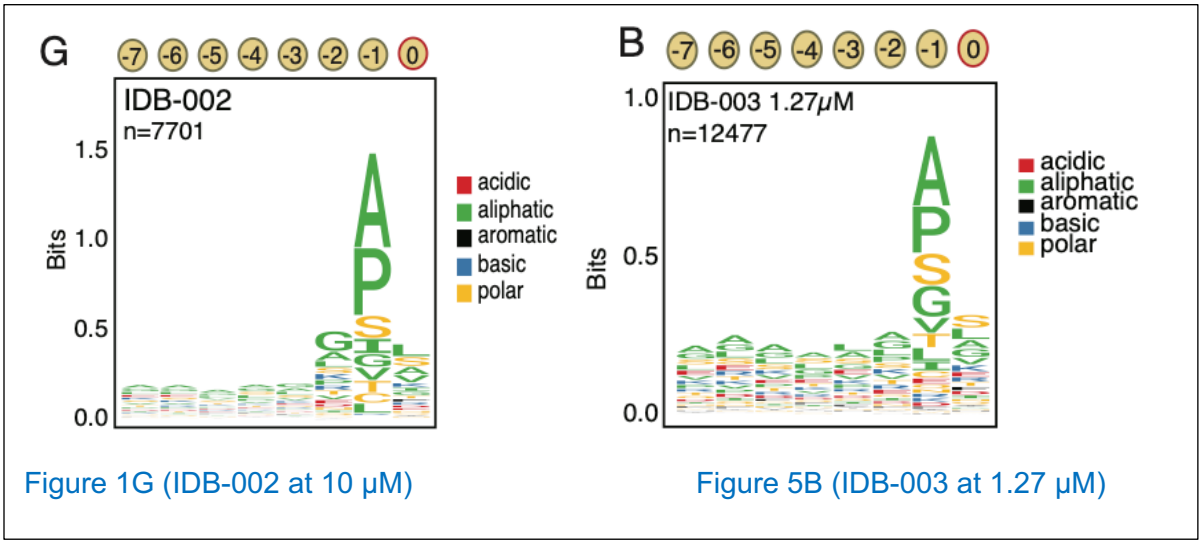


Figure 1G (IDB-002 at 10 μM)

Figure 5B (IDB-003 at 1.27 μM)

• IDB-003 seems less specific than IDB-001 and -002, and in general, only showing specificity for small amino acids in position -1. This suggests that IDB-003 may affect most genes. Please clarify if and why you think that this inhibition relates to MYC.

We believe the previous responses, as well as the general revision of the text surrounding MYC and context-dependent activity can clarify this point. Briefly, we share observations for context-dependent activity from these designed synthetic PTC-binding molecules in human cancer cells, but the selectivity is not 100% for MYC. MYC, as a short half-life oncogene, is especially vulnerable to translation inhibition like other short half-life oncogenes as others have noted (Robert, *et al.* 2009; Myasnikov, *et al.* 2017). Disruption of protein synthesis has a large impact to MYC levels because upon inhibition, the cellular 'pool' of MYC rapidly depletes. We added a panel in Figure 5 with Cyclin D1, another short half-life oncogene, as well as CDK4 (a longer-lived oncogenic protein) to better illustrate these points. We believe the text revisions better communicate the context-dependent activity of these molecules and observations to short half-life proteins that result from translation inhibition.

• Figure 6: Please spell out SEM in the legend.

Revised.

Page 11-13:
• There are many buzz-words in the discussion. Please reduce these vague statements, e.g. “a more advanced tool molecule”.

We have revised the discussion and limited our use of forward-looking statements to the final paragraph which speaks to the broad potential applicability of this modality in drug discovery and compares it with other modalities for greater context.

• The authors indicate that IDB-003 is highly effective at “well-tolerated oral doses”. Where is this shown? In the methods section it is indicated that all animals were monitored for changes in several parameters. Is there such data to support this claim?

This sentence has been revised. We intended this note to summarize that during the course of the study we made no observations that would signal animal stress from drug exposure. This has been noted in the methods instead.

• The authors indicate that “these data validate our approach of sequence-selective interdiction of protein synthesis as a powerful therapeutic strategy for MYC-driven cancers”. As mentioned above, this needs to be better supported.

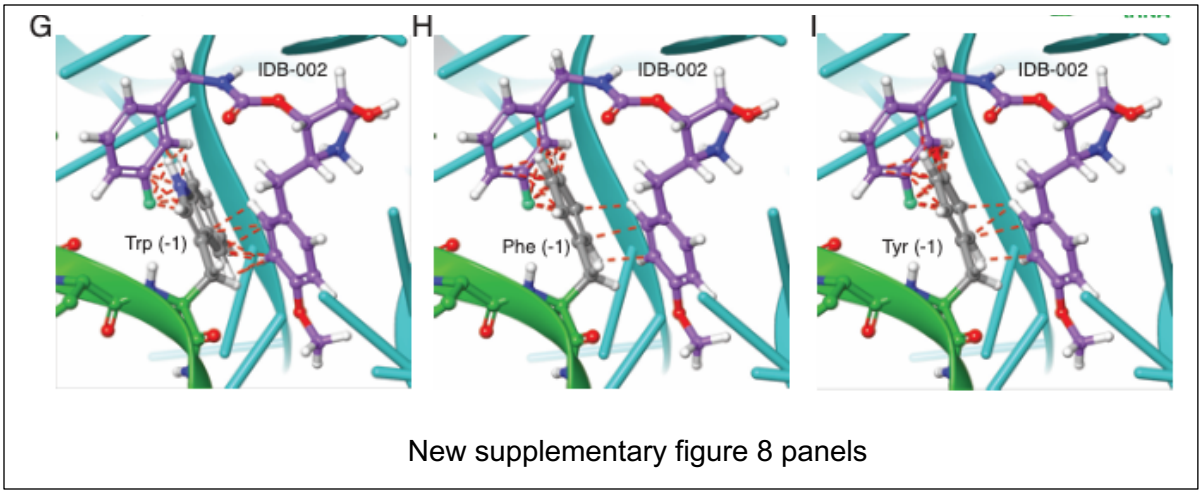
We have revised this in the text as “interdiction of translation elongation is a viable approach.”

• The authors mention again that the approach is sequence-selective to MYC, but this would need to be supported by data.

We thank the reviewer for this comment. We have made revisions throughout the manuscript to better clarify this point, that these molecules are not precision selective for MYC, and instead have context-dependent activity with regard to specific sequences engaged in the PTC.

• The statement ”Similar considerations also explain the observed deenrichment of certain sequences that contain bulky aromatic residues, as they are likely to clash sterically with the molecule in the PTC (Figure 1I-J).” would need to be supported by a supplementary figure visualizing how such bulky residues would make clashes.

We thank the reviewer for the thoughtful suggestion to address the statement in the paper. As suggested, we added 3 images to Supplementary Figure 8 that clearly show the steric clashes between aromatic residues Trp, Tyr, and Phe in the (-1) position of the polypeptide with IDB-002 The new figure panel is also included here below. The red lines indicate unfavorable steric clashes between IDB-002 and these amino acids.



New supplementary figure 8 panels

• The authors make a statement eluding to similarities between interdictors and PROTACS in ”substoichiometric or catalytic mode of action.” Please clarify this sentence; it is unclear which stoichiometry the authors refer to. Here it would actually be interesting to discuss the stoichiometry of inhibited and trailing ribosomes, interdictors and different nascent chains.

As the reviewer suggested, this statement was meant to elude to the stoichiometry of inhibited and trailing (5´) ribosomes. We have revised the text to clarify this more directly.

Methods:
• Page 16: The authors indicate the luminescence assay was performed in a luminescence-capable plate reader from BMG Labtech. Please specify the specific instrument used.

Resolved.

• Page 17: Please spell out IRES.

Resolved.

• Page 17: Why is the cryo-buffer in square brackets when the authors used parenthesis before?

This has been revised.

• Page 19: The authors indicate two numbers for relative humidity, likely a typo.

This has been revised.

• Page 19: A “2-day holiday” does not seem like a correct term to use here.

To be more specific we have changed this language to “2-day dosing holiday”. “Holiday” is commonly used to describe a temporary pause in dosing.

• Chemical synthesis section: The authors use calc’d to specify the m/z. Is this a typo?

This has been fixed.

Other comments:
• In the abstract the authors use “~” next to the reported resolution. This could be removed.

This has been removed.

• Supplementary Figures are not mentioned in order in the text. There is a jump from Supplementary Figure 6 to 9 and 7 and 8 are mentioned much later only in the methods.

We have resolved this error.

• Supplementary Figure 2 is very low resolution and difficult to judge.

We apologize and have tried to resolve this. We can provide a .pdf if the figure is still not legible.

• Supplementary Figure 8: The local resolution should be provided for the main map, not only the locally refined. The inset is misleading, as the resolution for the nascent chain is clearly not 1.9 when looking at this region of the map (looks more like 3.5 Å).

The local resolution is now being shown for the main map and not the locally refined maps. While the nascent chain is indeed not at 2 Å resolution as judged by the quality of the density, the local resolution calculation output is still useful to judge the overall quality of the surrounding PTC residues. Changing the window size of the local resolution calculation and other parameter changes did not yield substantially different results regarding the nascent chain.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank this reviewer for their insightful comments addressed above.

Reviewer #4 (Remarks to the Author):

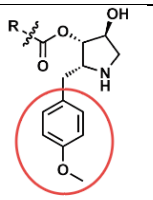
In this study, P.D. Diamond and colleagues used structure-based drug design to propose small molecules called interdictors, that selectively slow translation elongation of specific sequences in human ribosomes. They used ribosome profiling, cryo-EM analyses and in vitro translation assays to characterize and cross-validate the stalling sequences that are specific to their designed pharmacophores. They also show that translation of the pro-proliferation MYC gene can be specifically impeded by these interdictors in vitro (on purified ribosomes), in cellulo within Myc-dependent cancer cell line and finally in vivo, using human triple-negative breast cancer xenografted mice.

Overall, the study appears well designed, with many complementary techniques that convincibly validate the authors' findings, both in vitro and in animal models. Their work represents yet another solid proof that ribosomes and translation are worth investigating as therapeutic targets, and the molecules they designed might prove extremely promising to fight against the vast panel of MYC-related cancers. Since I am not an expert in chemistry, I can not evaluate the drug design process herein presented. However I believe that this manuscript is worth publishing in Nature Communications, provided that the following comments are addressed:

- The first part of the manuscript is about the extensive in vitro/in cellulo characterization of the two compounds IDB-001 and IDB-002, which seem to be efficient to stop the translation of specific sequences, including MYC's acidic stretch DSEEEQEDE. However, the in vivo validation on the xenografted mouse was performed using a third interdictor, IDB-003, which only appears late in the results section and has not undergone the same exhaustive validation as the other two compounds. This is confusing, and readers may wonder if IDB-001 and IDB-002 were tested on mouse models and found to be ineffective. If this is the case, I believe the authors should clearly justify their choice of the third compound IDB-003, and re-shuffle the corresponding results section so that the ribosome profiling and MYC expression assays (shown in figure S10) are presented earlier in the text. Otherwise, the current elliptical style slightly undermines the strength of the reported study.

We agree that the transition to the *in vivo* work was not sufficiently described in the original submission. IDB-003 is a second-generation analogue derived from IDB-002 to enable oral dosing and adequate exposure in mice. It retains the same PTC-binding scaffold and nascent-chain-interacting substituent, and we demonstrate the same -1 context-preferential activity observed in ribosome profiling (new Figure 5). IDB-001 and IDB-002 were not tested for xenograft efficacy because the plasma pharmacokinetic exposure was not sufficient based on studies of related molecules (see below). We added more cell and ribosome profiling data into the main results section immediately before the xenograft section, as well as more explanation to clarify the exposure-driven decision to move forward with IDB-003.

Table to illustrate why we did not use IDB-001 and IDB-002 for in vivo experiments because of poor bioavailability for related compounds:

ID	SD Rat bioavailability (%F)	 Southern Group
IDB-A (related to IDB-001)	2.4%	Methoxyphenyl
IDB-B (related to IDB-002)	5.4%	Methoxyphenyl
IDB-001	Not determined	Methoxyphenyl
IDB-002	Not determined	Methoxyphenyl
IDB-003	21%	Oxazole

Rat PK for methoxy-bearing IDB-A and IDB-B (first generation designs) had low oral bioavailability. We attribute this characteristic to the metabolically labile methoxy substituent resulting in high first pass hepatic clearance which is a common feature in IDB-001 and IDB-002 as well. Although we do not have a matched molecular pair showing that this change is responsible for poor %F, the data for IDB-A and IDB-B was sufficient to inspire the replacement of the southern group for subsequent *in vivo* tool compounds, such as IDB-003 with a significantly improved bioavailability.

- A CryoEM analysis of the third compound represents a significant amount of additional work and is probably out of the scope of this manuscript, thus, to reinforce their findings, could the authors also test the effect of IDB-003 on RSR, by WB experiments?

We agree that a cryo-EM analysis of another compound feels out of scope. However, we added a deeper characterization of the effects of IDB-003 in cells. This includes the depletion of short half-life oncogenic proteins (Myc and Cyclin D), and dose-responsive activation of the RSR (Figure 5, Supplementary Figure 10) to support that IDB-003 retains context-dependent activity, RSR activation, and efficacy in cells. We hope this addresses the concerns of this reviewer.

Minor comment:
The composition of the tetrapeptide/4-mers sequences should be described earlier (p.5) to improve clarity.

We hope the revised text addresses this comment. We limited our use of the term '4-mers' until their identification in Figure 2. In Figure 1 we are observing the amino acid preferences observed in specific nascent polypeptide positions, but in aggregate across all ribosome footprints, rather than pulling out specific 4-mers, which we do in Figure 2.

Reviewer #5 (Remarks to the Author):

Diamond, Sauer, et al. report the exciting discovery of first in class molecules that can inhibit protein synthesis in a specific manner dependent upon the polypeptide sequence in the peptidyl-transferase center (PTC). The findings presented in this manuscript are an important step toward selectively targeting the translational machinery with the potential to specifically inhibit the growth of tumors, which could be programmed to combat other diseases. The authors conduct transcriptome-wide ribosome profiling to demonstrate the selective stalling of the ribosome, which, coupled with structural studies, illuminates the potential selective mechanism of action at the molecular level. They show that the compounds can inhibit cancer viability potentially through repressing of MYC translation and/or ribosomal stress response. Finally, they demonstrate in vivo efficacy of tumor growth inhibition with a third orally available compound. This study is of interest to the broad readership of Nature Communications. However, while this work is a valuable step towards harnessing the power of targeting selective translation, it requires additional experimentation to solidify the proposed claims mechanism of action and target specificity.

Major Points:

1. While the authors provide insight into the specificity of the stall sequences with interdictor treatment, there is a lack of validation at the proteome level. This is key to demonstrating that their compounds result in selective and specific changes at the protein level, which may mechanistically underpin the phenotype on cancer cell line viability. The authors should show global proteomic changes in their cell line models and analyze how those changes overlap with the observed stall sites transcriptome-wide.

We agree with the reviewer that a proteomics validation would be a great experiment, generating rich data, however we feel it is both out of scope for this work given the biochemical selectivity we show, and technically challenging for the mechanism of action.

First, we want to clarify the scope of selectivity we report in the manuscript. The sequence preferences we define operate at the motif level (e.g. -3 to -1 context in the PTC) and are shared by many transcripts (Figure 1-2). While we can observe some kinetic selectivity, these presented molecules are not selective for single mRNAs, or even 10s or 100s of mRNAs as many transcripts have the motifs we identify. This is also evident in the global shift in ribosome occupancy we observe (Figure 1 & Supplemental Figure 2).

Second, a proteomics experiment performed with an interdictor requires careful selection of the time point for observation. As interdiction only limits new synthesis of proteins (and does nothing to the native ‘pool’ of proteins), the observable effects by proteomics are limited to the intrinsic half-life of proteins. If we performed this experiment 4 hours after interdictor treatment (similar to the exposure kinetics we achieve in animal models), we would only be able to observe effects on protein abundance for proteins with < 4-hour half-lives (to see a > 2-fold change in those proteins). Performing proteomics at longer time points is possible, but given the biochemical selectivity discussed, we anticipate we will in fact find many proteins impacted.

While an unbiased proteomics time course is beyond the scope of the current study, we implemented a targeted validation focused on endogenous short-half-life oncoproteins (CCND1 and MYC) that are clinically relevant, compared with longer half-life proteins (CDK4 and Tubulin). These new time-course immunoblots with IDB-003 support this kinetic selectivity for short half-life oncogenes and supports the efficacy we observe in the *in vivo* model (Figure 5).

2. The authors fail to show the kinetics and dynamics of interceptor therapy on endogenous targets. Together, the experiments detailed below are key for supporting their claim on p. 12 that the selectivity of the compounds for specific peptides in the PTC will be more effective against oncogenes with short half-lives over essential housekeeping genes.

a. Specifically, the authors should test the kinetics of inhibition of Myc or other endogenous targets (these will be better defined by the above proteomics experiments). Currently, the earliest time point of treatment is 2hrs at which time Myc protein level is already quite suppressed.

We have added several experiments to the manuscript addressing this comment. First, we quantified MYC levels in MYC-dependent TNBC cells (Figure 5E) and identified that MYC depletion in these cells closely parallels the concentration-dependent viability defects. Furthermore, we added an analysis of short vs. long half-life proteins measured in MCF7 breast cancer cells at shorter time points to better visualize the kinetics of short half-life protein (MYC and CCND1) depletion from these cells upon interdictor treatment (Figure 5 and Supplemental Figure 10).

b. Also the authors should test the inhibition of endogenous protein expression across a drug titration as shown for RSR signaling in Fig 5. Are the short-lived driver oncogenes inhibited at a lower concentration? This can help discriminate between the contributions of ribosomal stress response vs loss of driver oncogene expression for the compounds' efficacy.

We now provide dose-responsive measurements of endogenous MYC protein across compound titration (Figure 5E). In HCC1143 cells, MYC reduction occurs with an IC_{50} of 0.11 μM after 2 hours of IDB-003 treatment. These concentrations are in the same range at which we first detect JNK phosphorylation (Supplemental Figure 10) and are similar to the 72h cell-viability IC_{50} values for IDB-003 (Table 3, Figure 5E). Thus, for IDB-003, efficacy coincides with both MYC depletion and RSR engagement.

In contrast, the cell viability IC_{50} value for IDB-001 in HCC1143 cells is determined as ~800 nM but only a minor impact to MYC protein levels is observed at 10 μM concentration (Supplemental Figure 10). Without significant MYC loss and with only weak JNK phosphorylation, this supports a distinct mechanism (beyond MYC and RSR) for IDB-001 activity in inhibiting cell viability.

Other points:

1. The authors should demonstrate the "on-target" effects of IDB-003 in the in vivo tumorigenesis study. That is, does Myc protein level decrease in the treated tumors?

We thank the reviewer for this comment and chance to add this data to the manuscript. We assessed target engagement in the xenograft study by RNA sequencing (Figure 5). Analysis of the MYC protein levels from these terminal tumor samples was not reliable enough for us to have confidence in the protein abundance detected, either because of inter-animal variability, the time point chosen for tumor harvesting, or the lysis buffer we used which did not include translation or proteosome inhibitors to reliably capture MYC protein levels). However, RNA-seq of the same tumors revealed significant suppression of MYC transcriptional programs (Hallmark MYC Targets v1). We have revised the text to include these data, methods, and deposited the data to GEO (GSE309226).

2. The manuscript would benefit from additional discussion of potential rules or guidance on how to evolve the compounds to increase selectivity based on the structural data. Additionally, discussion of the relative contribution and interdependency of peptide binding and the A site inclusion would be appreciated and further illuminate the specificity of the molecules for their targets.

We thank the reviewer for this helpful suggestion. We modified the paragraph in the discussion section beginning with “Chemical library expansion …” to hopefully more clearly outline the structural learnings and guidance for improving selectivity. We provide a more detailed description of the interdependency of rRNA binding, nascent peptide-facing interactions, and A-site occlusion and elaborate more on how these collectively contribute to tuning selectivity towards various peptide motifs/targets.

3. The authors should discuss the literature on the translation of Myc mRNA and how their work is relevant in this context.

MYC translation is tightly controlled at the level of initiation and is responsive to changes in the availability of eIF4F and activity of mTOR/4EBPs (Pelletier, *et al.* 2015). Under conditions that dampen cap-dependent initiation, MYC translation may be dependent on non-canonical translation initiation mechanisms, such as usage of upstream CUG start codon or reported IRES (Hann, *et al.* 1988; Stoneley, *et al.* 1998). Our ribosome profiling suggest that Interdictors cause increases in ribosome occupancy early in the MYC CDS and concomitantly increases in ribosome footprints within reading frames in the 5’ UTR. This may be consistent with occlusion of the canonical (and CUG) start codons that diverts scanning complexes to uORFs. We have included an explanation of the relevance of this in the results.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

My concerns and comments were addressed sufficiently!
All the best!

We thank the reviewer for their feedback and the improvement of our manuscript resulting from their comments.

Reviewer #2 (Remarks to the Author):

The revised version of the manuscript "Context-dependent translation inhibition as a novel cancer therapeutic modality" by Diamond et al. has addressed all our comments on the previous version of the manuscript. The updated structures show reasonable validation statistics and are now adequately described in the text and the figures. Overall, the text is more stringent and clear.

With access to all the maps in a clearly organized folder, we identified an additional problem that needs to be addressed. Other than that, we find the revisions to be satisfactory.

A fast inspection makes us question whether the P-site tRNA modeled in the FTED RNC + IDB-001 structure (code 9O3X) correctly reflects what is mainly present in the complex. The anticodon density suggests that the residue 35 on the P-tRNA should be a purine (currently modeled as a pyrimidine), at the same time, residues 31 and 39 appear to be purine and pyrimidine respectively (currently modeled in the opposite way). We recommend the authors to take a look again at the tRNA density of this structure to confirm which tRNA it is mainly present in the density, and to adjust their interpretation of the tRNA and the nascent peptide accordingly. The other structures appear to be correct.

We thank the reviewer for their positive comments, and their note about the FTED-RNC structure. Re-examining the experimental density for this structure, we agree that for the tRNA residues mentioned by the reviewer, base densities could potentially be interpreted in several ways. We have chosen to be conservative in our interpretation of the exact base sequence, considering the biochemical setup of the experiment and the density of the mRNA in addition to the tRNA density itself, for several reasons:

First, tRNAs are significantly more dynamic, and less rigid, than the surrounding ribosomal RNA and their local resolution is commonly lower than the overall resolution of the structure. Further, the distinction between purines and pyrimidines can be difficult to determine as base pair hydrogen bonding can contribute to density, and base modifications common in tRNAs can distort base shape.

Second, while we have done our best to biochemically isolate a homogeneous cryo-EM sample, and performed extensive particle sorting, the particle set could still include a small fraction of ribosomes containing unintended tRNAs from the lysate. Even a small fraction of such ribosomes could contribute ambiguity in the tRNA density.

Third, in this structure the density for the mRNA extends towards the 5’ end but not past the decoding site. This suggests that the structure, like the other 3 RNC structures, is stalled on the last codon of the message, in line with the experimental design.

However, after reviewing the referee's concerns and re-examining the experimental maps, we have decided to move the FTED structure to the supplement of the manuscript (Supplementary Figure 8A) and include a note about the limitations of this map in the text. Since all conclusions derived from the FTED structure in the presence of IDB-001 overlap with those from the QEDE structure plus IDB-001, this modification does not affect any conclusions or results presented in the study.

Minor comments:

The uploaded LSU focus map of 9O3V (MYC without IDB) appears to be shifted and does not fit to the model. The authors should correct this.

Thank you for the comment. The shift is a result of 3D refinement during cryo-EM data processing, and, while influenced by the chosen reference, can shift during the iterative refinement process. The only way to shift the map without a loss of resolution (which could be done using the volume resampling function in ChimeraX) would be to re-run the refinement with an adjusted reference. However, at this stage of the manuscript review process we would like to avoid changing primary experimental data + analysis because it would risk introducing difficult to follow-up inconsistencies for our PDB deposition, reported map statistics and overall model validation. Because the map is shifted by only a few Å compared to the consensus map, a simple map fitting procedure (for example the fit-in-map function in ChimeraX) can easily be carried out by interested readers.

In the cryo-EM methods and perhaps in Table 1 and Supplementary Table 1, please clarify which classes from 3D classification are used for the final reconstructions and modelling. Based on the numbers that Supplementary Figure 6 shows for the MYC:IDB-001 RNC complex, this seems to be the POST state. In Supplementary Table 1, it is unclear what "best of" means for the POST states of IDB-001+FTED and IDB-002+FPAK - please clarify.

We have added the requested clarifications. Indeed, the POST state was used for the final reconstruction and modelling. The 'best of' means that final classes were classified again, to discard all lower-quality particles to obtain better maps.

In the methods, it is stated that "All data processing was carried out in Cryosparc v4.584 645 and is 646 schematically outlined in Supplementary Figure 7." This should probably be Supplementary Figure 6.

Thank you for catching this, we have corrected it.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank this reviewer for their insightful comments addressed above.

Reviewer #4 (Remarks to the Author):

With this revised version, P. Diamond and colleagues have appropriately addressed my previous comments. I believe their work should be published in Nature Communications, although the following sentences should be modified:

- Lines 331-332: “IDB-002 treatment showed a dose-response to ANS, while IDB-001 displayed modestly weaker activity (Figure 4A)”. I suggest rephrasing this sentence as follows: “IDB-002 treatment showed a dose-response similar to that of ANS, while IDB-001 displayed weaker activity (Figure 4A).” I am not sure that the term “modestly” is appropriate here, since there is a tenfold difference between the doses of IDB-001 and ANS/IDB-002 required to reach 50% cell viability.

We agree with the reviewer and thank them for catching this important edit which we have revised accordingly.

- Lines 371-373 “Indeed, treatment of MCF7 cells with 1 μM IDB-003 led to rapid depletion of MYC and CCND1, with substantial loss evident by 1-2 hours and near-complete depletion by 4-8 hours (Figure 5D).” While this statement is accurate for CCND1 and CHX, the Western blot in Figure 5D does not show a significant decrease in MYC signal after 1–2 hours, nor near-complete depletion after 4–8 hours, when compared to the tubulin signal. However, this description does hold true when using 10 μM IDB-003, as shown in Supplementary Figure 10D. Therefore, I recommend that the authors rephrase this sentence accordingly.

We thank the reviewer for bringing this to our attention. We have adjusted the text as follows: “Indeed, treatment of MCF7 cells with 1 μM IDB-003 led to rapid depletion of CCND1 evident by 1-2 hours and reduction in MYC levels observable after 2 hours of treatment (Figure 5D) while treatment with 10 μM IDB-003 led to rapid depletion of MYC in <4 hours (Supplementary Figure 10C).”

Reviewer #4 (Remarks on code availability): There was no code to review.

Reviewer #5 (Remarks to the Author):

The authors argued that the validation of their ribosomal profiling by mass spectrometry or other means is out of the scope of this work, but I do not agree. It is still unclear how these compounds reduce cancer cell viability in culture and in vivo. Is this due to the reduction of translation of specific oncogenes or the activation of deadly stress? In fact, the title of the manuscript itself, “Context-dependent translation inhibition as a novel cancer therapeutic modality”, emphasis that the context-dependent translation is important as a cancer therapeutic. This indeed would be very exciting, but there is no data to substantiate such a claim without additional experiments to address this important distinction between translation-specific effects versus a general stress response.

It appears that the authors are unaware of the existing literature on the role of translation control and cancer, as well as the numerous efforts aimed at targeting translation specificity, which is more prevalent in cancer cells than in normal cells.

While targeting ribosome and translation control is an emerging field in cancer biology, and it is important to pursue, this work still falls short of being published in Nature Communications.

We thank this reviewer for their feedback which has greatly improved the manuscript. We believe we have thoroughly characterized the context-dependent activity of the interdictors presented in this study through a combination of ribosome profiling, *in vitro* kinetics, and cryo-EM. As we demonstrate, this selectivity operates at the short-peptide motif-level and is driven primarily by the -1 nascent polypeptide position. We have further demonstrated that these sequence preferences can be rationally engineered by chemical modification of the interdictor molecules. However, as we acknowledge throughout the text, this level of selectivity is not yet sufficient to limit the effect of these molecules to single mRNAs, or even 10s or 100s of mRNAs.

Therefore, in a putative proteomics experiment, changes to the proteome on interdictor treatment would be driven by: 1) The intrinsic half-life of the proteins in the proteome, 2) direct translation inhibition driven by the interdictor, and 3) downstream transcriptional and translational responses. Such experiments would generate large and complex datasets that would provide detailed and expansive insights into the physiological consequences of interdictor treatment. However, we believe that such data would likely not answer the reviewer’s specific question on the mechanistic basis of cell killing by our molecules – hence we suggest that such exploratory experiments are outside the scope of the present study.

We are also eager to disentangle the precise mechanism of action by which cancer cells die following interdictor treatment. As the reviewer aptly asked, is it because of depletion of rapid turnover oncogenic proteins, like MYC, CCND1, MCL1, etc. or from a protein-synthesis dependent stress response induced by the molecule e.g., the RSR or the ISR? Both depletion of oncogenic proteins from addicted cancer

cells and induced stress responses are well documented in the literature as mechanisms that can impact cancer cell viability. Further, these responses occur on similar time scales and may overlap in their molecular pathways.

That said, we have performed some cell viability experiments for interdictor molecules co-treated in the presence of inhibitors of the RSR (Nilotinib) and ISR (GCN2iB) pathways, based on the concentrations reported in Sinha *et al.* 2024 in MCF7 cells. For all three interdictors and anisomycin, co-treatment with stress-response inhibitors did not alter the cell viability EC_{50} compared to the interdictor alone, implying that the two main canonical ribosome-mediated stress response pathways are not the sole contributors to the cell-killing effect of interdictors. We have now included these results in a new supplementary figure panel (Supplementary Figure 10E), as well as a note about our continued characterization of this MOA in the discussion and look forward to publishing our findings in a separate study when those investigations are complete.

We have also identified several citations we may have neglected to include from the literature regarding other translation inhibitory mechanisms being investigated as cancer treatments, such as targeting eIF4A, eIF4E and mTOR, to more comprehensively survey all the ongoing work in this area. We have revised the introduction to include the sentence beginning with “Beyond elongation inhibitors…”. In addition, we now cite genetic studies demonstrating that translational output is a critical dependency in MYC-driven cancers (see sentence beginning with “Notably, genetic studies have shown…” in the discussion. As we continue to dive into the precise mechanism more deeply and look forward to continued interactions with the scientific community and this reviewer to strengthen our understanding of the interdictor mechanism of action, and the basic understanding of these ribosome surveillance pathways.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

All minor comments have been addressed in a satisfying manner.

However, the major comments on the FTED structure have not been addressed in a satisfying manner by moving this structure to the supplement and stating that "However, some ambiguity remained in the density-based distinction of three purine/pyrimidine bases in the aspartyl-tRNA of the FTED RNC, possibly because of impurities in the sample." The authors write in the answers that "We have chosen to be conservative in our interpretation of the exact base sequence, considering the biochemical setup of the experiment and the density of the mRNA in addition to the tRNA density itself", which does not at all make sense.

It has to be clarified that in this structure, the peptide sequence in the PTC and its interactions with the inhibitor (i.e. the focus of this study) are inferred from the tRNA species, not the other way around. While there may be some ambiguity, the cryo-EM map clearly shows that the major tRNA present in the complex is different from the one modelled; i.e. stalling does not mainly occur where they expect. This in turn means that the peptide modelled is not the dominating peptide present in the PTC of this complex. Thus, the current description is misleading. The authors have the choice to either re-model and re-interpret the structural data based on the density (i.e. purine-pyrimidine pattern) for the tRNA and mRNA to figure out where this complex has stalled and build the resulting peptide in the PTC or to remove this structure from the manuscript.

We thank the reviewer for their comments and have decided to remove the FTED RNC structure from the manuscript out of an abundance of caution. All conclusions drawn from the FTED RNC structure overlap with those from the QEDE RNC structure, so its removal does not affect the conclusions or main results of the study. We thank the reviewer for their detailed comments here and throughout the revision process.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their feedback and the improvement of our manuscript resulting from their comments.